Published in final edited form as:

Curr Pharm Des. 2012 ; 18(22): 3255–3259.

Therapeutic Options for Transfusion Related Acute Lung Injury; the Potential of the G2A Receptor

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Abstract

Priming of polymorphonuclear leukocytes (PMNs) enhances their adhesion to endothelium, the release of their granule content and their production of reactive oxygen species. These effects are etiological in transfusion related acute lung injury (TRALI) and many clinically important mediators of TRALI prime PMNs. A priming activity that develops over time in stored blood products has been shown to be due to the accumulation of lysophosphatidylcholines (lyso-PCs) and has been found to be related clinically to TRALI. Lyso-PCs prime PMNs activating the G2A receptor and several inhibitors of this receptor, which could potentially be therapeutic in TRALI, have been identified. Recent work has described early steps in the signaling from the G2A receptor which has revealed potential targets for novel antagonists of lyso-PC mediated priming via G2A. Additionally, characterization of the process by which lyso-PCs are generated in stored blood products could allow development of inhibitors and additive solutions to block their formation in the first place.

Keywords

Transfusion-related acute lung injury; lysophospholipid; lysophosphatidylcholine; G2A receptor; G-protein coupled receptor; clathrin-mediated endocytosis

INTRODUCTION

Neutrophils/polymorphonuclear cells (PMNs) are the first line of defense against microbial pathogens and their normal role involves release of cytotoxic, microbicidal molecules. These include superoxide formed by the NADPH oxidase, other ROS formed from superoxide, and the cytotoxic contents of secretory granules. In pathological states, these molecules have the capacity to damage host tissues [1–5]. In transfusion related acute lung injury (TRALI) the ROS and granule content are inappropriately released and damage the pulmonary endothelium. In the two event model of TRALI [6; 7] the first event involves the activation of the pulmonary endothelium resulting in its production of chemokines (IL-8, GRO α and ENA-78) which promote increased expression of adhesion molecules (primarily intracellular adhesion molecule-1) on endothelial cells and cause β_2 -integrins on PMNs to undergo conformational changes; subsequent binding of the adhesion molecules to the β_2 -integrins results in firm adherence of PMNs to the pulmonary endothelium [6–8]. The second event of

the two event model involves infusion of blood products containing biologic response modifiers (BRM) that cause the adherent PMNs to release their complement of cytotoxic molecules and trigger endothelial damage, capillary leak, and acute lung injury (ALI) [6; 7; 9].

Neutrophils are characterized by three functional states, resting, primed and activated [10; 11] which are relevant to the mechanism of TRALI. Active neutrophils release ROS and granule content. Priming, which is reversible, does not cause the release of the microbicidal arsenal but instead involves the changes, discussed above, which cause PMNs to become firmly adherent. Priming also causes PMNs to become functionally hyper-reactive i.e. they are activated by molecules that do not activate resting neutrophils [10; 11]. Priming agents are of significance in TRALI because many clinically relevant mediators of TRALI, e.g. endotoxin, chemokines, leukotrienes and other 5-lipoxygenase products prime PMNs [12–15]. Lysophospholipids have also been implicated in TRALI; these molecules accumulate in stored blood products, signal through the G2A receptor, and are the focus of this review.

