Review of: "Loss of soluble guanylyl cyclase in platelets contributes to atherosclerotic plaque formation and vascular inflammation"

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Review of: ”Loss of soluble guanylyl cyclase in platelets contributes to atherosclerotic plaque formation and vascular inflammation (2021/22)” ;

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This study (Mauersberger C et al. [1]) reports that atherosclerosis-prone *Ldlr*-/- mice with additional platelet-specific deletion of soluble guanylyl cyclase (sGC; Pf4-*Cre*+*Gucy1b1*flox/flox*Ldlr*-/-mice) display larger atherosclerotic plaques in the aortic roots with an increased content of monocytes and macrophages. Intravital fluorescence microscopy studies with these mice showed an enhanced leukocyte adhesion to atherosclerotic plaques. In subsequent *in vitro* experiments, also with human platelets, evidence was collected that a reduced angiopoietin-1 release from sGC- deficient murine platelets or human platelets with the GUCY1A1 risk genotype was responsible for the inflammatory endothelial responses. Pharmacological sGC stimulation by BAY-747 increased angiopoietin-1 release from murine and human platelets and reduced atherosclerotic plaques in the murine model.

The major and very important conclusion of this paper is that a genetically induced loss of the platelet sGC contributes to enhanced plaque formation in atherosclerosis-prone mice. These data and conclusions represent an important advancement of previous murine/human studies of this group which linked augmented sGC expression to a lower atherosclerosis risk.[2] Ideally (as further proof of concept) it would be important to show in the future that platelet specific restoration of sGC/ cGMP signaling would reverse the proatherogenic phenotype of sGC loss or sGC impairment.

However, there are some unsolved problems in this study which need to be addressed. Hopefully, these comments will be helpful to advance this important project.

1. **Pharmacological sGC stimulation.** In the experiments reported, the mechanisms and specificity of sGC stimulation is not well described and supported by the data available. In most experiments, the Bayer sGC stimulator BAY-747 was used, but it is not clear whether BAY-747 stimulates platelet sGC *in vitro* or *in vivo*. This could be easily established. A related Bayer sGC stimulator, riociguat, effectively stimulated the sGC/cGMP pathways in washed human platelets but less effectively in whole blood platelets.[3] But this has not been followed up in *in-vivo* studies. The authors discuss that cells other than platelets could be targeted by BAY-747 in their experimental system which are also important for

atherosclerosis, for example in monocytes, macrophages and other immune cells. The authors could address this in future investigations. For example, if the protective BAY-747 effect is primarily due to platelet sGC stimulation, this effect should be essentially absent in mice with sGC-deficient platelets. Similarly, the Bay-747 simulated Angiopoietin-1 release observed with WT platelets (Fig. 4A) should be absent in sGC-deficient platelets. The discovery of another major sGC containing cell as important BAY-747 target would be very important.

2. **Increased Angiopoietin-1 release by platelet sGC stimulation.** The authors invested significant efforts to show that sGC/cGMP-mediated increase of angiopoietin-1 release from murine and human platelets is, at least in part, the mechanism for the protective effects of sGC stimulation (summarized in their Fig. 6). However, the data presented do not allow this conclusion, which should be considered as interesting hypothesis. By a proteome profiler Mouse XL Cytokine Array (ARY028, R&D), they identified angiopoietin-1 as factor released from platelets which was reduced in sGC-deficient platelets, but was increased by the sGC-stimulator BAY-747 in wild type platelets. These data were also confirmed by ELISA measurements. There are several problems/uncertainties with the data. The hypothesis that platelet-released factors play a major role in their system, is well founded. However, activated platelets release hundreds of factors (proteins, peptides, neurotransmitters, nucleotides, (poly)phosphates secreted from α/δ granules) and additional factors by other mechanisms. Such factors are important for wound healing and may play a major role in inflammation, cancer progression/metastasis.[4] [5] It appears unlikely that angiopoietin-1 alone is the major factor responsible for the effects observed by the authors. It is also highly unusual that sGC stimulation and increased cGMP levels in platelets would enhance the release/secretion of angiopoietin-1 from granules (presumably α-granules). Platelet granules, including α-granules and dense (δ)-granules, store hundreds of factors and secrete these mediators in response to platelet activating signals via elevation of intracellular Ca^{++} and activation of several protein kinases such as PKC, myosin light chain kinase, tyrosine kinases and MAPKs.[6] If BAY-747 would increase angiopoietin-1 release from platelets by a cGMP-dependent pathways (as suggested here), the authors could easily provide hard evidence for this suggestion:

