

Progranulin inhibits platelet aggregation and prolongs bleeding time in rats

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Abstract. – OBJECTIVE: Several adipokines secreted by adipose tissue have an anti-thrombotic and anti-atherosclerotic function. Recently identified adipokine progranulin was found to play a protective role in atherosclerosis. Bearing in mind the central role of platelets in inflammation and atherosclerosis, we aimed, in this study, to examine the effect of progranulin on platelet function and coagulation profile in rats.

MATERIALS AND METHODS: Healthy male albino Wistar rats weighing (250-300 g) were divided into 4 groups. Three groups were given increasing doses of progranulin (0.001 μ g, 0.01 μ g, and 0.1 μ g) intraperitoneally, while the control group received phosphate-buffered saline (PBS). Bleeding time, prothrombin time, activated partial thromboplastin time and platelet aggregation responses to adenosine diphosphate and arachidonic acid were assessed.

RESULTS: Administration of progranulin resulted in a significant inhibition of platelet aggregation in response to both adenosine diphosphate, and arachidonic acid. Bleeding time, prothrombin time and activated partial thromboplastin time were significantly prolonged in all groups that received progranulin, in particular, the 0.1 μ g dose, in comparison to the control group.

CONCLUSIONS: This preliminary data is first suggesting that the antiplatelet and anticoagulant action of progranulin could have a physiological protective function against thrombotic disorders associated with obesity and atherosclerosis. However, these results merit further exploration.

Key Words:

Adipokines, Progranulin, Platelets aggregation, Coagulation profile, Aggregation agonists, Bleeding time, Prothrombin, Activated partial thromboplastin time, Cardio-protection.

Introduction

Adipose tissue is considered an active endocrine organ that secretes adipokines, which plays an important role in cardiovascular function^{1,2}. The adipokine progranulin (PGRN), which was first purified as a growth factor from conditioned tissue culture media³, is a protein composed of 593 amino acid residues with seven and a half-domains, each consisting of tandem repeats of the 12-cysteine motif⁴. PGRN has a significant role in health and disease, including cell growth^{5,6}, wound healing⁷, tumorigenesis^{6,8} and neuro-degenerative disease such as fronto-temporal dementia⁹. In the context of heart disease, PGRN has a protective role; as it was shown, in atherosclerosis PGRN reduced inflammation by inhibiting monocyte chemotaxis, and the release of interleukin-8 from the vasculature¹⁰. Furthermore, it attenuates lipopolysaccharide-mediated pro-inflammatory signaling in endothelial cells through the activation of the Akt/eNOS pathway and attenuation of the NF- κ B pathway¹¹. PGRN knockout mice, are more susceptible to atherosclerosis due to increased expression of inflammatory cytokines, adhesion molecules, and reduced expression of endothelial nitric oxide synthetase¹². Our interest in PGRN stems from the confirmed observations that platelets play a central role in thrombotic, inflammatory and cardiovascular diseases¹³, and that platelet dysfunction is a common feature of numerous acute cardiovascular events^{14,15}. Besides, platelets gather and interact with leucocytes at the vascular endothelium, thus enhancing the release of pro-inflammatory and pro-thrombotic factors that promote atherosclerosis and its complications¹⁶. We took these observations further

and the present study aimed to find out the effect of PGRN on platelet function and coagulation profile, an area of research not touched before.

Materials and Methods

Animals

Male albino Wistar rats weighing 250 to 300 g were obtained from the Animal House, College of Medicine, King Saud University. The rats were maintained under standard conditions (ambient temperature 21-23°C; with a 12-h dark-light cycle) and were fed with a standard diet with free access to tap water.

Study Groups and Experimental Design

The study included four groups (6 animals in each group) that were divided as follows: Group (1) control group, received phosphate buffered saline; Group (2) received PGRN (0.001 µg/rat); Group (3) received PGRN (0.01 µg/rat); Group (4) received PGRN (0.1 µg/rat). PGRN was administered intraperitoneally. Human recombinant PGRN was obtained from R & D Systems (Minneapolis, MN, USA), and the assigned doses were selected according to previous preliminary experiments performed by our group (unpublished). Intraperitoneal urethane anesthesia was used (1.25 g/kg), and every effort made to minimize animal suffering. The study protocol was approved by the Institutional Review Board of the Faculty of Medicine, King Saud University.

