Aspirin, but not clopidogrel, reduces monocyte plaque content through a modulatory action on endothelial-derived netrin-1 via a COX-dependent epigenetic modification

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Introduction: The neuroimmune guidance cue netrin-1 is an important regulator of atheroma development by virtue of its ability to inhibit monocyte motility. Endothelial-derived netrin-1, which is reduced under pro-inflammatory conditions, repels monocyte vascular infiltration from the bloodstream; on the other hand, monocyte netrin-1, which is enhanced by pro-atherogenic stimuli, compromises their egress from plaques. Hence, an important and unresolved question is whether systemic therapeutic manipulation of netrin-1 might produce detrimental rather than beneficial anti-atherogenic effects due to an indiscriminate action on different cell types with opposite effects on monocyte arterial accumulation.

Aims: We aimed to identify whether i) aspirin, which is used as anti-platelet agent in cardiovascular prevention but also exerts anti-inflammatory properties on the endothelium, modulates the endothelial expression of netrin-1 *in vitro* and ii) what is the net effect of aspirin on netrin-1 production and atherosclerosis progression *in vivo*.

Methods: Human umbilical vein endothelial cells (HUVECs) were incubated overnight with TNFα (10 ng/ml) either alone or in combination with aspirin (0.5 mM), the cyclooxygenase (COX)-1 inhibitor SC-560 (30 nM), the COX-2 inhibitor NS-398 (10 μM) or indomethacin (100 μM). Netrin-1 was measured in cells and tissues by RT-PCR and immunofluorescence staining and by ELISA in cell supernatant. Monocyte migration toward treated endothelial cells was evaluated using a Boyden chamber system. Given its crucial role in regulating TNFα-dependent gene expression, histone acetylation was studied by western blotting and immunofluorescence staining. Histone deacetylase (HDAC) and histone acetyltransferase (HAT) activities were measured by enzyme immunoassay. The HDAC inhibitor trichostatin A (TSA, 400 nM) was used as a positive control. $ApoE^{-/-}$ mice on 8-weeks high fat diet were treated with aspirin (5 mg $kg^{-1}day^{-1}$) or clopidogrel (25 mg $kg^{-1}day^{-1}$) and compared to untreated animals. Netrin-1 was measured in the plasma by ELISA and within the arterial wall by immunofluorescence staining. Circulating and plaque monocytes were characterized by flow cytometry.

Results: TNFα down-regulated the synthesis of secreted netrin-1 by HUVECs (86.25±57.2 ng/ml vs 295.5±64.4 ng/ml in control; $p<0.01$), thus increasing monocyte chemotaxis toward the endothelium (122.4±20.6 vs 66±30 cells/field in control; $p<0.01$). Aspirin restored netrin-1 secretion by TNFα-stimulated cells (199.6±30 ng/ml; $p<0.01$) and reduced monocyte chemotaxis (83±35.7 cells/field; $p<0.01$). Aspirin, in co-treatment with TNFα, increased histone acetylation (ratio acetylated/total histone: 0.2±0.01 with aspirin/TNFα co-treatment vs 0.02±0.02 in untreated cells; $p<0.05$ and vs 0.05±0.02 in TNFα-treated cells; $p<0.05$). Among the other COX-inhibitors, only indomethacin reproduced the effects of aspirin. TSA stimulation either alone (618.3±77 ng/ml; $p<0.01$ in control) or in combination with TNFα (993.6±61 ng/ml; $p<0.01$ vs control), enhanced endothelial netrin-1 secretion. In $ApoE^{-/-}$ mice, aspirin, but not clopidogrel, increased the plasma level (170.7±56.13 ng/ml vs 53.73 ng/ml in control mice; $p<0.01$) and endothelial expression of netrin-1. Unlike clopidogrel, aspirin reduced plaque monocyte content (26.6±3.7% vs 44.2±7.1% in untreated animals; $p<0.01$), despite both drugs suppressing the blood monocytosis that accompanies plaque development in $ApoE^{-/-}$ mice (monocytes: 29±3% and 28.4±4.5% in aspirin and clopidogrel groups respectively vs 42.8±6% in control mice; $p<0.01$ and $p<0.05$ respectively).

Conclusions: Aspirin restores endothelial-derived netrin-1 production under pro-inflammatory conditions through a platelet-independent action on the endothelium sustained by COX-inhibition and subsequent histone hyper-acetylation. Aspirin-dependent increase in netrin-1 beneficially affects plaque composition by reducing monocyte accumulation.