LYSOPHOSPHOLIPIDS ACCUMULATE IN STORED BLOOD PRODUCTS AND PRIME THE NEUTROPHIL NADPH OXIDASE

When plasma from packed red blood cells (PRBCs), whole blood (WB) and platelet concentrates (PC) at outdate (the last day on which they may be transfused; 42, 35 and 5 days respectively) was applied to isolated PMNs (at 10% plasma, volume: volume), the cells produced superoxide 2–3 times more rapidly than buffer treated controls when exposed to formyl-Met-Leu-Phe (fMLP), a soluble activator of the NADPH oxidase [16]. Fresh plasma or stored plasma that was frozen when fresh did not exhibit priming activity suggesting that the priming activity was generated during the storage of cellular blood components. The observed priming was shown to be inhibited by WEB2170, a member of a family of heterazepine compounds which inhibit platelet activating factor (PAF) priming of PMNs via antagonism of the PAF receptor [17]. However, in a subsequent study [18], GC/MS of chloroform extractable (lipid) material from the plasma of WB and PRBCs at outdate demonstrated that there was no detectable PAF. The nature of the priming activity was eventually revealed [19] when chloroform extracts from the plasma of WB and PRBCs at outdate (which caused WEB2170 inhibited priming of PMNs) were separated by normal-phase HPLC; two peaks of priming activity were observed, an early peak at the retention time of neutral lipids and a later one at the retention time of lysophosphatidylcholines (lyso-PCs). Fast atom bombardment mass spectroscopy of the material in the later peak identified several species, C₁₆ lyso-PAF, C₁₈-lyso PAF, palmitoyl lyso-PC (16: 0/OH), oleyl lyso-PC (18: 1/OH) and steroyl lyso-PC (18: 0/OH). Quantitation by GC/MS demonstrated increases in the concentrations of all these species during PRBC storage and defined the ratios and absolute concentrations of these species at the time of PBRC outdate. A mixture of commercially available versions of these molecules in the defined ratio resulted in WEB2170 inhibited priming when applied to PMNs at 10% of the concentration found in the PRBCs: This concentration could plausibly be reached in a transfused patient who received two units of PRBCs, the standard PRBC transfusion administered to adult patients [19]. Later work confirmed that the mixture of commercial molecules was not contaminated with PAF since pretreatment with two different PLA₂ enzymes did not alter the ability of the mixture to prime PMNs but did reduce priming by PAF [20]. Similar to the results obtained with WB and PRBCs, chloroform extracts from apheresis and whole blood platelet (WB-PLT) plasma was also found to prime PMNs at outdate but not the start of storage [21], WEB 2170 inhibited the priming and separation by normal-phase HPLC resulted in a peak of priming activity at a retention time characteristic of lyso-PCs (in this case a neutral lipid peak was not noted); GC/MS identified palmitoyl lyso-PC and C₁₆ lyso-PAF in the peak.

Although a range of techniques are used in banking of PCs, a recent study [22] of the effect of different isolation, storage and treatment conditions indicated that priming activity developed under all conditions examined and is thus likely to be a widespread and general phenomenon.

Lastly, a recent study [23] has demonstrated that prestorage leukoreduction of PRBCs prevents the accumulation of lyso-PCs due to the removal of ~3 logs of contaminating platelets by the leukoreduction filters (this is in addition to the >3 logs of contaminating leukocytes removed). Since apheresis platelets (which are leukoreduced) produce large amounts of lyso-PCs [21], the recent data [23] suggests that platelet removal during leukoreduction (rather than leukocyte removal) blunts lyso-PC formation and that the lyso-PCs which accumulate in stored blood products, even WB and PRBCs, are actually platelet-derived. The pre-storage leukoreduction of PRBCs decreased but did not abrogate the plasma priming activity that appeared during storage [23]. Additionally, priming assays using normal-phase HPLC fractions of chloroform extracted material from the leukoreduced PRBCs revealed priming activity at the retention time of neutral lipids. Thus, while lyso-PC priming activity appears to be platelet derived, neutral lipid priming activity appears to be red cell derived and is not reduced by leukoreduction of PRBCs. The neutral lipids isolated by normal-phase HPLC were identified as 4 moieties by GC/MS/MS: arachidonic acid, and 5-, 12- and 15- hydroxyeicosotetraenoic acids (HETE) [23].

CONTRADICTIONARY DATA

A recent study conducted in the Netherlands by Vlaar *et al* [24] indicated that storage of red blood cells (RBC) in either saline-adenine-glucose-mannitol (SAGM) or plasma did not result in accumulation of lyso-PCs (as measured by HPLC/MS/MS) or PMN priming over time; also there were less lyso-PCs and priming activity at all time points in the SAGM stored samples relative to the plasma stored samples. Additionally, Vlaar *et al* reported that lyso-PC accumulation was reduced in PCs as the ratio of an additive solution (SSP+) to plasma in the storage medium increased but PMN priming was not reduced in concert. Further, lyso-PC accumulation was observed in cell free plasma stored at 22°C (but not 4°C) and this accumulation did not coincide with increased PMN priming activity of this plasma. It was concluded that lyso-PC accumulation in stored blood products is plasma rather than cell derived and does not account for the PMN priming effect of aged blood products.