Blood Collection and Processing

Blood was collected by cardiac puncture and added to sodium citrate solution (3.2%) in a plastic tube in a ratio of 1:9 (1 part anticoagulant to 9 parts blood). Blood samples were transferred without delay (within 2 hours of collection) to the Coagulation Research Laboratory located at the Physiology Department, College of Medicine, King Saud University.

Preparation of Platelet-Rich and Platelet-Poor Plasma for Platelet Aggregation

Platelet-rich plasma (PRP) was prepared by the centrifugation of citrated whole blood at 1000 rpm for 5 min, at 20°C. PRP was removed and the remaining sample was centrifuged at 3000 rpm for 15 min to obtain platelet-poor plasma (PPP), which was used as a standard for the aggregation studies.

Platelet Aggregation Studies

Platelet aggregation studies were undertaken using the Platelet Aggregation Profile® Model PAP-4 (BioData Corporation, Horsham, PA, USA), using adenosine diphosphate, 20 µmol/l (BioData Corporation, Horsham, PA, USA) and arachidonic acid, 5 mg/ml (BioData Corporation, Horsham, PA, USA). The aggregometer micro-cuvette (7.25 × 55 mm, BioData Corporation, Horsham, PA, USA), was used in the entire study. Using plastic tips, 0.2 ml of PRP was pipetted into the cuvette, followed by 0.02 ml of the aggregating agent and the recording started. The machine automatically registers the aggregation result as maximum aggregation (%), against the control platelet-poor plasma (PPP), which represents the zero scale of the machine.

Coagulation Tests

Bleeding Time Measurement

Bleeding time (BT) was measured 30 min after the administration of PGRN. The terminal 1-mm tip of the tail of the anesthetized rats was excised using a sterile razor blade. The resultant wound gently blotted with filter paper at 30-sec intervals until bleeding stopped. The time when no blood could be blotted was recorded as the BT¹⁷.

Prothrombin Time, and Activated Partial Thromboplastin Time

The STA®-Néoplastine® CI plus kit (Diagnostica Stago, Asnières sur Seine, France) was used for the determination of the Prothrombin time (PT), and the STA®-PTT Automate 5 kit (Diagnostica Stago, Asnières sur Seine, France) for determination of activated partial thromboplastin time (aPTT) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using SPSS21 software (SPSS Inc., Chicago, IL, USA), and data expressed as the mean ± SD. Normally distributed data were analyzed by one-way analysis of variance (ANOVA) followed by LSD Post-Hoc test for multiple comparisons. Kruskal-Wallis test was used to compare data that were not normally distributed, and the Mann-Whitney test was applied to find the significant difference between any two groups. A *p*-value less than 0.05 was considered statistically significant.

