ijlpr 2020; doi 10.22376/ijpbs/lpr.2021.11.2.L97-106

International Journal of Life science and Pharma Research

Research Article

Biochemistry for Better Diagnosis and Therapy

Effect of Acute Phase Protein, Alpha – 1 – Acid Glycoprotein (AGP-1), On Platelet-Activating Factor (PAF) – Induced Pro-Inflammatory Responses

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Abstract: Alpha-1-acid glycoprotein (AGP-1), an acute phase protein with ill defined functions, circulates in blood (sAGP-1) at basal levels, but levels can go up several fold upon inflammatory stimuli. Very recently, AGP-1 is shown to play a critical role in the inflammatory process by interacting with inflammatory molecules and/ or cells of the immune system. However, the interactions of sAGP-1 with endogenous inflammatory lipid mediators like Platelet-activating factor (PAF) are not well characterized. PAF is a potent autocoid with implications in several inflammatory disorders and is known to activate various cells of the innate immune system. To address this issue, we looked into the effect of sAGP-1 in PAF-induced sudden death models using Swiss Wistar (albino) mice established previously in our laboratory, to find sAGP-1 neither augmented nor inhibited the lethality of PAF *in vivo*. To dissect the mechanism behind this, we employed primary immune cell type namely, human neutrophils. We found that both sAGP-1 and PAF are potent activators of neutrophil adhesion and in fact, sAGP-1 even augmented PAF-induced neutrophil adhesion. Although sAGP-1 is a potent activator of neutrophils, for some responses such as PAF-induced NETosis, sAGP-1 was without any effect. These results shed light on the pro-inflammatory actions of sAGP-1 and its implications in some of the hyper-inflammatory disorders where involvement of PAF is also suspected.

Keywords: Alpha-1-acid glycoprotein; Platelet-Activating Factor; Neutrophil Activation; NETosis; Murine model

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Recieved On 22 September 2020
Revised On 22 October 2020
Accepted On 30 October 2020
Published On 03 December 2020

Funding This work is supported by University Grants Commission, India [BSRF] Grant no. F4-1/2006(BSR)/7-366/2012(BSR).

Citation Mosale Seetharam Sumanth and Gopal K. Marathe , Effect of Acute Phase Protein, Alpha – 1 – Acid Glycoprotein (AGP-1), On Platelet-Activating Factor (PAF) – Induced Pro-Inflammatory Responses..(2020).Int. J. Life Sci. Pharma Res.10(5), L97-106 http://dx.doi.org/10.22376/ijpbs/lpr.2021.11.2.L97-106

1. INTRODUCTION

Inflammatory process involves an intricate interplay between various immune cells, soluble factors and their specific receptors[1]. The soluble factors include acute phase proteins, chemokines, cytokines and lipid mediators[1,2]. One of the major acute phase proteins and lipid mediators are alpha – 1 – acid glycoprotein (AGP-1) and platelet-activating factor (PAF) respectively[3,4]. AGP-1 is often considered as one of the inflammatory biomarkers and its functions are not very well understood[3,5,6]. Although hepatocytes are the primary site of synthesis of AGP-1, extra hepatic cells like neutrophils, epithelial cells, monocytes and lymphocytes are known to secrete AGP-1[6,5,7]. However, AGP-1 demonstrates opposing effects on the isolated cells of the innate immune system, although its receptor is yet to be defined[8,7]. Hence we need to understand how AGP-1 modulates the effects of PAF, for which receptor is well characterized on neutrophils. PAF is chemically defined as 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. PAF is a key pro-inflammatory lipid mediator, present in low levels in resting cells but its synthesis by innate immune cells and vascular cells increases upon appropriate stimulus[4,9]. PAF exerts its action via PAF-receptor (PAF-R), a typical GPCR[10]. PAF is known to activate various cells of the innate immune system including neutrophils and platelets[4]. PAF induces neutrophil adhesion mediated by activated β2 integrins. Additionally, dysregulation of PAF synthesis and degradation is known to be involved in various other inflammatory disorders including asthma, sepsis and many more[4,9]. Previous studies have suggested that AGP-1, in the absence of albumin, binds PAF and prevents activation of granulocytes[11,12]. In contrast, Libert C et. al. have shown that the AGP-1 fails to inhibit PAF-mediated lethality in a murine model[13]. Besides, we have previously demonstrated that hepatocyte derived serum AGP-1 (sAGP-1) shows preferential augmentation of TLR-2 – mediated mortality, while TLR-4 – induced mortality was delayed in murine model of endotoxemia[8]. Neutrophils when activated/ stimulated adhere to the endothelium, migrate to the site of inflammation and either undergo degranulation or NETosis (Neutrophil Extracellular Traps)[14,15]. We have previously shown that sAGP-1 can induce pro-inflammatory responses like neutrophil adhesion, migration and NETosis by itself[7]. We now show that the sAGP-1 and PAF act independently and for some responses sAGP-1 even augments PAF-mediated responses both *in vivo* and *in vitro*. Our results suggested the possible role played by sAGP-1 together with PAF in various inflammatory disorders.

