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Low-dose aspirin (acetylsalicylate) prevents increases in brain PGE_2, 15-epi-lipoxin A4 and 8-isoprostane concentrations in 9 month-old HIV-1 transgenic rats, a model for HIV-1 associated neurocognitive disorders

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Abstract

Background—Older human immunodeficiency virus (HIV)-1 transgenic rats are a model for HIV-1 associated neurocognitive disorders (HAND). They show behavioral changes, neuroinflammation, neuronal loss, and increased brain arachidonic acid (AA) enzymes. Aspirin (acetylsalicylate, ASA) inhibits AA oxidation by cyclooxygenase (COX)-1 and COX-2.

Hypothesis—Chronic low-dose ASA will downregulate brain AA metabolism in HIV-1 transgenic rats.

Methods—Nine month-old HIV-1 transgenic and wildtype rats were given 42 days of 10 mg/kg/day ASA or nothing in drinking water; eicosanoids were measured using ELISAs on microwaved brain extracts.

Results—Brain 15-epi-lipoxin A4 and 8-isoprostane concentrations were significantly higher in HIV-1 transgenic than wildtype rats; these differences were prevented by ASA. ASA reduced prostaglandin E_2 and leukotriene B_4 concentrations in HIV-1 Tg but not wildtype rats. Thromboxane B_2, 15-HETE, lipoxin A_4 and resolvin D_1 concentrations were unaffected by genotype or treatment.

Conclusion—Chronic low-dose ASA reduces AA-metabolite markers of neuroinflammation and oxidative stress in a rat model for HAND.

Keywords

HIV-1; transgenic; PGE_2; 15-epi-lipoxin A_4; 8-isoprostane; brain; chronic aspirin; rat; low dose; neuroinflammation; HAND

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Introduction

Human immunodeficiency virus (HIV)-1-infected patients are at risk of developing HIV-1 associated neurocognitive disorders (HAND) [1, 2]. These disorders progress over time, ranking from mild neurocognitive impairment to HIV-1-associated dementia, which involves severe cognitive dysfunction in multiple domains [3]. The introduction of antiretroviral therapy (ART) has reduced the prevalence of dementia. However, as the lifespan of HIV-1 infected patients has been prolonged by ART, the prevalence of HAND with aging remains high [4].

HAND probably arises from direct and maintained viral invasion of the central nervous system (CNS), which is an important reservoir for HIV-1 virus regardless of plasma viral suppression or cumulative time on ART. In the North-East AIDS Dementia consortium, over 50% of viremically controlled HAND patients had detectable virus in the cerebrospinal fluid (CSF) [5]. Aberrant macrophage and T-lymphocyte activation in the CSF continued despite viremic control by ART [6], and brain atrophy correlated with the CSF level of quinolinic acid, evidence of activated CNS macrophages and microglia associated with neuroinflammation and excitotoxicity [7].

HIV-1 infection of the brain stimulates both the innate and adaptive immune systems.. Brain damage and neuronal loss are associated with activation of microglia, astrocytes and invasive macrophages, which release toxic quantities of agents such as nitric oxide, tumor necrosis factor (TNF)-α, interleukin (IL)-6, and interleukin (Il)-1β [8]. Additionally, preclinical evidence indicates that there is secondary activation of phospholipases A_2 (PLA_2) that release of the n−6 polyunsaturated fatty acid arachidonic acid (AA) from membrane phospholipids [9]. AA and many of its eicosanoid products are proinflammatory. They include prostaglandin (PG)E_2 formed preferentially by cyclooxygenase (COX)-2, thromboxane (TX)B_2 formed preferentially by COX-1, 15 (S)-HETE formed by 15-lipoxygenase (LOX), lipoxin (LX)A_4 formed from 15-HETE by 5-LOX [10], leukotriene (LT)B_4 also derived from AA by 5-LOX [11], 15-epi-LXA_4 synthesized from 15 (R)-HETE by acetylated COX-2 [12, 13], and isoprostanes produced by non-enzymatic pathways [14, 15]. COX-2 expression is increased following injection of HIV-1 Tat or gp-120 proteins into rodent brain, and in other HIV-1 rodent models [16–20].