- BAY-747 should elevate platelet cGMP levels and further cGMP-dependent events
- Similar effects should be observed with other agents which definitively elevate platelet cGMP levels (sodium nitroprusside, other NO-releasing compounds)
- Under sub-maximal conditions, the effects should be enhanced by a PDE-5 inhibitor
- Angiopioietin-1 release should be compared with established α/δ-granule secretion also induced by classical platelet agonists, and not only by shaking

It would be novel and exciting if angiopoietin-1 is released from by platelets by a sGC/cGMP-dependent mechanism. At present, the evidence for this is not very strong. In fact, there are data that classical platelet agonists such as thrombin induce angiopoietin-1 release from platelets.[7]

1. **Platelet sGC as an endogenous brake on platelet aggregation.** The authors leave the reader with the impression that the platelet sGC has only recently been recognized as endogenous platelet brake.

Since the initial discovery that NO or NO-releasing drugs potently inhibit ADP-induced platelet activation[8] , it has been well established by numerous investigators that elevation of platelet cGMP [by sGC activation, inhibition of cGMP degradation (PDE5) or both] strongly inhibits agonist-induced Ca^{++}-release from intracellular Ca^{++}-stores and prevents the activation of several protein kinases (e.g. PKC, MLCK, MAPKs). All of this leads to inhibition of major platelet functions including aggregation, granule secretion, thromboxane A2 release (reviewed [9] [10-12]).

1. **Interaction of sGC/cGMP signaling with other platelet pathways**

The authors briefly touch the interaction of the sGC/cGMP pathway with other important pharmacological pathways (aspirin effect in only homozygous carriers of the GUCY1A1 risk allele; comparison of PGE1 treatment with the PDE5 inhibitor/sildenafil treatment). This may also be of interest here. The potent synergistic interaction of the prostacyclin (PGI_2)/ AC/ cAMP pathway and the NO/sGC/cGMP signaling with respect to platelet inhibition including inhibition of secretion is well established and a hallmark of platelet pharmacology.[10, 11, 13] It would be important if the authors also consider the PGI_2/cAMP pathway as platelet brake in their system which cooperates with the sGC/cGMP brake. For example, are the BAY-747 effects on platelets enhanced by a PDE5 inhibitor or by platelet AC stimulators? Would atherosclerosis-prone effects of sGC loss/ impairment be worsened if the platelet AC/cAMP pathways are impaired? The exciting part of such considerations is that his may lead to novel therapeutic possibilities.

1. **Additional minor points**

Platelet-specific sGC KO mice are prepared as published previously by the authors. This results in the platelet specific deletion of the sGC-ß1 subunit and, as consequence, down-regulation/loss of the sGC α1 subunit and loss of platelet sGC activity. In this paper, the authors only analyzed the loss of the sGC-ß1 subunit (Suppl. Figure S1). It may be advisable to analyze the protein expression of the sGC α1 subunit and sGC activity in platelets, since they study a new and important model.

Concentration of BAY-747. The authors used PPM as units which is fine. Many biological readers of this work may prefer to see conventional units (µM/mM) as reported for the other reagents.

Platelet aggregation. The authors discuss platelet function primarily as “platelet aggregation”. A more sophisticated discussion and analysis of platelet functions such as secretion, procoagulant activity and others would be helpful.

Downstream cGMP targets. It is recommended that the cGMP pathway is described more precisely. IRAG1 is not a direct cGMP effector system but an effectors system of PKG1ß. In fact, cGMP, via PKGi1ß, affects multiple important substrate proteins/effector systems[11], which substantially overlap with the effects of cAMP/PKA. PDE3A is one important link between the two major inhibitory pathways in platelets.

Platelet activation (Fig.3). To obtain platelet releasates, platelets were activated by orbital shaking (30 min/1000 rpm) and subsequently removed by centrifugation (12,000 g /10 min). The supernatants were used for further assays including cytokine profiling and ELISA. There are some open questions: Why were

platelets resuspended in RPMI-medium? Why were classical platelet agonists not used? What is the general platelet activation profile of “shaken” platelets ?

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