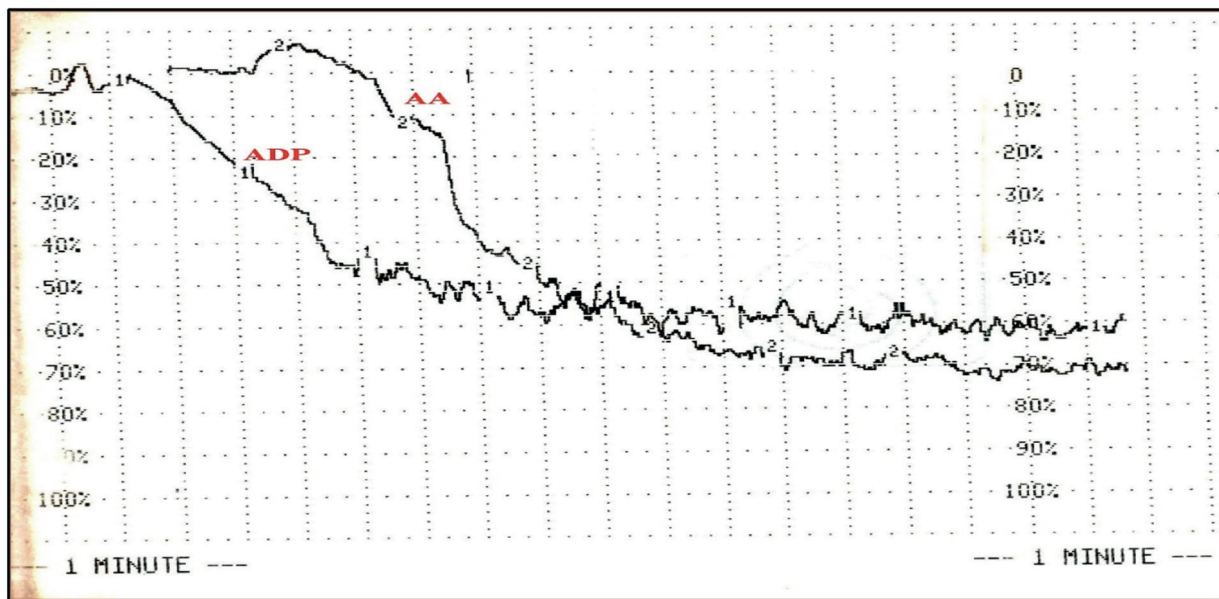


Figure 1. Optical platelet aggregometry performed in control rat platelet rich plasma, in response to adenosine diphosphate (ADP), showing maximal amplitude (MA%) of 58%, while the response to arachidonic acid (AA)-induced aggregation is 71%.

Results

Effect of Progranulin Administration on Platelet Aggregation

Figure 1, 2, 3, and 4 show typical examples of the aggregation responses to ADP and AA before and after the administration of PGRN. PGRN caused significant dose-related inhibition of the

platelet aggregation responses to ADP ($p, 0.004$) after administration of the three doses of PGRN ($0.001 \mu\text{g}$, $0.01 \mu\text{g}$, and $0.1 \mu\text{g}/\text{rat}$), when compared to the aggregation responses of the control group (Figure 5A). The maximum aggregation (MA%) response (mean \pm SD), in the control was $41.3 \pm 10.0\%$, while after PGRN administration, the MA% dropped to $4.5 \pm 2.6\%$ for the $0.001 \mu\text{g}$

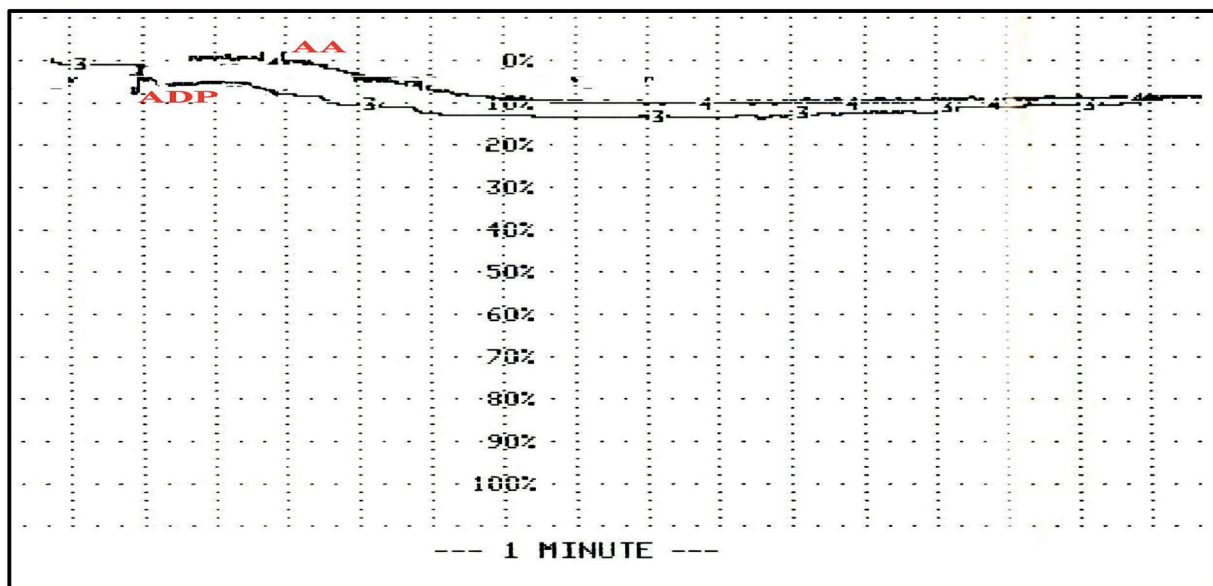


Figure 2. Optical platelet aggregometry performed in rat platelet rich plasma in response to adenosine diphosphate (ADP) after the administration of PGRN $0.001 \mu\text{g}$, showing maximal amplitude (MA%) of 8%, while the response to arachidonic acid (AA)-induced aggregation is 9%.