2. MATERIALS AND METHODS

2.1 Reagents

Phospho-specific antibodies to p38, JNK and ERK, β-actin and anti-rabbit IgG-HRP were procured from Cell Signaling Technology, Danvers, MA, USA. Complete Mini EDTA-free protease inhibitor cocktail tablets were obtained from Roche Diagnostics, Mannheim, Germany. PVDF membrane was from BioRad Laboratories, Hercules, California, USA. Cibacron Blue F3GA, DEAE-Cellulose and anti-human AGP-1 IgG were procured from Sigma Aldrich, St. Louis. MI, USA. Calcein 2 AM, DAPI and Sytox orange were procured from Invitrogen, Carlsbad, CA, USA. Micrococcal DNase was from Sigma Aldrich, St. Louis, MI, USA. Platelet-Activating Factor (PAF) was from Avanti polar lipids, Alabaster, AL, USA.

2.2 Animals

Swiss Wistar (albino) mice, 8-10 weeks old (both male and female), weighing 20-25g were obtained from Central Animal Facility, University of Mysore, and all the experiments were approved by Institutional Animal Ethics Committee, University of Mysore, Mysore, India (Approval No: UOM/IAEC/12/2016). The animals were maintained with adequate ventilation, food, and water (available *ad libitum*) and were monitored daily over the experimental period.

2.3 Serum collection

Permission to draw blood from human volunteers was also obtained from the Institutional Human Ethics Committee (UOM No. 104 Ph.D/2015-16). Blood was drawn from apparently healthy human volunteers after obtaining informed consent. Briefly, the blood was collected and allowed to coagulate. The coagulated blood was then centrifuged at 600 x g for 20 min at 25□C to collect serum and stored at -20□C until further use[8].

2.4 Isolation and purification of AGP-1 from human serum

AGP-1 from pooled human serum was isolated and purified using conventional chromatographic techniques (Cibacron blue-pseudo-affinity column and DEAE-cellulose anion exchange column) as described previously[8]. The purity of the isolated sAGP-1 was checked by SDS-PAGE, immunoblotting for AGP-1 and the endotoxin content was tested as described in our previous study [8].

2.5 Effect of AGP-1 on PAF-induced sudden death in Swiss Wistar (albino) mice

PAF-induced sudden death was induced in Swiss Wistar (albino) mice by administering a lethal dose of PAF (5µg/ mouse)[16]. In some experiments, AGP-1 (5, 10 and 25 mg/Kg) was administered before injecting a lethal dose of PAF (5µg/ mouse) to Swiss Wistar (albino) mice intraperitoneally. Survival and clinical signs were monitored for 24 hours. All animals were euthanized 24 hours post-treatment with PAF.

2.6 Neutrophil Adhesion Assay

Neutrophils were isolated freshly from healthy human volunteers with informed consent. Permission to draw blood from human volunteers was also obtained from the Institutional Human Ethics Committee (UOM No. 104 Ph.D/2015-16). The neutrophils were isolated by dextran sedimentation and separation over ficoll density gradient centrifugation[17]. Neutrophil-rich pellet obtained was suspended in 1ml of Hank's-Balanced salt solution (HBSS) containing 0.2% human serum albumin (HBSS/A). For assessment of adhesion, the neutrophil suspension was loaded with calcein-AM to a final concentration of 1 µM prior to incubation for a period of 45 min at 37 ^{0}C. The labeled neutrophils ($1x10^6$ cells/ml) were incubated with PAF (10^{-7} M) with or without AGP-1 (10, 25, 50, and 100 µg/ml) in triplicate wells in twelve well cell culture plates (Nest Biotechnology Co. Ltd., China) pre-coated with 0.2% gelatin. The adherent neutrophils were visualized and photographed at a magnification of 10x under a fluorescent microscope[18]. The quantification of adhesion assay was performed by counting the cells adhered in 10 randomly chosen fields using

ImageJ software and then calculating the average number of cells adhered per field.

2.7 *Neutrophil Extracellular Traps (NETosis)*

1 million freshly isolated human neutrophils were treated with PAF (10^{-7} M) and/ or then with sAGP-1 (25, 50 and 100 µg/ml) before being transferred to poly-L-Lysine coated coverslips. The neutrophils were incubated at 37 °C in 5% CO_2 for 1 hour before adding DAPI (cell-permeable) and Sytox orange (cell-impermeable) fluorescent dye mixture and visualized by fluorescent microscopy (EVOS Fluorescence microscope, Thermo Scientific, USA)[7]. The two dye images were merged using ImageJ software. High-throughput NET quantification[19] was employed to quantify neutrophil NETs. Neutrophils were added to 24 well plates pre-coated with poly-L-lysine followed by stimulation for 1 hour by defined agonists at 37 °C under 5% CO_2. The extracellular traps were recovered by treating the neutrophils with Micrococcal DNase and the DNA stained with cell-impermeable Sytox Green dye. These were measured with a fluorescent plate-reader with excitation at 485 nm and emission at 530 nm.