An explanation for the divergent conclusions of Vlaar *et al* [24] and the body of work outlined in the previous section awaits definitive experimental clarification, but may be due to the differences in the priming assays employed. Vlaar *et al* used 30 minute priming incubations whereas in the series of earlier studies shorter, 5 minute, incubations were employed. Priming lipids, e.g. PAF, leukotriene B₄, lyso-PCs, 5-HETE, and arachidonic acid rapidly cause maximal priming in 3–5 minutes, priming then begins to subside at 15 minutes and is reduced at 30 minutes (unpublished observations and, for PAF, as published [25]). Thus prolonged 30 minute incubations may not detect priming activities present in stored blood components.

In the study by Vlaar *et al* [24] accumulation of lyso-PCs during RBC storage was not observed, however leukoreduced RBCs were used and, as outlined above, another recent study [23] demonstrated that leukoreduction of PRBCs prevents lyso-PC accumulation because it is platelets, that are significantly removed by leuko-reduction filters, that generated lyso-PCs in non-leukoreduced PBRC. Thus [23] and the work by Vlaar *et al* are consistent in terms of lyso-PC accumulation in leukoreduced RBCs.

A striking finding from the study by Vlaar *et al* is that SAGM used for the storage of RBCs and SSP+ used for the storage of platelets correlated with reduced of lyso-PCs. The use of

new additive solutions may thus decrease the lyso-PC levels in stored blood products and could be a welcome addition to TRALI mitigation strategies.

LYSO-PCS FROM STORED BLOOD PRODUCTS ARE IMPLICATED IN CLINICAL TRALI

The association of lipids with clinical cases of TRALI was demonstrated in a retrospective study of transfusion recipients in the State of Colorado that examined 10 patients with TRALI and control group of 10 patients with uncomplicated febrile, non-hemolytic transfusion reactions or urticarial reactions [26]. Priming of PMNs, measured as enhancement of superoxide generation in response to fMLP revealed no differences between pre- and post-transfusion sera from the control group, pre-transfusion sera from the TRALI group and healthy donor sera; however there was significantly higher priming in the post-transfusion sera from the TRALI group compared to all other groups. This significant increase was inhibited by WEB 2170 and, strikingly, normal phase HPLC of chloroform extracts of sera from three of the TRALI patients gave peaks of priming activity corresponding to the neutral lipid and lyso-PC peaks described above [19; 21]. Thus, based on identical retention times on normal-phase HPLC, the PMN priming lipid species initially observed in stored blood products were also found clinically in patients who had developed TRALI suggesting that they had been infused at high levels during transfusion. Alternatively the lipids may have been generated as a result of the lung damage, this is especially true of the neutral lipids which could contain leukotriene B₄, a known marker of lung injury released following ALI which primes PMNs [27].

In a much larger study [28], involving several hospitals in Alberta, Canada, plasma from WB-PLT units that were given to patients who developed TRALI was tested for its ability to prime fMLP-stimulated superoxide production by PMNs. In comparison to non-TRALI-implicated units stored for the same length of time, the TRALI-implicated units caused more priming. Moreover, the post transfusion plasma from patients who developed TRALI contained significantly more priming (which was WEB2170 inhibited) than their pre-transfusion plasma, similar to the earlier study discussed above [26]. Also in concordance with earlier data, chloroform extractable material from pre and post transfusion plasma from six TRALI patients was separated by normal phase HPLC and peaks of priming activity were found at retention times corresponding to neutral lipids and lyso-PCs in post transfusion material but was less (neutral lipids) or absent (lyso-PCs) in pre transfusion material.

