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Low-Dose Aspirin Protects the Neurovascular Unit through Lysine Acetylation of Histone in an Oxygen–Glucose Deprivation Model of Cerebral Ischemia

Deepthi Ann Thomas
Yenepoya (Deemed to be) University
Abhina Gangadhar
Yenepoya (Deemed to be) University
Manju Babu
Yenepoya (Deemed to be) University
Rajas M Rao
Yenepoya (Deemed to be) University
Anaekshi Gogoi
Yenepoya (Deemed to be) University
Riyama Shirin
Yenepoya (Deemed to be) University
Arnab Datta
arnabdatta@yenepoya.edu.in

Yenepoya (Deemed to be) University

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Abstract

Low-dose aspirin is widely used as a low-cost prophylactic against atherosclerotic cardiovascular disease and for secondary prevention of ischemic stroke due to its anti-platelet action that is mediated by serine acetylation of cyclooxygenase (COX) enzymes. However, whether beneficial effects of low-dose aspirin are mediated by direct protective effect on the cells of the neurovascular unit that is platelet-independent, remain unclear. Here, we investigated the effects of chronic low-concentration (10 μM) aspirin pretreatment in cultured brain vascular endothelial cells and neurons subjected to sub-lethal in vitro cerebral ischemia via 4 hoxygen-glucose deprivation (OGD). Chronic aspirin pretreatment abolished the OGD-induced increase in Mcp1/Ccl2 transcript expression suggesting attenuation of inflammatory signalling. This was accompanied by anincreased lysine acetylation on a protein having same molecular weight as histone 3 (H3). Molecular docking calculations showed that aspirin can indeed bind to the lysine residues of H3, with the acetyl group of aspirin being in close contact with side chain lysine residues. Together, these observations indicate low-dose prophylactic aspirin can produce brain cell type-specific anti-inflammatory effect in response to ischemic stress that is associated with lysine acetylation of histone protein.

INTRODUCTION

Aspirin (acetylsalicylic acid) remains one of the most commonly prescribed drugs in acute ischemic stroke following the lapse of the narrow therapeutic window for thrombolysis. Aspirin is also used as a secondary prevention strategy to reduce the risk of recurrent vascular events including atherosclerotic cardiovascular disease in the surviving population. Healthy individuals with vascular risk factors such as hypertension are routinely treated with low-dose aspirin (75–150 mg/day) as a prophylactic against stroke or cardiovascular disorder (Awtry and Loscalzo, 2000). Thus, a large section of mid-to-high risk population for stroke or cardiovascular disorder is exposed to low-dose aspirin for months-to-years. In platelet, low-dose aspirin irreversibly acetylates serine residues of cyclooxygenase-1 (COX-1) and persistently inhibits its activity. This prevents the conversion of arachidonic acid to thromboxane A2, which is a vasoconstrictor and potent lipid-inducer of platelet aggregation. This way it is known to produce its anti-thrombotic action.

Beyond the classical COX enzyme inhibition in platelets, relatively less is known about the prophylactic mechanisms of chronic aspirin treatment on various brain cell-types, particularly in the context of cerebral ischemia. In one study on primary rat cortical neurons exposed to oxygen-glucose deprivation (OGD), aspirin protected neuron by reducing glutamate excitotoxicity induced by ATP depletion and caused an elevation in the neuronal ATP levels (De Cristóbal et al., 2002). Additionally, in rat forebrain slices exposed to OGD conditions, aspirin inhibited glutamate release, induced NF-kB translocation to the nucleus and iNOS expression (Moro et al., 2000). In human umbilical vein endothelial cells (HUVECs), aspirin has been shown to suppress TNFα mediated vascular inflammation by reducing the transcript levels and secretion of chemokines such as IL-8, monocyte chemoattractant protein-1 (Mcp1) (Yang et

al., 2004). However, none of these studies investigated chronic aspirin treatment at low micromolar concentrations that more closely recapitulate clinically relevant aspirin exposure for prophylaxis.

Notably, apart from serine acetylation on COX enzymes, aspirin can acetylate many proteins particularly on lysine resides as reported by different high-throughput studies (Tatham et al., 2017, Guo et al., 2020). However, it is unclear if low concentrations of aspirin can acetylate lysine residues of cellular proteins on different brain cell-types that are associated with its favourable response following ischemic stroke. Here, we have tested cerebroprotective action of chronic aspirin pretreatment using a combination of cell-based experiments under *in vitro* cerebral ischemia followed by *in silico* molecular docking.

METHODS

The details can be found in the Supplementary Materials.