2.8 *MAPK Signaling*

Freshly isolated human neutrophils (1 x 10^6 cells/ml) were stimulated for 30 min with sAGP-1 (25, 50, and 100 µg/ml). PAF (10^{-7} M) served as positive control[7]. Unstimulated neutrophils served as negative control. Lysates were prepared using Radio-Immunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail and immunoblots were developed using specific primary and secondary antibodies for phospho-p38, phospho-JNK, phospho-ERK and β-actin (1:1000 v/v).

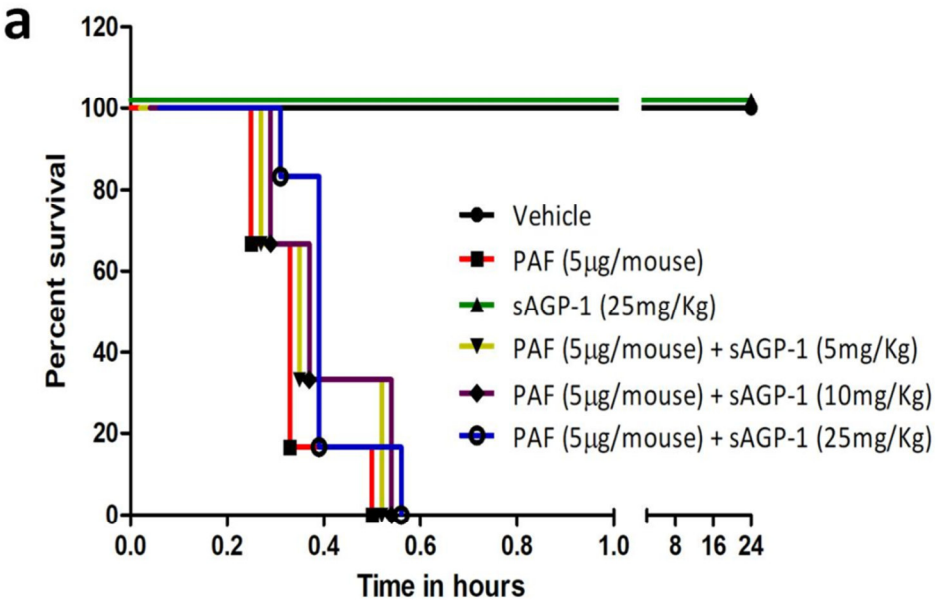
3. STATISTICAL ANALYSIS

The animal experiments are representative of more than 2 independent experiments and the statistical significance among groups was determined by the log-rank test. Unpaired two-tailed t-test was used to compare the mean for each treatment group with the mean of the control group. All other results were analyzed using one-way analysis of variance (ANOVA) where applicable. All statistical analyses were performed using GraphPad Prism 5.0 software.

4. RESULTS

4.1 *sAGP-1 does not inhibit PAF-induced mortality in Swiss Wistar (albino) mice*

Previously in our laboratory, we had established a PAF-induced lethality murine model[16]. Since, sAGP-1 showed differential effects on the immune-receptors TLR-2 and TLR-4[8], we were intrigued to check the effect of sAGP-1 on PAF-receptor mediated PAF-induced lethality in our murine model. The sAGP-1 was isolated and purified in our laboratory from healthy human volunteers as described previously[8]. Animals were injected with a lethal dose of PAF (5µg/mouse) with or without sAGP-1 (5 – 25 mg/Kg) (Fig 1). 100% of the animals that received PAF, died within 15-30 min. We found no change in the mortality rate with sAGP-1 on board as 100% mortality was seen in all the groups that received PAF along with various concentrations of sAGP-1. Previously, although, sAGP-1 showed inhibition of TLR-4 and augmentation of TLR-2 mediated mortality, here sAGP-1 had neither inhibited nor augmented PAF-induced lethality. Although we do not know the specific receptor of sAGP-1, yet, our data suggests AGP-1 circulating in serum has limited ability to protect mice from PAF-induced lethality. Ideally one should use mouse AGP-1 rather than human AGP-1 for these experiments.



b

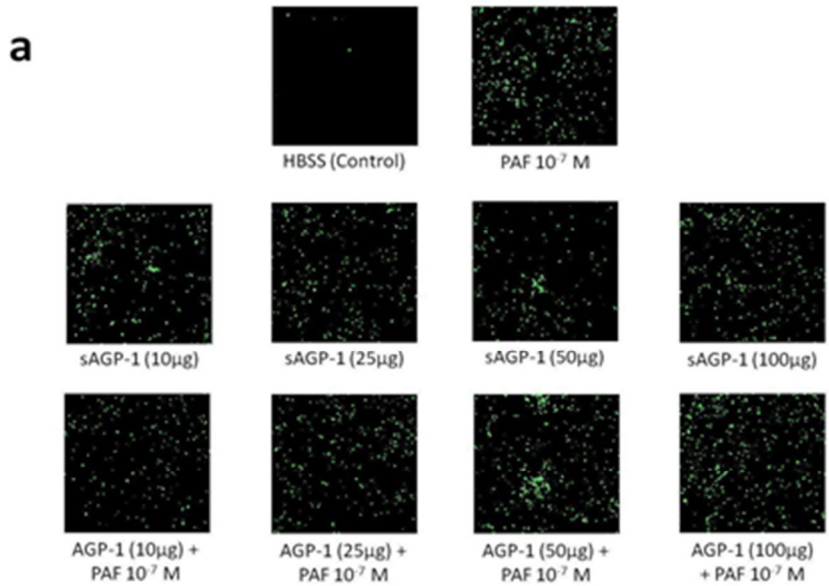
Groups	Treatment	Percent survival
Group I	Vehicle	100
Group II	PAF (5µg/mouse)	0
Group III	sAGP-1 (25mg/Kg)	100
Group IV	PAF (5µg/mouse) + sAGP-1 (5mg/Kg)	0
Group V	PAF (5µg/mouse) + sAGP-1 (10mg/Kg)	0
Group VI	PAF (5µg/mouse) + sAGP-1 (25mg/Kg)	0