The pathogenesis of TRALI has been related to the infusion of antileukocyte antibodies or their presence in the blood product recipient [29–34]. A further feature of the data from the large study of clinical cases of TRALI [28] was the observation that a possible antibody-mediated etiology was of low frequency in the cases examined. A further discussion of evidence supporting the notion that an immunological mechanism cannot account for many reported cases of TRALI is reviewed elsewhere [35].

A case study of TRALI caused by autologous transfusion [36] that describes WEB2170 inhibited PMN priming activity in chloroform extractable material from the implicated unit and the patients post transfusion plasma, but not the patients plasma drawn 5 weeks post TRALI, further supports the role of priming lipids in TRALI.

Thus several studies from disparate sites provide clinical evidence of the relative importance of lipids (and other BRM) as causative agents in TRALI. Additionally, clinical data supporting the importance of lyso-PCs in TRALI is consistent with their biological activity in animal models of TRALI [27; 37].

THE LYSO-PC MIX OF STORED BLOOD PRODUCTS PRIMES NEUTROPHILS VIA AN INCREASE IN CYTOPLASMIC Ca^{2+} MEDIATED BY A PERTUSSIS TOXIN SENSITIVE RECEPTOR

PMN priming by the lyso-PC mix and its component lipids was confirmed in a more extensive study of their effects on PMNs [20]. It was also shown that, as with other priming agents, the lyso-PC mix and its component lipids induce increases in the cytosolic concentration of Ca^{2+} in isolated PMNs. Pretreatment of neutrophils with PAF caused increased cytosolic Ca^{2+} but did not inhibit Ca^{2+} flux induced by subsequent application of the lyso-PC mix, likewise pre-treatment with the lyso-PC mix did not desensitize Ca^{2+} flux in response to subsequent PAF; the lack of cross desensitization supported the emerging theory, outlined above, in which the lyso-PC mix and PAF prime neutrophils via different receptors. In contrast, individual components of the lyso-PC mix and the mix itself did cross desensitize Ca^{2+} flux. Additionally, PMN priming and Ca^{2+} flux induced by the lyso-PC mix, but not PMA, were blocked by pertussis toxin, an inhibitor of $\text{G}_{\alpha i}$, implicating a G-protein coupled receptor (GPCR) distinct from the PAF receptor in the response of PMNs to the lyso-PC mix. Application of the lyso-PC mix caused a host of other functional changes in neutrophils that are associated with priming, i.e. increased expression of the $\beta 2$ integrin CD11b and the fMLP receptor, reduced chemotaxis, changes in morphology, and enhanced fMLP induced degranulation. Since the intracellular Ca^{2+} chelator BAPTA blocked priming, Cd11b expression and morphology differences, these functional changes appeared to be downstream of Ca^{2+} flux. Since lyso-PC application was also found to cause serine phosphorylation of a 68kD PMN protein, which was also inhibited by BAPTA, downstream phosphorylation cascades induced by changes in cytosolic Ca^{2+} were implicated in mediating the effects of the lyso-PC mix on PMN function.

THE GPCR G2A MEDIATES PMN PRIMING BY THE LYSO-PCS MIX

G2A was first identified as a lyso-PC receptor by a candidate based approach due to its homology to OGR1, a high-affinity receptor for a lysophospholipid structurally similar to lyso-PC [38]. This study showed that activation of G2A by lyso-PC increased the intracellular Ca^{2+} concentration in a pertussis toxin inhibited manner, activated ERK mitogen-activated protein kinase, and modified migratory responses of Jurkat T lymphocytes, however the study incorrectly determined that lyso-PC acted as a classical ligand binding directly to G2A [39]. Later work however demonstrated that G2A is spontaneously internalized to endosomal compartments and its surface expression is stabilized by lyso-PC [40; 41], thus lyso-PC appears to act by changing the balance between cell surface and internal distribution of G2A. A model to explain activation of G2A by lysophospholipids in the absence of direct binding is looser packing of membrane lipids due to insertion of cone shaped lyso-phospholipids into the membrane, this perturbation may cause dimerization or oligomerization and thus functional changes of G2A [41]. This model is supported by increased FM 1-42 staining of PMN membranes after lyso-PC treatment [41] (FM 1-42 preferentially inserts into loosely packed membranes) and membrane association of fluorescent NBD labeled 1-O-lauroyl lyso-PC [20]. Further supporting the role of G2A in the PMN response to lyso-PCs, neutrophil cytoplasmic Ca^{2+} flux [41], ROS production and *in vivo* bacterial clearance [42] are blocked by an antibody against G2A.

