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Inhibition of the biosynthesis of prostaglandin E₂ by low dose aspirin: implications for adenocarcinoma metastasis

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Abstract

Meta-analyses have demonstrated that low dose aspirin reduces the risk of developing adenocarcinoma metastasis, and when colon cancer is detected during aspirin treatment, there is a remarkable 83% reduction in risk of metastasis. As platelets participate in the metastatic process, the anti-platelet action of low dose aspirin likely contributes to its anti-metastatic effect. Cyclooxygenase-2 (COX-2)-derived prostaglandin E₂ (PGE₂) also contributes to metastasis, and we addressed the hypothesis that low dose aspirin also inhibits PGE₂ biosynthesis. We show that low dose aspirin inhibits systemic PGE₂ biosynthesis by 45% in healthy volunteers ($p < 0.0001$). Aspirin is found to be more potent in colon adenocarcinoma cells than in the platelet, and in lung adenocarcinoma cells its inhibition is equivalent to that in the platelet. Inhibition of COX by aspirin in colon cancer cells is in the context of the metastasis of colon cancer primarily to the liver, the organ exposed to the same high concentrations of aspirin as the platelet. We find that the interaction of activated platelets with lung adenocarcinoma cells up-regulates COX-2 expression and PGE₂ biosynthesis, and inhibition of platelet COX-1 by aspirin inhibits PGE₂ production by the platelet-tumor cell aggregates. In conclusion, low dose aspirin has a significant effect on

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Disclosure of potential conflict of interest

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Clinical Trial Registration

This investigation has been registered at www.clinicaltrials.gov (identifier NCT 00761891).

extraplatelet cyclooxygenase, and potently inhibits COX-2 in lung and colon adenocarcinoma cells. This supports a hypothesis that the remarkable prevention of metastasis from adenocarcinomas, and particularly from colon adenocarcinomas, by low dose aspirin results from its effect on platelet COX-1 combined with inhibition of PGE₂ biosynthesis in metastasizing tumor cells.

Introduction

Both observational studies (1–5) and controlled clinical trials (6) support the concept that aspirin prevents mortality and metastasis from adenocarcinomas. Importantly, aspirin use reduced the development of metastases by 69% in patients who develop cancer while receiving aspirin (7). The anti-metastatic benefit of aspirin occurred even at low doses in the ranges used for its anti-platelet effect (75 to <300 mg daily) (7, 8).

A convincing body of evidence supports the concept that platelets are integral to the process of metastasis, and this forms the basis for considering that the anti-platelet effect of low dose aspirin contributes importantly to the prevention of metastasis (9–16).

In addition to the platelet, cyclooxygenase-2 (COX-2) and its product PGE₂ are known to contribute to both tumorigenesis and metastasis. COX-2 inhibitors reduce the incidence of colorectal adenomas (15,16). PGE₂ exerts multiple effects that promote tumorigenesis and metastasis (17–19), including induction of the transition from epithelial to mesenchymal phenotype (20), increased angiogenesis and cell growth (21, 22), invasiveness (23) and down regulation of the immune response (24–27).

Given these effects of the COX-PGE₂ pathway on tumorigenesis and metastasis, an important question is whether low doses of aspirin can act directly on extra-platelet cyclooxygenases to inhibit PGE₂ biosynthesis in humans. The strong inhibition of platelet COX-1 by low doses of aspirin results from at least 3 determinants. First, there is virtually no turnover of COX-1 in platelets, and daily administration of aspirin inhibits platelet COX-1 cumulatively over the platelet life span (28). Second, because of pre-systemic clearance of aspirin, exposure of platelets to the high concentrations of the drug in the portal circulation leads to greater effects on the platelets than on systemic targets (29). Finally, aspirin is particularly potent in inhibiting COX-1 in the platelet (30).

We previously discovered that there is cellular selectivity in the potency of aspirin. High concentrations of hydroperoxides in cells accelerate redox cycling in the peroxidase site of the enzyme to generate a protoporphyrin radical, and in this higher oxidative state, the acetylation of the COXs by aspirin is inhibited (31). Thus, COXs in cells with robust anti-oxidant defenses and low levels of peroxides, such as the platelet, are highly sensitive to inhibition by aspirin. Epithelial cells of the lung (32–35) and colon (36–38) also are protected from elevated levels of peroxides by extensive anti-oxidant systems. Accordingly, in the research reported here, we tested the hypothesis that aspirin would potently inhibit COX activity in epithelial cancer cells *in vitro*. The potency of aspirin as a cyclooxygenase inhibitor in cancer cells was found to be as great (lung adenocarcinoma) or greater (colon adenocarcinoma) than the potency in platelets.