RESULTS

The aspirin pretreatment of cultured vascular endothelial and neuronal cells for 24 h and 48 h respectively in the concentration range of 1 nM to 10 mM did not cause a decline in the metabolic activity of either cell-type as seen in MTT assay **(Supplemental Fig. 1)**. Based on this, 10 μM aspirin (0.1% of max concentration) was chosen for all subsequent

experiments. To test the effect of chronic aspirin pretreatment on cultured bEnd.3 cells under ischemic conditions, the cells were aspirin-treated once daily for up to 9 days before inducing ischemia by 4 h OGD. Similarly, for N2a cells, the cells were cultured up to 80% confluency in every passage, during which they were treated with 10 μM aspirin once daily for 14 consecutive days (4 passages). Serum deprivation for 48 hours combined with aspirin pre-treatment was followed by 4 h OGD on 14th day. We first checked the transcript levels of Ccl2/Mcp1 in our continuous culture model of endothelial and neuronal cells exposed to 4 h OGD with or without aspirin pre-treatment. Ccl2/Mcp1, a chemokine, which is known to be upregulated in cerebral ischemia, is responsible for attracting monocytes and macrophages at the site of ischemic injury on the vascular endothelium. Ccl2 transcript was elevated after 4 h of OGD in both cell-types that was restored to the NGM levels by chronic aspirin pretreatment **(**Fig. 1A, D**)**. Immunoblotting in bEnd.3 and N2a whole-cell lysates showed that 4 h of OGD causes a decrease in the lysine acetylation of a target protein (15–20 kDa) compared to the NGM condition. This OGD-induced decrease in the lysine acetylation was rescued in the aspirin-pretreated cells **(**Fig. 1B-C, E-F**)**. Overall, these results show that OGD is accompanied by aberrant lysine acetylation that can be corrected by low-dose aspirin pretreatment. Further, in addition to serine residues of COX enzymes, aspirin can also acetylate lysine residues on a target protein of 15–20 kDa.

Considering the molecular weight of the acetylated target protein matches that of histone3 (H3), we hypothesized that aspirin may directly interact and acetylate histone H3. Notably, previous studies also documented lysine acetylation of H3 by aspirin (Passacquale et al., 2015). To verify this, histone octamer complex was blind-docked with the ligand aspirin. We found that aspirin bound to theH3.1 (chain A and

E) alone in the large nucleosome complex. In the top-ranked complex (binding free energy = -7.2 kcal/mol among 2,000 docked conformations), we found that aspirin bound to a wedge created by the interface of two H3.1 monomers (Fig. 2A). Interestingly, this wedge is the same site where DNA molecule coils around the histone octamer, indicating that aspirin binding may sterically block DNA binding with H3.1. We further identified the H3.1 residues forming the interface with aspirin. The wedge where aspirin binds to H3.1 is created by both the K115 residues of H3.1, which forms a groove for DNA binding (Fig. 2B). The aspirin formed contact with the K115 residue of both the H3.1 chains. Further, this contact is formed by the methyl moiety of aspirin, suggesting that this methyl group may be transferred to either of the lysine molecules, resulting in its acetylation. From mapping the residue interactions, we found that the binding resulted from a combination of hydrophobic contacts and hydrogen bonding. While the residues H113, A114, K115 and K122 formed hydrophobic contacts with aspirin, the carboxyl moiety of aspirin formed hydrogen bond with the R116 residue. This hydrogen bond appears to act as an anchor, holding aspirin stably for the transfer of the methyl group to the lysine (i.e., K115) (Fig. 2C).

DISCUSSION

In this study, we have designed a cellular model to recapitulate chronic low-dose aspirin pretreatment as prophylactic in the context of acute cerebral ischemia. We have selected two major brain cell-types, vascular endothelial cells and neurons for performing the continuous aspirin treatment for 10–14 days before inducing in vitro cerebral ischemia (i.e., 4 h OGD). The inner wall of brain vasculature is the primary site for thrombus development during acute ischemic stroke, while neuron, being the functional workhorse is the most affected cell-type. Hence, fully confluent bEnd.3 (vascular endothelial) cells that has formed an impermeable monolayer and N2a (neuron) cells were selected for this chronic model. The aspirin concentration was kept at a lower level (i.e., 10 μM), similar to what has been observed in human plasma in pharmacokinetic studies with low-dose aspirin treatment (Rosenkranz and Frölich, 1985). Notably, this is far lower compared to the supraphysiological concentration that is commonly used in primary cell culture or slice culture-based OGD studies where it varied between 30 μM – 10 mM (De Cristóbal et al., 2002, Moro et al., 2000). Here, our chosen concentration (i.e., 10 μM) will favour high-affinity interaction and likely rule out any off-target effects that might happen in higher doses in the above-mentioned studies.