Swiss Wistar (albino) mice were divided into 6 groups containing six animals each and the mice were injected intraperitoneally with the stated concentrations of PAF (5µg/mouse) in the presence/absence of sAGP-1 (5, 10 and 25 mg/Kg body weight) in a total volume of 500 µl. The survival time was monitored for 24 hours. (a) The graph represents the percentage of survival with respect to time of the groups of mice receiving PAF with or without sAGP-1. (b) The table indicates the number of groups, the respective treatment that each group received and the percent survival. We did not observe significant change between the groups of mice receiving either PAF alone or PAF with sAGP-1 as determined by log-rank test.

Fig 1: Exogenous sAGP-1 does not inhibit PAF-induced mortality in Swiss Wistar (albino) mice

4.2 sAGP-1 augments PAF-induced neutrophil adhesion

PAF is a potent inducer of intracellular Ca^{2+}, thereby activating neutrophils[20]. AGP-1 is known to have many immunomodulatory effects on various cells of the immune system including neutrophils. To determine whether sAGP-1 has any effect on PAF-stimulated neutrophils, freshly isolated human neutrophils were stimulated with PAF with or without various concentrations of sAGP-1 (10–100 µg/ml). Although, we found that both PAF and sAGP-1 were agonists for neutrophil adhesion dose dependently, the combination of PAF and sAGP-1 augmented the adhesive responses of neutrophils to the gelatin-coated glass surface (Fig 2). Furthermore, the highest dose of sAGP-1 (100µg/ml) was as potent as the optimal dose of PAF (10^{-7} M) in activating neutrophil adhesion (Fig 2).



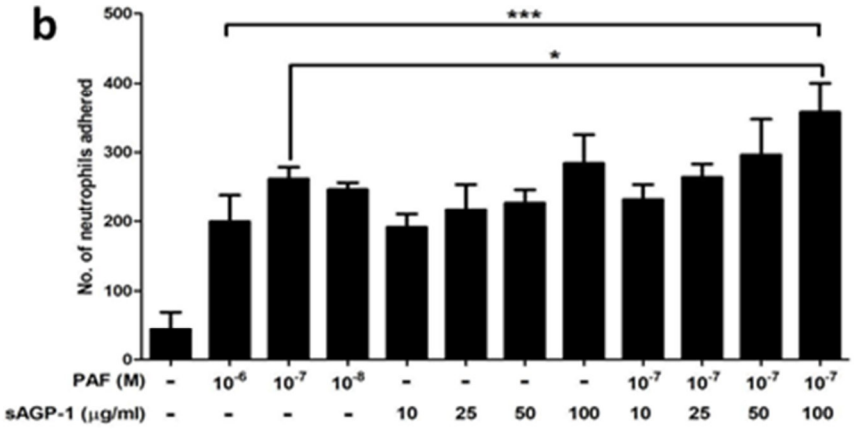
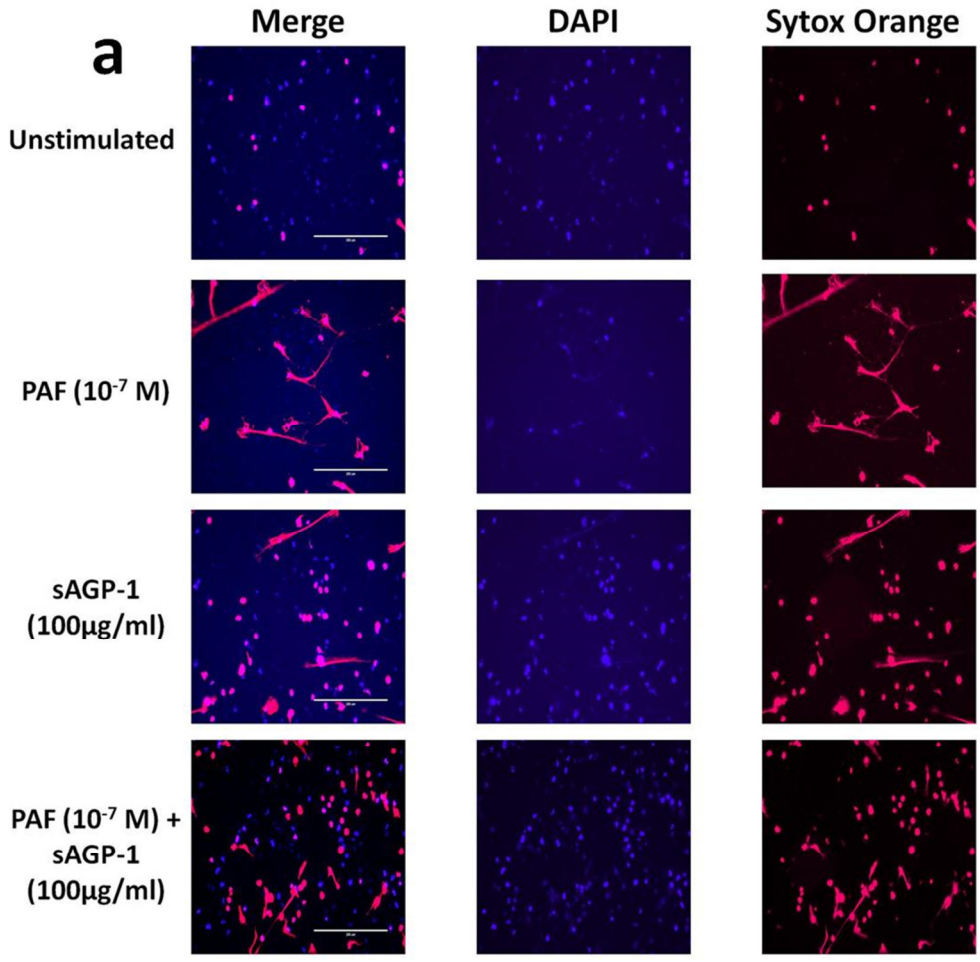