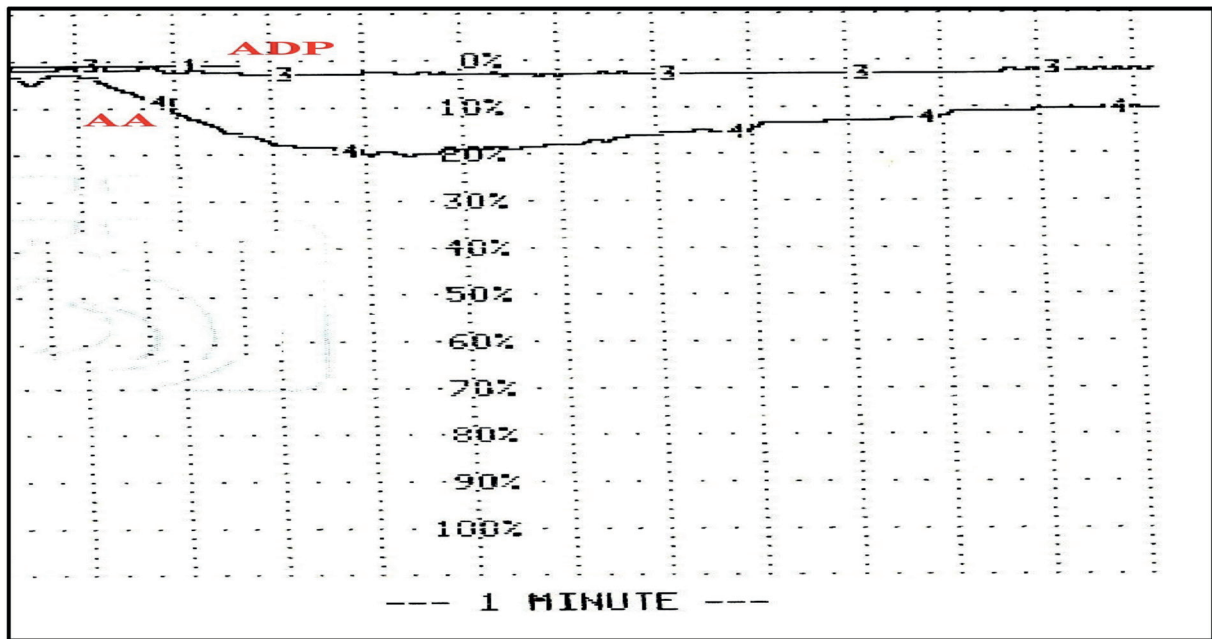


Figure 3. Optical platelet aggregometry performed in rat platelet rich plasma in response to adenosine diphosphate (ADP) after the administration of PGRN 0.01 µg, showing maximal amplitude (MA%) of 2%, while the response to arachidonic acid (AA)-induced aggregation is 10%.

dose, $9.8 \pm 11.7\%$ for the 0.01 µg dose, and $13.5 \pm 10.0\%$ for the 0.1 µg dose. However, the differences between doses were insignificant; 0.001 vs. 0.1 µg dose ($p, 0.05$), 0.01 vs. 0.1 µg ($p, 0.4$),

and 0.001 vs. 0.01 µg ($p, 1.0$). Similarly, PGRN caused marked ($p < 0.001$) inhibition of the platelet aggregation responses to AA (Figure 5B). The MA% (mean \pm SD) in the control group was 59.3