Considering anti-inflammatory and anti-thrombotic effects of aspirin are primarily linked to serine acetylation of COX enzymes in platelets, we examined lysine acetylation using anti-acetyl lysine antibody under ischemic condition (i.e. OGD). Here we show for the first time that a reduced lysine acetylation likely on H3 protein in both neuron and endothelial cells following 4 h OGD can be corrected by low-dose chronic aspirin pretreatment (Fig. 1B-C, E-F). The molecular docking study confirmed strong interaction between aspirin and H3 with 43 hydrogen-bonding contacts and a low binding energy of around − 7.2 kcal/mol. Further, aspirin is able to bring down the Ccl2 transcript level significantly, which was upregulated following 4 h OGD condition (Fig. 1A, D). Previous study showed that aspirin co-treatment (55.5 μM up to 2.78 mM) overnight can reduce MCP1 protein secretion by HUVECs in a TNFα-induced model of inflammation that was accompanied by a corresponding reduction in the U937 monocyte cell

adhesion, while aspirin significantly reduced monocyte migration at the highest concentration (i.e., 2.78 mM) (Yang et al., 2004). In another study involving TNFα-treated HUVECs, high concentration of co-incubated aspirin (500 μM) hyperacetylated H3 protein, which was associated with increased transcript levels of secreted isoform of nectrin-1. Furthermore, aspirin-induced H3 hyperacetylation was accompanied by a marked reduction of histone deacetylase/histone acetyltransferase (HDAC/HAT) ratio indicating an epigenetic regulation (Passacquale et al., 2015).

Our finding that chronic aspirin pretreatment even in very low concentration attenuates post-OGD inflammatory signalling that is accompanied by increased lysine acetylation of H3 protein raises the question of whether these two effects are mechanistically linked. It is possible that aspirin-induced changes in chromatin acetylation at specific Ccl2/Mcp1 regulatory regions, including promoter and/or enhancer elements, could adversely influence chromatin accessibility and transcription-factor occupancy, thereby contributing to the observed reduction in the Ccl2/Mcp1 transcription. Our molecular-docking analysis provides supportive evidence for a possible interaction between aspirin and H3 but does not confirm that the acetylated protein is H3 or that such an interaction mediates the observed transcriptional effect. Alternatively, aspirin may inhibit Ccl2/ Mcp1 expression by inhibiting COX enzymes as seen previously in cultured activated hepatic stellate cells where inhibiting COX-2 enzyme by indomethacin or ibuprofen markedly reduced MCP1 protein expression that was caused by TNFα or interleukin-1α exposure (Efsen et al., 2001). Further studies are needed to delineate if aspirin attenuates OGD-induced inflammation through its classical serine acetylation-mediated COX inhibition or through an alternative epigenetic pathway through direct lysine acetylation of histone proteins.

CONCLUSION

We established a chronic model of low-dose prophylactic aspirin treatment using cultured brain cells and showed aspirin-driven acute reversal of OGD-induced deacetylation of H3 protein that was accompanied by a decrease in the Ccl2/Mcp1 transcript levels. The observed lysine acetylation in neuron and endothelial cells may therefore represent a serine acetylation-independent effect that defines aspirin's classical mechanism of COX-inhibition on platelets.

Abbreviations

C-C motif chemokine ligand 2
Ccl2
Cyclooxygenase
COX
Histone 3
H3
Human umbilical vein endothelial cells
HUVECs
Monocyte chemoattractant protein-1

MCP1
Normal growth medium
NGM
Oxygen glucose deprivation
OGD.

Declarations

Supplementary Information

The supplementary material is available in the supplementary file accompanying this article.

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None.

Declarations of competing interest

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Author Contributions

Conceptualization & Study Design: A.D.; Funding Acquisition: A.D., R.M.R.; Data collection & analysis: D.A.T., A.Ga., M.B., R.M.R., A.Go, R.S., A.D.; Writing - Original Draft: D.A.T., R.M.R., A.D.; Writing – Review & Editing: A.D. All authors approved the final version of the manuscript. The authors declare that all data were generated in-house, that no paper mill was used and that no AI tool has been used for the generation of text or figures.