Evidence for neuroinflammation in HIV-1 patients, and the recognized relation between neuroinflammation and upregulated AA metabolism in animal HIV-1 models and human neurodegenerative disease [21–23], suggest that antiinflammatory drugs that target the brain AA cascade might be of clinical relevance. One such drug is aspirin (ASA, acetylsalicylic acid), whose antiinflammatory actions in peripheral inflammation are well described [24, 25]. ASA inhibits COX-1, which converts AA to TXB_2, while acetylating and inhibiting COX-2, which converts AA to PGE_2 and also becomes capable of converting docosahexaenoic acid (DHA) to antiinflammatory 17R-hydroxy-containing di- and tri-hydroxy-docosanoids termed resolvins [26]. Evidence in animals and humans indicates that even low dose aspirin can exert behavioral and other biological effects in the intact CNS [27–32].

To provide a basis for testing potential efficacy of ASA in HIV-1 patients with HAND, we examined in the present study the effect of low-dose ASA on brain AA and DHA metabolism in a transgenic (Tg) rodent model of HIV-1, and in control rats. The human HIV-1 provirus, carrying seven out of the nine HIV-1 genes after functional deletion of the infectious genes *Gag* and *Pol* is constitutively expressed by the HIV-1 Tg rat. The HIV-1 Tg rat develops neuropathology as it ages, thus may be an animal model for HAND [33, 34]. It shows reduced spatial learning at 5 months of age. At 7–9 months, neuroinflammation and synaptic loss occur, associated with increased expression of AA-metabolizing cytosolic $cPLA_2$ IVA, secretory $sPLA_2$ IIA and COX-2 [19] and changes in fatty acid composition [35].

We treated 9-month old HIV-1 Tg and wildtype rats with 10 mg/kg/day ASA in drinking water for 42 days, using ASA-free water as a control. This ASA regimen is equivalent to a low therapeutic dose of 100 mg in human (for a 70 kg subject) [36–38]. We used ELISA assays to measure brain concentrations of PGE_2, thromboxane TXB_2, leukotriene LTB_4, lipoxin LXA_4, 15-epi-LXA_4, 15-hydroxyeicosatetraenoic acid (HETE), 8-isoprostane and resolvin D1.

Materials and Methods

Chemicals

ASA was purchased from Sigma-Aldrich (Saint Louis, MO). Hexane and isopropanol (Reagent Grade) were obtained from Fisher Scientific (Pittsburgh, PA). Ultra-pure water was purchased from KD Medical (Columbia, MD).

Animals

The experiments were conducted under an approved NICHD animal protocol (12–027) in accordance with the NIH Guidelines on the Care and Use of Laboratory Animals. Age-matched male HIV-1 Tg or Fischer 344/NHsd wildtype rats (9 months-old), purchased from Harlan Laboratories (Madison, WI) were housed in an animal facility under a 12 h/12 h light–dark cycle with *ad libitum* access to water and an identical Teklad global 18% protein 2018S diet. The diet was 2018S (sterilized) for controls and 2918 (irradiated 2018S) for HIV-1 Tg rats (Teklad Harlan, Madison, WI). The diets were processed in identical ways, except that the 2018S diet was further gamma irradiated to minimize the risk of infection in the HIV-1 Tg colony. The diet contained (as % of total fatty acid) 16.7% saturated, 21.8% monounsaturated, 54.8% linoleic acid, 6.2% α-linolenic acid, 0.03% AA, 0.02% eicosapentaenoic (EPA, 20:5 n−3) and 0.06% docosahexaenoic acid (DHA, 22:6 n−3) [35]. After 42 days on the diet, the rats were anesthetized with Nembutal (40 mg/kg, i.p.), and subjected to head-focused microwave irradiation at 5.5 kW for 3.4 sec (Cober Electronics, Stamford, CT). The brain was immediately removed, placed on dry ice, and stored at −80 °C.

ASA treatment

Before going on the diet, rats were separated into four different groups (n = 12): untreated wildtype, untreated HIV-1 Tg, ASA-treated wildtype, and ASA-treated HIV-1 Tg. Untreated

groups received regular drinking water. The ASA-treated groups received ASA in water (10 mg/kg/day) for 42 days. Fresh ASA drinking water was prepared and provided every two days, as was control drinking water. Rat weight and water intake were monitored weekly. According to an interspecies conversion factor based on body surface, the 10 mg/kg/day dose used here was equivalent to a dose of 100 mg for a 70 kg person [36].