Fig 2: sAGP-1 augments PAF-induced neutrophils adhesion

(a)Neutrophils labelled with calcein-AM in HBSS/A were treated respectively with vehicle, PAF (10^{-7} M) in the presence/absence of AGP-1 (10 – 100 µg/ml). The reaction mixture was incubated for 60 min at 37 °C. The adhered neutrophils were visualized under a fluorescent microscope at a magnification of 40X. Although AGP-1 alone stimulated PMN adhesion dose-dependently, it augmented PAF-induced β2-integrin-mediated adhesion of neutrophils to the gelatinous substrate. (b)Neutrophil adhesion was quantified by counting the cells per field using ImageJ software as explained under "Methods". ***p < 0.0001 and *p < 0.01 as determined by ANOVA.

4.3 sAGP-1 does not suppress PAF-induced NETosis

We determined whether sAGP-1, which is an agonist for neutrophil activation, can also augment PAF-induced NETosis (Fig 3). In fact, in accordance to our previous result, sAGP-1 by itself induced NETosis[7]. Although sAGP-1 induces NETosis, we found that there was no significant augmentation of PAF-induced NETosis unlike neutrophil adhesion at all the concentrations tested (Fig 3), suggesting that sAGP-1's inability to suppress or augment PAF-induced NETosis.