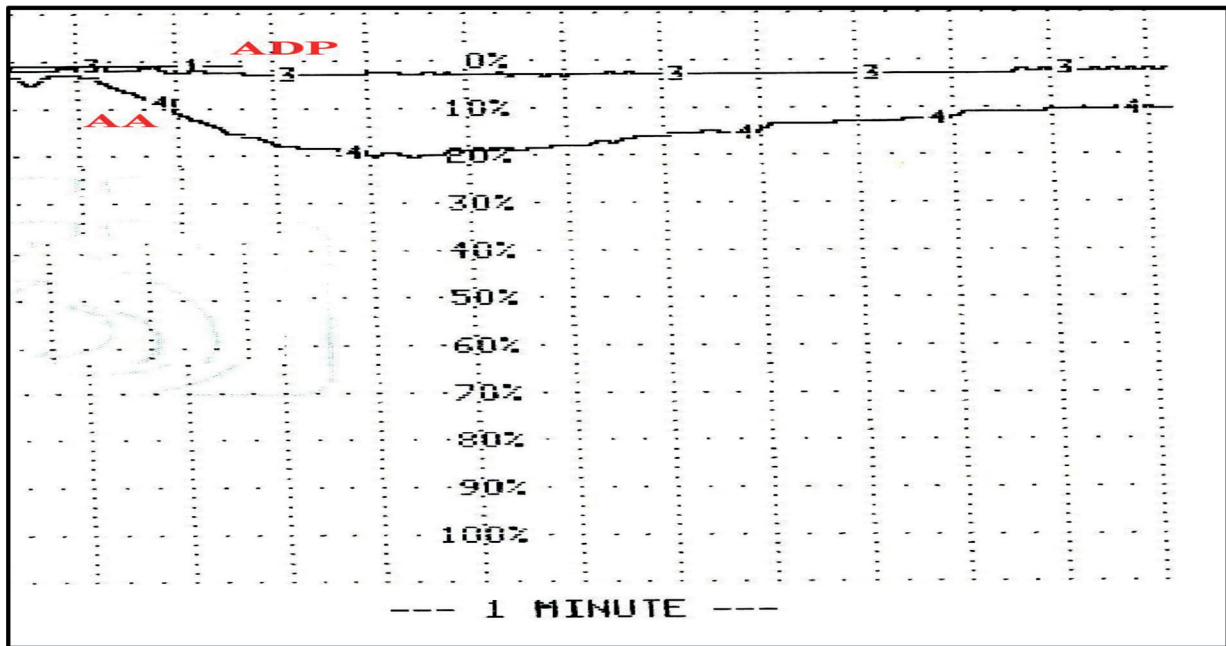


Figure 4. Optical platelet aggregometry performed in rat platelet rich plasma in response to adenosine diphosphate (ADP) after the administration of PGRN 0.1 µg, showing maximal amplitude (MA%) of 14%, while the response to arachidonic acid (AA)-induced aggregation is 27%.

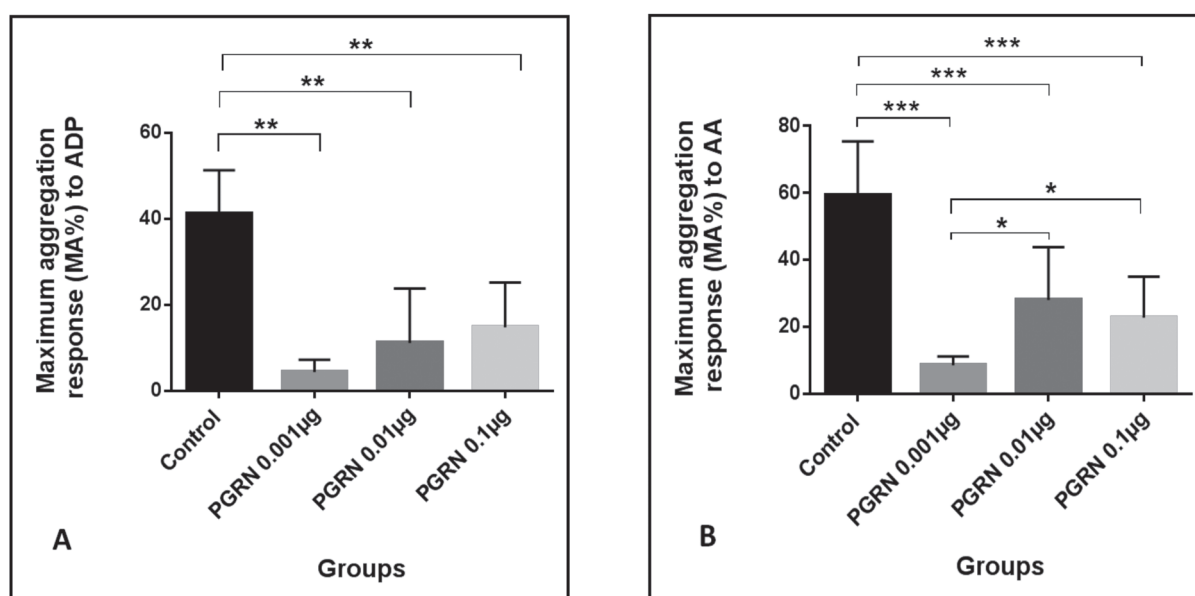


Figure 5. Platelet aggregation studies in rat platelet rich plasma (n=6 in each group) in response to **(A)** adenosine diphosphate (ADP) and to **(B)** arachidonic acid (AA) after the administration of different doses of progranulin (0.001 µg, 0.01 µg, and 0.1 µg), expressed as the mean±SD of the MA% of the aggregation responses, * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) designates statistical significance. PGRN: progranulin.