Sample preparation

Extraction of oxygenated metabolites of AA and DHA was performed according to the Radin method [39]. Half-brains were homogenized in a glass Tenbroeck homogenizer in hexane-isopropanol (3:2 v:v, 18 ml/g brain). The homogenate was transferred to a glass centrifuge tube and the homogenizer was washed twice with 4 volumes of hexane-isopropanol solution. The pooled homogenate was centrifuged at 1500 rpm for 5 min at room temperature, and the organic supernatant was collected. The pellet was re-extracted twice in 5 ml hexane-isopropanol. The pooled extracts were dried under N_2 at 45 °C, resuspended in 3 ml hexane-isopropanol and stored at −80 °C.

Measurement of brain eicosanoids and docosanoids by enzyme immunoassay

To perform an enzyme immunoassay, 1 ml of the sample in hexane-isopropanol was dried under N_2 and resuspended in 500 μl buffer. Concentrations of eicosanoids or docosanoids were determined with commercially available ELISA kits in accordance with the manufacturer's instructions. PGE_2 and 15-epi-LXA_4 kits were obtained from Oxford Biochemical (Oxford, MI) and TXB_2, LTB_4, 15-HETE, LXA_4, 8-isoprostane and Resolvin D1 kits were from Cayman Chemicals (Ann Arbor, MI).

Statistical analyses

All data are expressed as mean ± SEM (n = 10–12 per group). In some groups, an outlier was identified using Grubbs' test, and removed from the data set (as indicated in the figure legend). For 8-isoprostane quantification, n = 10 for controls and n = 11 for HIV-1 Tg rats due to sample shortage. A two-way ANOVA was performed to identify global effects of genotype and treatment (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA), and was followed by Least Significant Difference (LSD) post-hoc tests for multiple comparison. The level of significance was set at $p < 0.05$.

Results

Body weight

There was a significant main effect of genotype on body weight. HIV-1 Tg rats weighed significantly less than wildtype controls (−20%, $p < 0.001$). No effect of ASA treatment was observed.

Brain Metabolites

The brain concentrations of AA-derived metabolites and Resolvin D1 in HIV-1 Tg and wildtype rats are shown Figure 1.

PGE_2 – A two-way ANOVA showed a significant effect of ASA on brain PGE_2 levels ($p = 0.009$), but no effect of genotype. Post-hoc analysis by an LSD test indicated that the brain PGE_2 concentration was significantly lowered by ASA treatment in HIV-1 Tg rats, compared to nontreated HIV-1 Tg rats (−66%, $p = 0.006$).

TXB_2 – There was no effect of genotype or ASA treatment on brain TXB_2 concentration.

15-HETE – There was no significant effect of genotype or ASA treatment on brain 15-HETE concentration.

12-HETE – 12 HETE levels in most samples were below the limit of detection (196 pg/mL).

Statistical comparisons therefore were not performed.

LXA_4 – There was no effect of genotype or ASA treatment on brain LXA_4 concentration.

LTB_4 – Brain LTB_4 concentration did not significantly differ between untreated HIV-1 Tg and wildtype rats. However, a post-hoc test showed that LTB_4 concentration was significantly lower in HIV-1 Tg rats that received ASA, compared to untreated animals (−29%, $p = 0.03$).

15-epi-LXA_4 – A two-way ANOVA showed a significant effect of ASA on brain 15-epi-LXA_4 levels ($p = 0.02$), but no effect of genotype. Post-hoc analysis by LSD test showed that 15-epi-LXA_4 concentration was significantly (2.1. fold) higher in HIV-1 Tg compared to wildtype rats ($p = 0.04$). This increase was prevented by ASA treatment in HIV-1 Tg rats, compared to non-treated (−62%, $p = 0.01$).

8-Isoprostane – Two-way ANOVA showed a significant effect of HIV-1 Tg genotype ($p = 0.01$) on 8-isoprostane concentration, as well as a significant effect of ASA treatment ($p = 0.04$). There also was a significant HIV-1 genotype × ASA interaction ($p = 0.02$). Subsequent post-hoc tests indicated that the levels of 8-isoprostane in untreated HIV-1 Tg rats were significantly increased ($p = 0.02$) compared to levels in the wildtype rats. ASA treatment resulted in a lower 8-isoprostane level in HIV-1 Tg compared to wildtype, which approached statistical significance ($p = 0.06$).

Resolvin D1 – We found no significant effect of genotype or ASA treatment on brain resolvin D1 concentration.