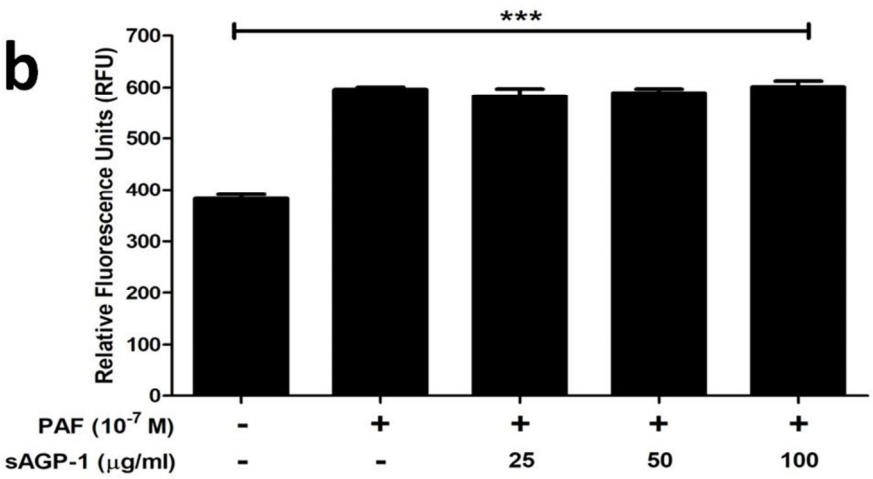
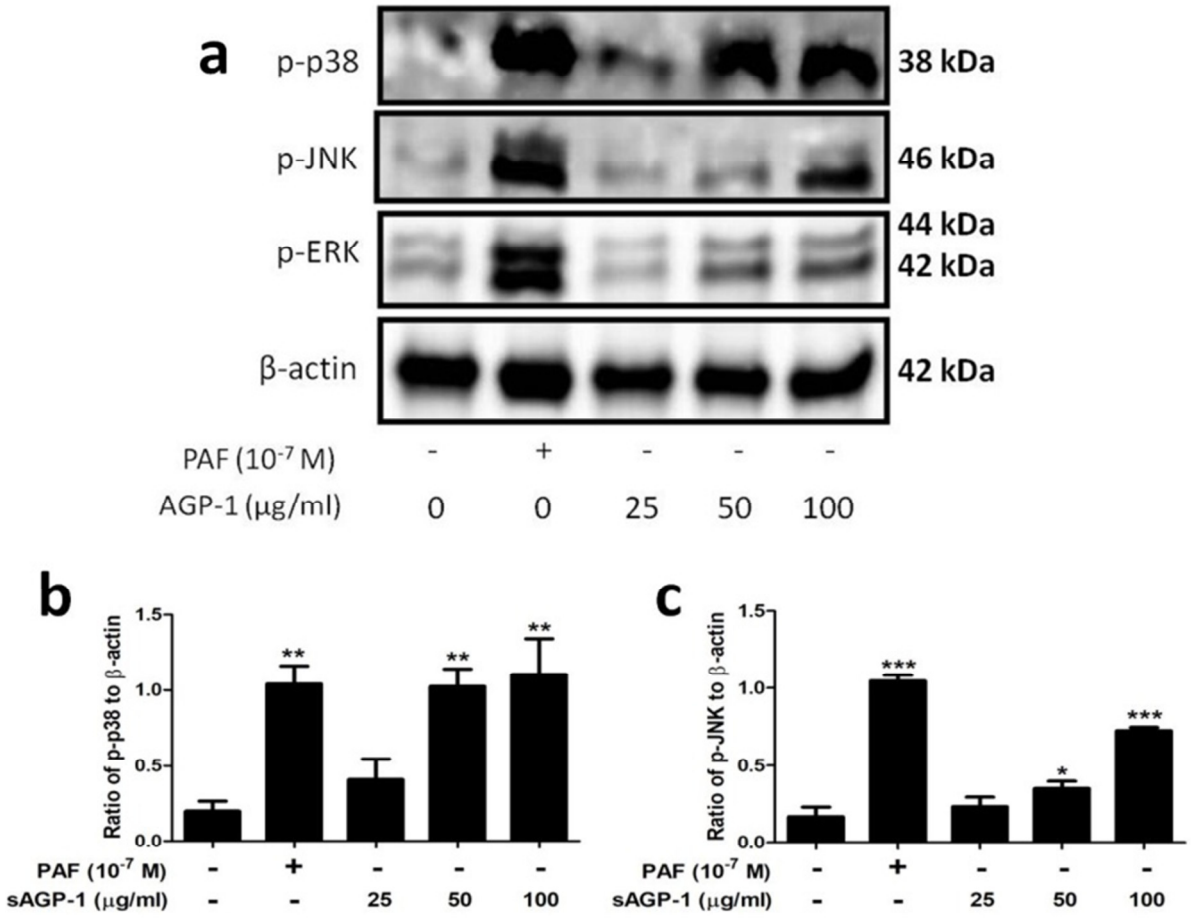


Fig 3: sAGP-1 does not suppress PAF-induced NETosis

(a)NET formation: Isolated human neutrophils adhering to poly-L-Lysine coated slides were incubated with media alone, or with PAF with or without sAGP-1 for 1 hour and were then stained with cell-permeable, DAPI, and/ or with cell-impermeable Sytox Orange fluorescent dyes. NETs were assessed by live cell imaging using fluorescence microscope at 20x magnification (Scale: 200µm). (b)Concentration-response relationships: NETs were quantified using high-throughput methods as explained in the "Methods" section. Neutrophils were stimulated with PAF (10^{-7} M) in the presence and absence of sAGP-1(25, 50 and 100 µg/ml) 1 hour before quantifying NETs by fluorimetry. The data shown are mean ± SEM (n = 3). ***=P <0.0001.

4.4 sAGP-1 activates p38, JNK and ERK MAP kinases

We showed that sAGP-1 activates neutrophils to adhere and induce NETosis. To understand the molecular mechanism behind the sAGP-1 – induced pro-inflammatory responses, we checked the activation of the inflammatory signaling cascade, the MAPK pathway. Although not to the extent of PAF, sAGP-1 dose dependently induced phosphorylation of p38, JNK and ERK MAP kinases (Fig 4).