± 16.0% in the PGRN groups; $8.5 \pm 2.6\%$ for the 0.001 µg, $26.8 \pm 12.7\%$ for the 0.01 µg, and $23 \pm 10.9\%$ for the 0.1 µg group, respectively. Furthermore, PGRN 0.001 µg had a significantly stronger antiplatelet action when compared to the 0.01 µg ($p, 0.01$) and 0.1 µg ($p, 0.04$) doses. The responses to the 0.01 µg and 0.1 µg doses did not differ significantly ($p, 0.5$) in the inhibition of platelet aggregation in response to AA. These results taken together, revealed significant blocking action of PGRN on platelet activation pathways of both ADP and AA.

Effect of Progranulin Administration on The Bleeding Time, Prothrombin Time and the Activated Partial Thromboplastin Time

The BT was significantly prolonged in all animal groups that received PGRN, in comparison to the control group (Figure 6). The PGRN 0.1 µg dose, in particular, caused the most marked prolongation of the BT, 27.3 ± 4.8 min in comparison to the control group ($p, 0.001$) and to the other smaller doses, 0.001 µg dose ($p, 0.004$) and 0.01 µg dose ($p, 0.005$). However, the other two doses of PGRN; 0.001 µg, and 0.01 µg also caused significant prolongation of the BT, in comparison to the control group ($p, 0.001$ and $p < 0.001$, respectively). The PT in rats treated with different doses

of PGRN was significantly prolonged ($p < 0.001$) compared to the control group, as shown in Figure 7A. The PT of the control group (mean ± SD) was 13.3 ± 1.0 sec, while after the administration

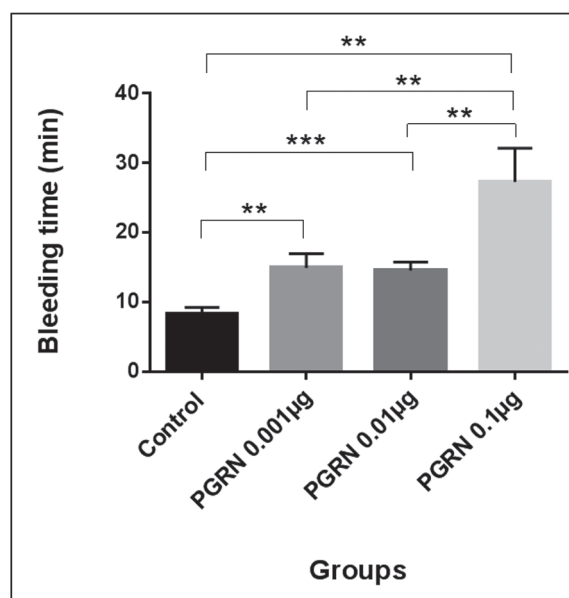


Figure 6. Bleeding time (min) in rats (n=6 in each group) after the administration of different doses of progranulin (0.001 µg, 0.01 µg, and 0.1 µg), expressed as the mean ± SD. **($p < 0.01$),***($p < 0.001$) designates statistical significance. PGRN: progranulin, min: minutes.