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Figures

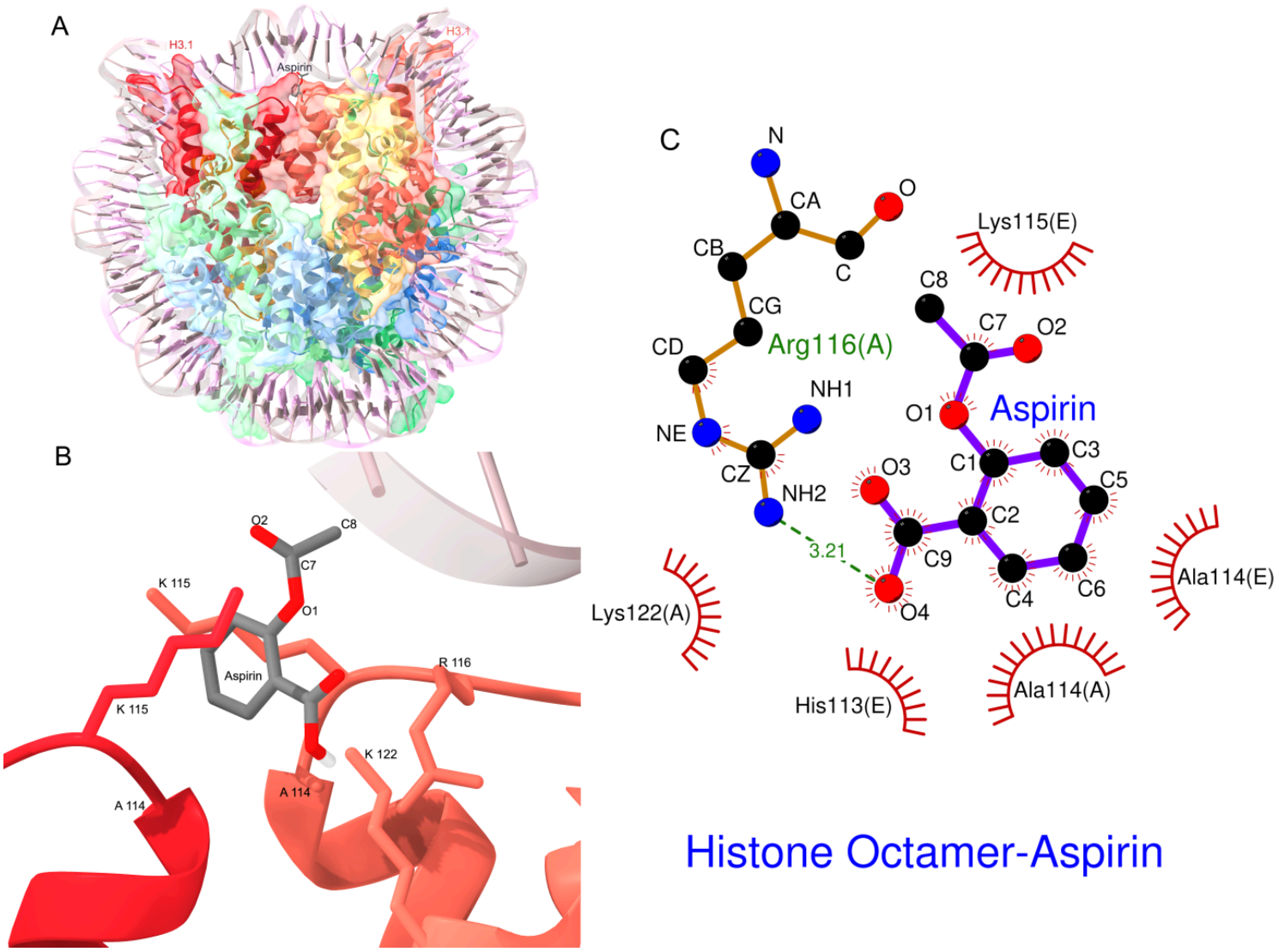

Figure 2

A) Top-ranked docked aspirin-histone octamer complex, showing interactions of Aspirin with Histone components. H3.1 is represented in red, Histone H4 in orange, Histone H2A in green and Histone H2B in blue. Proteins are represented as cartoons & surfaces. The orientation of DNA double helix in the experimental structure of Histone octamer (PDB:7VZ4) is structurally superimposed onto the docked complex. The DNA double helix is represented as pink ribbons. Aspirin molecule is represented as grey sticks. (B) Subfigure (A) zoomed in at the binding cavity showing the residues forming contacts with Aspirin. The contact forming residues are represented as sticks and labelled, Aspirin is represented as grey stick and colored according to heteroatoms (Carbons: grey; Oxygen: red; Hydrogen: white). (C) 2D Aspirin-Histone octamer interaction plot representing hydrophobic and hydrogen bonded interactions. Residues forming hydrophobic interactions are shown as spheres with radiating lines, hydrogen bonded interactions are shown as dashed lines with distances labelled.

Supplementary Files

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- SupplementaryMaterial.docx