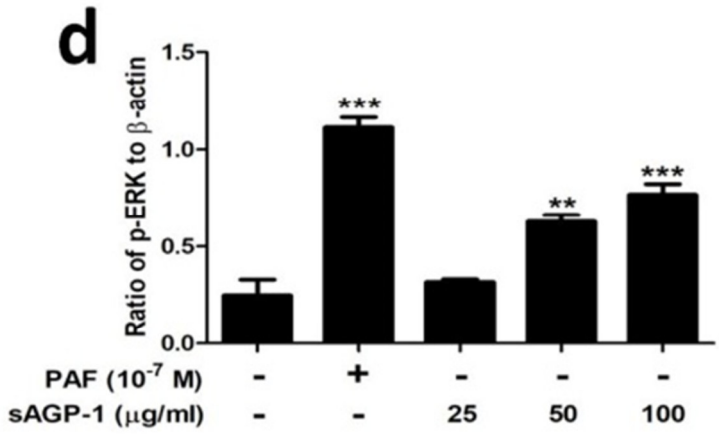


Fig 4: sAGP-1 induces activation of MAPK pathway similar to PAF

(a) sAGP-1 induces activation of phosphorylation of p38, JNK and ERK MAP kinases in human neutrophils. Human neutrophil lysates from the stated amounts sAGP-1 (25 – 100 μg/ml) treated cells were prepared using RIPA buffers and immunoblots were developed using specific primary and appropriate secondary antibodies (1:1000 v/v). PAF stimulated cells served as positive control. (b-d) Densitometric analyses of the blots. Densitometry was done using Image J software. The blots are representative of 3 different blots. The data shown are mean ±SEM ***p < 0.001; **p < 0.01 and *p < 0.05 when compared with negative control.

5. DISCUSSION

Inflammation is a defensive response against microorganisms, harmful stimuli, damaged cells, or irritants[21]. One group of proteins marking this process are acute phase proteins[2,22] like AGP-1, which is often considered as the marker of inflammation[3,23]. Although AGP-1 levels are elevated during inflammation, its biological function(s) remains not well understood. We isolated and purified the AGP-1 from human serum using the conventional chromatographic techniques in our laboratory, as described in our previous studies[8,7]. The AGP-1 purified from human serum is thus named as serum AGP-1 (sAGP-1). This sAGP-1 is a highly sialylated 43 kDa glycoprotein[8,7]. Using this sAGP-1, we sought to determine its effect on PAF-mediated inflammatory responses in neutrophils. AGP-1 or acute phase proteins are one of the integral parts of the inflammatory responses, whereas, PAF, a potent autocouid, is predominantly formed during inflammatory conditions[4,9]. PAF in turn leads to the upregulation of the synthesis of acute phase proteins which may or may not neutralize the pro-inflammatory effects of PAF[24,25]. In our previous studies we have shown that the sAGP-1, which is primarily produced in the hepatocytes, upon proper inflammatory stimulus can also be synthesised by the neutrophils (nAGP-1) and these two glycoforms have very opposing roles in an inflammatory milieu[7,8]. However, the effect of AGP-1 on PAF–induced inflammatory responses is not well understood. Hence, we focused our efforts on the effect of sAGP-1 on PAF-induced responses in neutrophils, the first responder of the immune system. In order to verify the effect of sAGP-1 on PAF-induced pro-inflammatory responses *in vivo*, we employed Swiss Wistar (albino) mice model of PAF-induced sudden death. PAF-induced sudden death model was established in our laboratory previously[16]. 100% mortality of Swiss Wistar (albino) mice was observed within 15-30 mins of administration of lethal dose of PAF (5μg/mouse)[16]. Although, sAGP-1 showed protection against TLR-4 agonist-induced lethality[8], sAGP-1 failed to inhibit/ protect PAF-induced lethality in Swiss Wistar (albino) mice. This data is in agreement with the study of Libert C et. al.[13]. In addition, the result suggests that sAGP-1 may have different effects on different families of receptors and their down-stream signaling. To understand the mechanism behind the inability of sAGP-1 to either inhibit or augment PAF-induced lethality *in vivo* as well as the translatability, we employed primary human neutrophils. Neutrophils are free flowing cells under normal circumstances; however, when stimulated by pro-inflammatory stimuli, they adhere to the endothelial layers with the help of cell adhesion molecules like β2-integrins[14,15,26]. It is well known that PAF is a potent pro-inflammatory stimulus that induces an increase in intracellular Ca^{2+} in neutrophils, an important event in the signaling cascade of neutrophil activation[20,27]. Here, we found that sAGP-1, by itself, is an agonist for neutrophils activation. This result is in accordance with our previous studies[8,7]. However, sAGP-1 was found to augment the effect of PAF on neutrophils adhesion, suggesting a pro-inflammatory role for sAGP-1, this might contribute to dysregulated inflammatory responses. Since, sAGP-1 induces intracellular Ca^{2+} mobilization, we hypothesized that sAGP-1 may interfere with PAF signaling. Neutrophils, once activated migrate to the site of infection/ inflammation and either degranulate to secrete proteins and lipids to neutralize inflammation or undergo a process called NETosis. NETosis is a process where neutrophils extrude their DNA along with antimicrobial proteins that form a net-like structure[28]. PAF with various other stimuli is known to induce NETosis[29]. We have recently for the first time shown that sAGP-1 is also capable of inducing NETosis[7]. Since sAGP-1, by itself, shows pro-inflammatory activity on neutrophils, sAGP-1 had no effect on PAF-induced NETosis. We next focused on determining the mechanism behind the pro-inflammatory effects of sAGP-1 on neutrophils. Previous studies have shown that AGP-1 inhibits neutrophil activation and migration via nitric oxide-dependent pathway[30,31]. However, only a few studies have reported that AGP-1 can induce Ca^{2+} spiking in the neutrophils[32,8,7]. We found that sAGP-1 activates the MAPK pathway similar to that of PAF[33], thereby activating neutrophils and hence amplifying the PAF-induced inflammatory responses. Put together, our results suggest the possible mechanism for the pro-inflammatory effect of sAGP-1 and the possible reason behind the augmentation of inflammatory responses induced by PAF. In an earlier study, Gunnarsson P et. al. have shown that the AGP-1-induces Ca^{2+} spike in neutrophils via a family of sialic acid binding immunoglobulin-like lectins (Siglecs) receptor, Siglec 5[32].