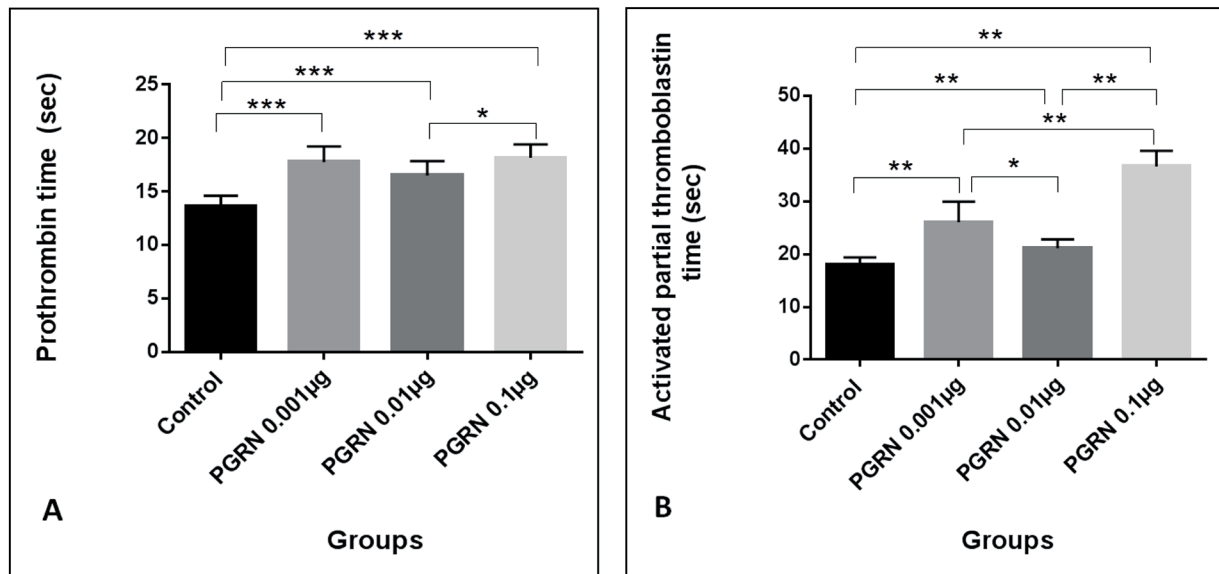


Figure 7. (A) Prothrombin and (B) activated partial thromboplastin time (sec) in rats (n=6 in each group), after the administration of different doses of progranulin (0.001 µg, 0.01 µg, and 0.1 µg), expressed as the mean ± SD. *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$) designates statistical significance. PGRN: progranulin, sec: seconds.

of PGRN 0.001 µg and 0.1 µg was 18.0 ± 1.4 sec, and 18.3 ± 0.9 sec respectively. The 0.01 µg dose also prolonged the PT to 16.6 ± 1.4 sec. Notably, there was a significant prolongation of the PT between the 0.01 µg and 0.1 µg doses ($p, 0.03$). On the other hand, the aPTT of all groups was significantly prolonged in comparison to the control ($p, 0.004$), (Figure 7B). Moreover, the differences between the other doses were significant; the 0.001 µg vs. 0.01 µg dose ($p, 0.01$), the 0.001 µg vs. 0.1 µg dose ($p, 0.004$), and the 0.01 µg vs. 0.1 µg dose ($p, 0.004$). These results are indicative of a potent anticoagulation action of PGRN.

Discussion

In the present study we reported two important new findings. First of all, we found *in vitro* evidence that the adipokine PGRN has a significant platelet inhibitory action based on its dose-related blocking of ADP, and AA-induced platelet aggregation. Secondly, PGRN has a coagulation inhibitory action, as it has caused a significant prolongation of the BT, PT, and aPTT. These findings are of important biological and medical interest. Platelet activation that follows vessel wall injury, results in platelet adhesion to the endothelium, secretion of the contents of platelet granules along with platelet shape changes, and platelet aggregation¹⁸. Several pathways lead

to platelet activation *in vivo*, including activation by collagen, adenosine diphosphate (ADP), thromboxane A₂, epinephrine, serotonin and thrombin¹⁹. ADP, which plays an important role in hemostasis and thrombosis, is secreted from the dense platelet granules. Other than activating platelets, ADP amplifies the *in vitro* platelet responses induced by other platelet agonists, such as thrombin and collagen. The amplifying effect of ADP on platelet activation and aggregation highlights the critical role played by ADP in hemostasis, and in the pathogenesis of arterial thrombosis²⁰. In this work we have shown that PGRN causes significant inhibition of platelet aggregation responses to both ADP and AA. It was reported previously that the platelet aggregation responses to ADP are reduced following incubation with the cardio-protective adipokine and adiponectin²¹, by attenuating oxidative/nitrative stress²². PGRN may exert its antiplatelet activity in a similar manner, which could represent its physiological action *in vivo*; however, this needs further exploration. It is now well established that platelet activation can be modified by interfering with the various pathways implicated in platelet activation and aggregation. For example, acetylsalicylic acid (aspirin) irreversibly inhibits the enzyme cyclo-oxygenase of the platelet prostaglandin activation pathway²³, thus decreasing the amplification of platelet activation induced by thromboxane A₂. The integrity