The involvement of G2A in PMN priming by the specific lyso-PC mix that accumulates in stored blood products was extensively studied by Khan *et al* [43]. Both the individual lyso-PC moieties and the mixture (16: 0/OH, 18: 1/OH, 18: 0/OH, C16 lyso-PAF and C18 lyso PAF) itself induced rapid internalization of the G2A receptor from the membrane to the cytosol of isolated PMNs at concentrations that would be present in a transfused host

(4.5 μ M for the mixture) [19]. In addition these lipids induced rapid serine phosphorylation of the receptor which is often associated with activation of GPCRs. Further, Western blot and fluorescence microscopy indicated that G2A in the cell membrane, but not the cytoplasm, was found to rapidly associate with the G protein subunits $G_{\alpha i-1}$, $G_{\alpha q/11}$ and $G_{\beta \gamma}$ after treatment with the lyso-PC mix; thus, in response to the lyso-PC mix, G2A seems to interact with G protein complexes composed of subunits $G_{\alpha i-1}$, $G_{\alpha q/11}$ and $G_{\beta \gamma}$ and presumably release these subunits prior to its internalization. $G_{\alpha i-1}$, $G_{\alpha q/11}$ and $G_{\beta \gamma}$ therefore appear to mediate signaling that promotes the neutrophil changes associated with the lyso-PC mix. An antibody directed against the extracellular domain of G2A blocked Ca^{2+} flux into the cytoplasm in response to the lyso-PC mix as did antibodies against $G_{\alpha i-1}$ and $G_{\alpha q/11}$ introduced into PMNs via an endosomal delivery system further indicating that these G proteins trigger the events leading to the Ca^{2+} flux characteristic of PMN priming. Positive fluorescence resonance energy transfer (FRET) observed by microscopy of PMNs treated with the lyso PC mix indicated close association between $G_{\beta \gamma}$ and hematopoietic cell kinase (Hck) as well as G_{β} and active Hck (i.e. phosphorylated on Tyr411). Therefore downstream effects of G2A activation might be mediated by this kinase. Internalization of G2A was abrogated by intracellular delivery of antibodies that neutralized α -adaptin and clathrin, components of the machinery that is involved in clathrin-mediated endocytosis (CME), demonstrating that G2A receptor uptake initiated by the lyso-PC mix is CME mediated, a characteristic of many GPCR signaling systems. Moreover the lyso-PCs caused interaction of β -arrestin-1 with clathrin and G2A, as demonstrated by FRET, and interaction of G2A and β -arrestin-1 with G-protein kinase 6 (GRK6) as demonstrated by co-immunoprecipitation. Since β -arrestins and GRKs are central players in GPCR signaling (β -arrestins activating signaling pathways and also desensitize GPCRs, GRKs cause functionally important phosphorylation of GPCRs) this data further supports the idea that G2A-mediated effects in PMNs exposed to the lyso-PC mix is characteristic of G protein coupled signaling. Thus, the work by Khan *et al* indicates that the lyso-PC mix causes release of $G_{\alpha i-1}$ and $G_{\alpha q/11}$ and $G_{\beta \gamma}$, internalization of the receptor in a CME-dependent manner and recruitment of GRKs and β -arrestin-1, molecules known to be important in G protein signaling. Thus the emerging picture is one in which the lyso-PC mix appears to cause increased cytosolic calcium in PMNs by activation of G-protein coupled signaling via the G2A receptor.