However, the action of Siglec 5 receptor signaling is still debated as it displays both activating as well as inhibitory inflammatory signaling in neutrophils[32,34-36]. Moreover, in our previous study, we have shown that sAGP-1 increases intracellular cAMP levels in platelets[7]. Platelets are known to express Siglec 7 and not Siglec 5[37]. Furthermore, there are very little to no evidence that Siglec 5 is involved in upregulation of cAMP levels. Hence, these findings point to the fact that sAGP-1 might act via its own specific cell surface receptor which may or may not be Siglec 5 or that it has different receptors on different cells (Fig 5).

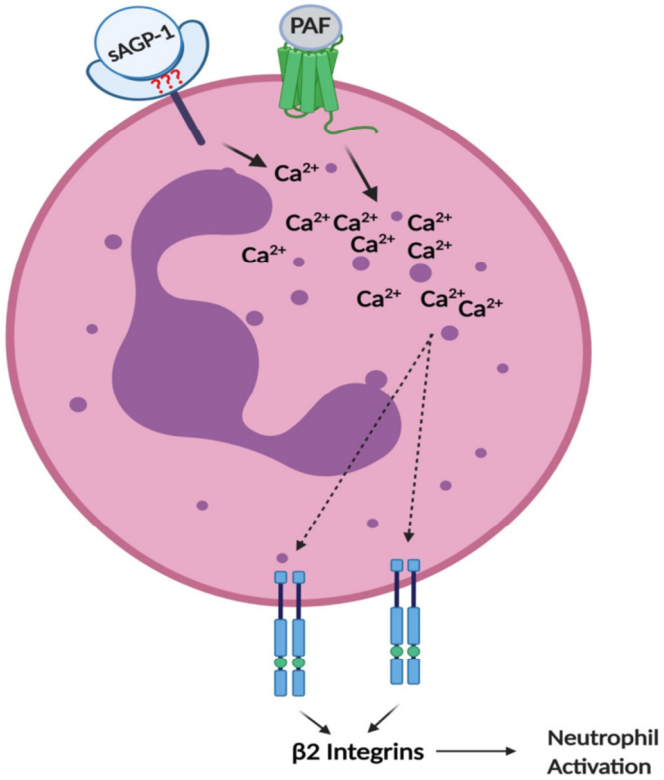


Fig 5: Probable mechanism of action sAGP-1- induced neutrophils activation

sAGP-1, like PAF, may induce intracellular Ca^{2+} mobilization. This event in turn increases the expression of β2-integris, thereby augmenting the PAF-induced neutrophils activation. We hypothesize that sAGP-1 mediates its action by a specific cell surface receptor. The illustration was created with BioRender.com Adding to the complexity, AGP-1 is present in multiple glycoforms in the human plasma, where some glycoforms have reduced sialic acid as their terminal sugar [38,3,7]. We have also shown in our previous study that the different glycoforms of AGP-1 have altered actions on cells of immune system[7]. Overall, all the disparities among the studies on AGP-1 and its receptor contributes to the poor understanding of this glycoprotein. Given the diversity of glycoforms of AGP-1, it might be worth exploring if glycoforms of AGP-1 mediate their effects via a specific yet common cell surface receptor or if each of the glycoforms has its own specific receptors. Hence, there is a need to fill this knowledge gap in the AGP-1 biology for better understanding of this glycoprotein in an inflammatory milieu.

6. CONCLUSION

In conclusion, our study demonstrates that sAGP-1 acts in unison with PAF in stimulating neutrophils and in fact, sAGP-1 augments the PAF-mediated effects. These findings also suggest that sAGP-1 is very likely a pro-inflammatory molecule that can activate neutrophils. In addition, sAGP-1 might be mediating its action via a specific receptor and identifying this receptor as well as studying different glycoforms of AGP-1 and their possible receptor(s) will shed some more light on the role of this enigmatic glycoprotein in the inflammatory processes.

7. FUNDING ACKNOWLEDGEMENT

The authors thank University Grants Commission (UGC), India [for Basic Science Research fellowship – F4-1/2006(BSR)/7-366/2012(BSR) to MSS and Vision Group of Science & Technology (VGST), Government of Karnataka, India. Authors also thank Dr. Christian Yost, Dr. Matthew Rondina and Prof. Andrew Weyrich, University of Utah, Salt Lake City, USA for their valuable inputs.

8. AUTHORS CONTRIBUTION STATEMENT

Gopal K Marathe envisaged the project. Sumanth Mosale Seetharam performed all the experiments in the manuscript. Both Gopal K Marathe and Sumanth Mosale Seetharam wrote and edited the manuscript.

9. CONFLICTS OF INTEREST

Conflict of interest declared none.

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