Discussion

Whole brain 15-epi-LXA_4 and 8-isoprostane concentrations were higher in 9 month-old HIV-Tg rats than in wildtype controls that had been administered drinking water for an additional 42 days. In contrast, treatment with low-equivalent dose ASA for 42 days significantly reduced the increases in 15-epi-LXA_4 and 8-isoprostane concentrations seen in the HIV-1 Tg compared to wildtype rats. Chronic ASA compared with aspirin also reduced brain PGE_2 and LTB_4 concentrations in HIV-1 Tg rats, but not in wildtype controls. In comparison, ASA treatment did not change the level of any other measured AA-derived eicosanoid in wildtype rats.

ASA directly inhibits COX-1 and limits COX-2 activity *via* acetylation, and therefore reduces the production of AA-derived prostaglandins [13, 24–26]. COX-1 and COX-2 catalyze the production of PGH_2 from AA, which subsequently is converted to PGE_2 by PG synthase or to TXB2 by TX synthase [40]. Our results showed a significant global effect of ASA on brain PGE_2 concentration, particularly in HIV-1 Tg rats, in which ASA treatment reduced PGE_2 concentration by 66%. This difference may correspond to upregulation of COX-2 in the brain HIV-1 Tg rats, whereas COX-1 is unchanged [19]. It suggests that ASA is more effective in a system where inflammatory processes are already activated. The ability of low-dose ASA to reduce the overproduction of brain PGE_2 confirms that low dose ASA has a central effect when administered peripherally, and agrees with prior preclinical and clinical evidence of a central action [27–32]. ASA crosses the blood-brain barrier and can directly target central PG synthesis, although the effective dose reaching the brain might increase if barrier integrity is altered by pathology [41, 42].

TXB_2 is measured as a marker for unstable TXA_2, which has potent vasoconstrictor function and facilitates platelet aggregation. ASA treatment inhibits TXA_2 synthesis in blood at doses as low as 40 mg/day in humans [43, 44], and is recommended in the prevention of cardiovascular complications. In this study, however, the brain TXB_2 level was not reduced by ASA.

AA can be converted to 15 (S)-HETE by 15-LOX, which is elevated in brain from 9-month HIV-1 Tg compared to wildtype rats [19]. Although its role in brain remains to be explored, 15-HETE has been detected in different brain regions [45] and shown to trigger constriction in piglet cerebral arterioles [46]. In this study, however, the concentration of 15 (S)-HETE was unchanged by the HIV-1 genotype or by ASA treatment. The synthesis of the stereoisomer 15 (R)-HETE is triggered by ASA *via* acetylation of COX-2 [25]. 15 (R)-HETE was not measured in this study because an ELISA assay method is not available to specifically determine its level.

Lower levels of 15-HETE were detected compared to levels of PGE_2. This might be explained by the use of commercial kits and possible difference in extraction efficiency depending on the metabolites. Additionally, negative interference may have affected detection of 15-HETE in the assay. Negative interference is associated with the composition of the sample and can arise from cross-reactants, heterophilic antibodies, or endogenous interferers [47]. Since our extraction process for lipid mediators removed proteins, the negative interference could have been caused by reduced binding of 15-HETE or lowered binding affinity of 15-HETE to its specific antibodies by interfering substances.

5-LOX is involved in the synthesis of LXA_4 from 15-HETE, or *via* the production of LTA_4 from 5-HETE [10]. LXA_4 is a mediator in the resolution of inflammation in various animal models, including stroke [48, 49], and also was increased in cultured HIV-1 infected monocytes and astroglia [50]. The brain LXA_4 level was not impacted by the HIV-1 genotype or by ASA treatment in the present study.

The brain 15-epi-LXA_4 concentration was significantly higher in untreated HIV-1 Tg rats than in wildtype controls, but this effect was absent following ASA treatment. 15-epi-LXA_4

is an ASA-triggered anti-inflammatory AA-derived mediator that is synthesized from 15 (R)-HETE by acetylated COX-2 [12, 13]. Increased 15-epi-LXA_4 concentration may reflect the brain response to increased pro-inflammatory eicosanoid production in HIV-1 Tg rats.

Brain LTB_4 was lowered by ASA in HIV-1 Tg rats. Derived from AA conversion by 5-LOX. LTB_4 is involved in pro-inflammatory signaling by promoting vascular permeability and leukocyte adhesion and activation [11]. LTB_4 also has been implicated in chronic inflammatory diseases such as asthma [51], rheumatoid arthritis [52] and irritable bowel disease [52].