of this pathway is tested *in vitro* by the platelet aggregation response to AA, which in this study is blocked by PGRN. Similarly, the ADP receptor antagonist, clopidogrel, interferes with the binding of ADP to its receptor (P_2Y_{12}) and, thereby, prevents changes within the platelet that lead to aggregation, including the amplification processes that releases stored ADP²⁴. Both aspirin and clopidogrel are antiplatelet drugs commonly used in the primary and secondary prevention of cardiovascular and cerebrovascular disease^{25,26}. In the current research we have shown that the action of PGRN simulates to a great extent the action of these two antiplatelet drugs. The results obtained show that the inhibitory action of PGRN on the BT is also of interest. The bleeding time is a test of primary hemostasis, involving the vessel wall, and platelet function²⁷. In the present investigation we demonstrate, for the first time, that administration of PGRN into rats causes marked prolongation of the BT, further supporting the suggested anticoagulant/antithrombotic action of PGRN. The results of the coagulation tests, PT and aPTT are in line. Both tests are used to monitor the coagulation mechanism; PT tests the integrity of the extrinsic and final common pathways of the coagulation cascade, while the aPTT is a measure of the integrity of the intrinsic and final common pathways of the coagulation cascade²⁸. The administration of PGRN resulted in significant prolongation of both PT and aPTT, suggesting that PGRN inhibits both intrinsic and extrinsic pathways, and/or the final common pathway. The findings detailed above suggest an important physiological function of PGRN in obesity. Obesity and thrombotic events are closely related²⁹, due to dysfunction of the vascular wall, hyperactive platelets, increased coagulability and reduced fibrinolysis³⁰. Besides, platelets are recognized to have a major role in the genesis of atherosclerosis and its complications¹⁶, as well as being central to the formation of blood clots³¹. Additionally, some adipokines, including adiponectin, and several of the C1q/tumor necrosis factor-related proteins possess protective actions in the vasculature and heart³², specifically, antithrombotic activity^{21,22,33,34}. Earlier studies in atherosclerosis have also shown that, PGRN is highly expressed in vascular smooth muscle cells, and macrophages, where it reduces inflammation¹⁰. This anti-inflammatory action is attributed in part, to blocking of the binding of tumor necrosis factor- α to its receptors^{35,36}. In addition to its anti-inflammatory action, PGRN

that is secreted from macrophages binds to Apo lipoprotein A-I, and forms a complex which may play a role in stabilizing atherosclerotic plaques³⁷. We found that PGRN ameliorated acute myocardial ischemia reperfusion injury in the normal, as well as the hyperlipidemic rat model. In line with these observations, the current study findings of the inhibitory action of PGRN on both platelet aggregation and blood coagulation in rats gives strong support to an atheroprotective role of PGRN. It is worth pointing out that in the atherosclerotic process, acute ischemic events occur as a result of rupture of atherosclerotic plaques, and sudden setting of thrombosis³⁸. Due to its antiplatelet, and anticoagulant effect, PGRN could play an important protective role against acute coronary events by inhibiting thrombosis at the atherosclerotic site. Our findings strongly suggest that PGRN may yet be another anticoagulant to be added to the other well studied natural coagulation inhibitors; protein S, protein C, anti-thrombin and tissue factor pathway inhibitor^{39,40}. Therefore, PGRN, which is generated by adipose tissue, may provide protection against obesity-related thrombosis and atherosclerosis in general.

Conclusions

The present work has shown that PGRN significantly attenuated ADP, and AA-induced platelet aggregation, and caused prolongation of BT, PTT, and aPPT. Given the diverse function of platelets both hemostatic, and non-hemostatic, particularly their involvement in inflammation and ischemic events, the physiological impact of these findings strongly suggest that PGRN may contribute to the protection against cardiac, and related thrombotic disorders associated with obesity. We hope our findings will encourage further research into the molecular mechanism of action of PGRN and its potential as dual antiplatelet/anticoagulant agent, and to address how this activity is related to its possible antithrombotic and cardio-protective function.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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