The work of Khan *et al* [43] indicated that the lyso-PC mix caused internalization of G2A and G-protein coupled signaling but the earlier work outlined above [40; 41] indicated that lyso-PC stabilizes G2A on the cell surface. This discrepancy is hard to understand definitively but may be due to differences in the precise experimental details that could include the method of presentation of the lipids (on human albumin verses bovine albumin or dissolved in methanol) and the methodology performed which is not congruous. Nevertheless, the work by Khan *et al* is relevant to the pathology of TRALI because it characterized mechanistic details of early signaling events triggered by the lyso-PC mix found in aged blood products when it is applied in a manner previously shown to mimic priming caused by these products.

THE POTENTIAL OF THE G2A RECEPTOR AS A THERAPEUTIC TARGET IN TRALI

A conceptually simple approach to reducing trail involves washing cellular blood products to remove the aged plasma and thus all potential mediators of TRALI (antibodies, lipid BRM and non lipid BRM) that might have accumulated. However this is time consuming and expensive and washing may be impractical in many cases due to the need for immediate transfusion of available units. Also even small amounts of residual plasma can cause TRALI [44; 45]. Thus it is attractive to consider approaches, admittedly highly speculative at this

time, that are based on administration of TRALI inhibitors to transfused patients, particularly those at increased risk from TRALI (for example patients with hematologic malignancies who are in the induction phase of chemotherapy and patients who have undergone cardiopulmonary bypass surgery [28]). As outlined above, WEB2170 inhibits priming of PMNs by the lyso-PC mix that accumulates in stored blood, at least in *in vitro* experimental settings, and could thus be considered in such an approach. Additionally A-79981.0, another molecule initially characterized as a PAF receptor antagonist was shown to behave like WEB 2170 and inhibit the lyso-PC mix induced priming of the fMLP response of PMNs [28]; thus, like WEB2170 it may also antagonize the G2A receptor and might be of utility in TRALI despite being unrelated to the tetrazepine-based WEB class of molecules [46]. Since two PAF receptor antagonists from different structural classes have been shown to also inhibit priming of PMNs by the lyso-PC mix, and since one of them (WEB 2170) is known to exert this effect by antagonism of G2A rather than the PAF receptor, cross antagonism of G2A by PAF antagonists may be relatively common. Therefore PAF antagonists could be a potentially rich source of structural diversity to explore for molecules which are also G2A antagonists that may be of utility in treatment of TRALI.

In addition to WEB2170 and functionally similar molecules, the recent elucidation of early events in G2A signaling initiated by the lyso-PC mix [43] reveals several molecular targets which could potentially be inhibited to block G2A activation and which might be therapeutic in TRALI; as outlined above, these are the G protein subunits $G_{\alpha i-1}$, $G_{\alpha q/11}$ and $G_{\alpha \beta}$, the kinase GRK6 and components of the CME system. Inhibitors of these components of signaling from the G2A receptor might thus lead to novel pharmacological agents to mitigate TRALI.

Another possible approach to “inhibit” the G2A receptor in a patient after a transfusion involves preventing the formation of lyso-PCs in stored blood products in the first place. Two enzyme activities can generate lyso-PCs, phospholipase A₂ (PLA₂) [47] and lecithin: cholesterol acyltransferase (LCAT) [48]. PLA₂ activity hydrolyses the sn-2 acyl bond of phospholipids where as LCAT catalyses the transfer of fatty acids from position sn-2 of phosphatidylcholine to free cholesterol in plasma. The role, if any, of these activities in formation of lyso-PCs in stored blood products awaits characterization. However, if a connection does exist inhibitors of these activities added to stored blood might minimize formation of G2A-activating lyso-phospholipids over time.

Despite some contradictions with earlier data that were outlined above and await full experimental resolution, a recent study by Vlaar *et al* [24] suggests another potential way to prevent lyso-PC accumulation in stored blood products; the observation that use of SAGM for storage of RBCs and SSP+ for storage of platelets will reduce lyso-PC concentrations suggests that the utilization of new additive solutions may decrease the lyso-PC accumulation in stored blood products and could be a valuable addition to TRALI mitigation strategies.

Acknowledgments

We would like to acknowledge support from Belle Bonfils Memorial Blood Center, the Stacy Marie True Memorial Trust and NIH Grants K07 HL088968 and GM049222.

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