The concentration of the PGF_2-like compound 8-isoprostane was significantly higher in HIV-1 Tg compared to wildtype brain, but the elevated 8-isoprostane level was reduced to a control level by ASA, suggesting that low-dose ASA reduced oxidative stress in the pathological brain. 8-Isoprostane is produced by non-enzymatic peroxidation of AA by free radicals and has been used as a marker of oxidative stress [53, 54]. Accumulation of free radicals and redox imbalance were observed in brain tissue from HIV-1 infected patients who died with dementia [55, 56]. The 8-isoprostane level also was increased by the HIV-1 tat protein in microglial cell culture [57]. F2-isoprostanes have been reported in CSF of patients with Alzheimer and other neuroinflammatory diseases [54, 58], and might be examined in CSF of HIV-1 patients.

Changes in brain AA-derived eicosanoid concentrations occurred despite the reported lack of change in brain esterified and unesterified AA concentrations in the HIV-1 Tg rat model [35]. These results are not inconsistent, since eicosanoid levels are 3 to 4 orders of magnitude lower than their AA substrate concentration, about 12000 nmol/g (25000 times the concentration of the most abundant eicosanoid detected, 8-isoprostane. Furthermore, a 3.6 fold increase in 8-isoprostane concentration in the HIV-1 Tg group would result in a < 0.5% change in brain AA concentration, which would not be detected using the gas-chromatography method [35].

Resolvin D1 is DHA-derived metabolite that is a precursor to 10(R), 17(S) hydroxyl-DHA, which has potent anti-inflammatory properties in brain [59, 60]. Neither ASA nor genotype affected the concentration of resolvin D1. According to the manufacturer, the kit that we used is selective for 17 (S)-resolvin D1 isomer. However, ASA can trigger the synthesis of protective 17-(R)-resolvin D1 in response to inflammatory stress [26] and thus it would be important in the future to quantify this isomer using LC-MS-MS.

A limitation of this study is that we used Fisher 344/NHsd controls from which the HIV-1 line was derived, instead of littermate controls. We did this to be consistent with our previous study on HIV-1 Tg rats [35]. We cannot rule out possible confounding effects of genetic drift in the Fisher 344/NHsd line, but we consider this unlikely. At an extreme, the limitation may affect our interpretation of whether HIV-1 Tg rats have higher eicosanoid concentrations than control littermates specifically, but it would not alter our conclusions with regard to ASA, which significantly reduced several eicosanoid concentrations in the HIV-1 Tg rats.

In summary, chronic low dose human equivalent ASA blocked the increases in AA-derived LTB_4 and 8-isoprostane concentrations that were produced in 9-month old HIV-1 Tg compared with wildtype rats, which show demonstrable pathology, behavioral changes and upregulated expression of AA metabolizing enzymes, including $cPLA_2$, $sPLA_2$ and COX-2. ASA also reduced 15 (S)-HETE and PGE_2 concentrations in HIV-1 Tg rats. Thus, treatment with low dose ASA may dampen brain inflammation associated with upregulated brain AA metabolism and potentially contributing to the presence and progression of HAND. ASA in HIV-1 patients is well tolerated, and chronic low dose ASA is recommended for reducing cardiovascular events in HIV-1 patients [61, 62], although only one in five HIV-1 patients are actually taking ASA [61, 62]. In this regard, other nonsteroidal inhibitors of COX-2 have been shown to be beneficial on peripheral markers of disease severity in HIV-1 patients [63–65].

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Abbreviations

AA	arachidonic acid
ART	antiretroviral therapy
ASA	acetylsalicylic acid
CNS	central nervous system
COX	cyclooxygenase
CSF	cerebrospinal fluid
DHA	docosahexaenoic acid
HAND	HIV-1 associated neurocognitive disorders
HETE	hydroxyeicosatetraenoic acid
HIV	human immunodeficiency virus
LOX	lipoxygenase
LT	leukotriene
LX	lipoxin
PG	prostaglandin
PLA	phospholipase
TX	thromboxane

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1. Even treated HIV-1 patients can develop neurocognitive disorders.
2. We studied a transgenic rat model of HIV-1, free of infectious Gag or Pol genes.
3. The model had high brain 15-epi-lipoxin A4 and 8-isoprostane concentrations.
4. These and other brain changes were prevented by chronic low-dose aspirin.
5. Low-dose aspirin enters brain and might be used to treat HIV-1 neuroinflammation.

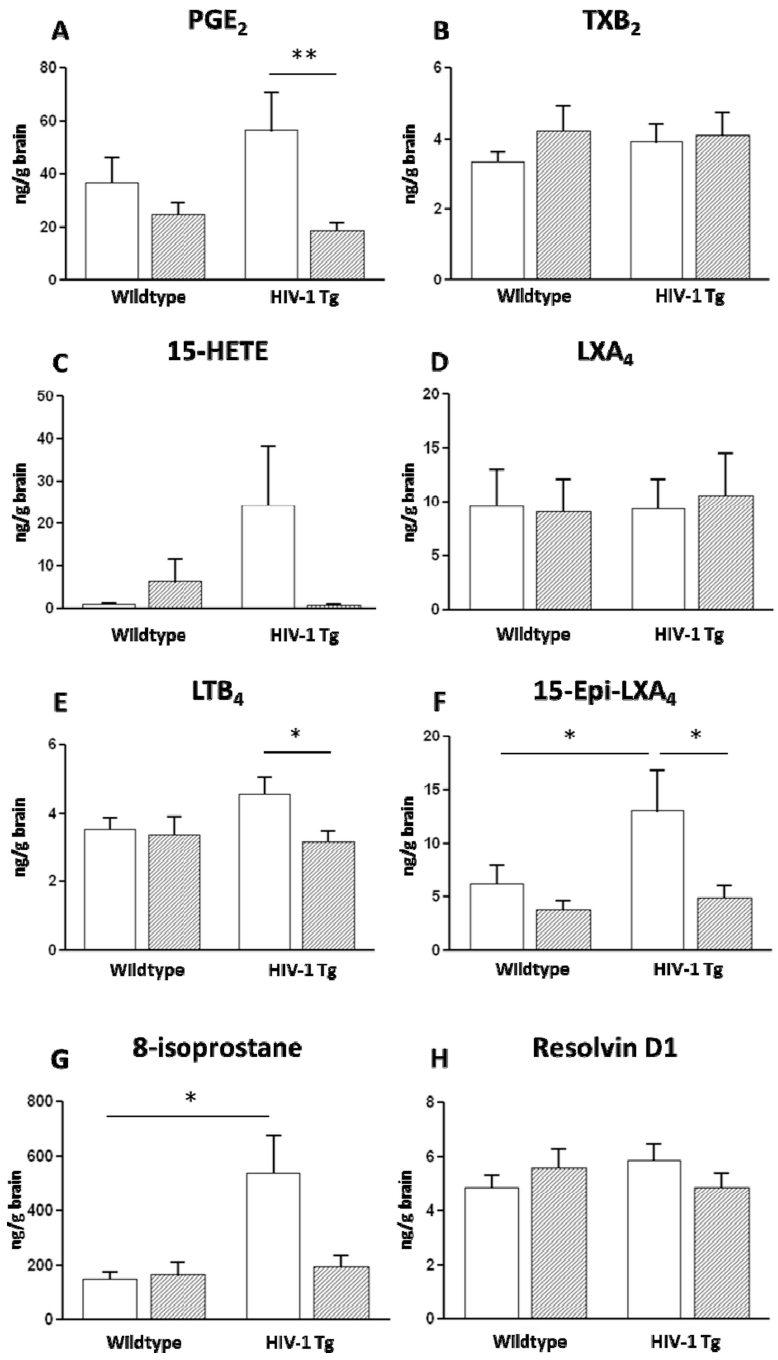

Figure 1.

Effects of HIV-1 genotype and low dose aspirin (ASA) on concentrations of PGE_2, TXB_2, 15-HETE, LXA_4, LTB_4, 15-epi-LXA_4, 8-isoprostane, and Resolvin D1 in microwaved rat brain. Data are means ± SEM (n=10–12) and were analyzed using a two-way ANOVA followed by LSD post-hoc test. * $p < 0.05$, ** $p < 0.01$. Some outliers were identified using the Grubbs' test, therefore n = 11 for PGE_2: wildtype ASA and HIV-1 ASA, LXA_4: wildtype water and HIV-1 ASA, LTB_4: HIV-1 ASA, epi-LXA_4: wildtype water, wildtype

ASA and HIV-1 ASA, Resolvin D1: HIV-1 ASA. Variability of the 15-HETE assay was high, with outliers removed from the four groups.