The hypothesis that low dose aspirin also can inhibit the catalytic activity of extra-platelet cyclooxygenase *in vivo* was tested, demonstrating that aspirin 81 mg daily reduced the biosynthesis of PGE₂ and prostacyclin significantly in healthy humans.

In addition to the action of aspirin to directly inhibit PGE₂ biosynthesis in tumor cells, it is well established that aspirin also can reduce tumor cell PGE₂ biosynthesis indirectly by blocking the PGE₂ production that results from the interaction of platelets with colon cancer cells (16, 39). In those investigations, the interaction of platelets with colon cancer cells increased the expression of COX-2 and production of PGE₂, and selective inhibition of the platelet cyclooxygenase was found to prevent the increase in PGE₂ biosynthesis (16, 39). Further, a PGE₂-dependent epithelial-to-mesenchymal transition of these platelet-activated colon cancer cells was demonstrated, supporting the importance of PGE₂ in metastasis. To determine whether this effect of platelets to elicit PGE₂ biosynthesis could be generalized to other tumor types, we evaluated the effect of the interaction of platelets with lung adenocarcinoma cells on PGE₂ production. We found that, like colon cancer cells, PGE₂ biosynthesis is increased by the exposure of lung adenocarcinoma cells to platelets. Moreover, the expression of COX-2 protein, while necessary, was not sufficient to account for the increased PGE₂ production, and evidence is presented that is consistent with the hypothesis that platelet adherence initiates calcium-activated cPLA₂ in the tumor cells.

Materials and Methods

Materials

Human A549, HCC827 and H2122 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Human HCA-7 were a generous gift from Dr. Robert Coffey. Written informed consent was obtained from study participants and human blood was obtained following a protocol approved by the Vanderbilt Institutional Review Board. Hanks' Balanced Salt Solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), aspirin (acetyl salicylic acid), Sepharose 2B column, tris(hydroxymethyl) aminomethane (Tris), and IL-1 β were purchased from Sigma-Aldrich (St. Louis, MO). Silica gel 60A thin layer chromatography plates were purchased from Whatman (Clifton, NJ). Antibiotic antimycotic solution was obtained from Life Technologies (Grand Island, NY). [²H₈] Arachidonic acid (AA) was obtained from Perkin Elmer Life Sciences (Boston, MA). Anti COX-1 and COX-2 antibodies were obtained from Santa Cruz Biotechnologies (Dallas, TX) and anti GADPH antibodies were obtained from Ambion (Austin, TX).

Participant Blood Sample Procurement

Blood samples were obtained from healthy participants and collected from a peripheral vein using a 21-gauge needle into vacuum tubes containing 3.2% sodium citrate and centrifuged at 300 \times g for 10 min at room temperature. The supernatant (platelet rich plasma, PRP) was used to prepare washed platelets as previously described (40). Volunteers were enrolled only if they could confirm lack of aspirin or non-steroidal inflammatory drug use for at least 10 days prior to sample procurement.

Preparation of washed platelets

PRP was acidified to pH 6.4 with 0.15 M citric acid, and centrifuged at $1000 \times g$ for 10 min at room temperature. The resulting pellet was resuspended with 24.4 mM sodium phosphate buffer, containing 113 mM NaCl and 5.5 mM glucose, pH 6.4 and platelets were purified on a Sepharose 2B column equilibrated with the same buffer. Platelets were counted with a Coulter counter and diluted with suspension buffer (110 mM NaCl, 4 mM K_2HPO_4 , 8 mM NaH_2PO_4 and 5 mM glucose, pH 7.2) to a final concentration of 300,000 platelets/ μ l.

Cell Culture

A549, HCC827 and H2122 non-small cell lung adenocarcinoma cells as well as HCA-7 and HT-29 colon cancer cells were cultured in 6-well plates at 37°C in a humid atmosphere containing 5% CO_2 . Cells were maintained in DMEM media supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution. All cells except HCA-7 were activated with 1 ng/ml IL-1 β once they were 90% confluent to induce expression of COX-2 for 24h prior to experiment of interest.

Relation of Aspirin Concentration to Inhibition of COX

To compare aspirin efficacy between cell types, washed platelets or activated adenocarcinoma cell lines, prostanoid production was assayed using similar experimental conditions: 1) all cells were incubated in albumin free media (41), 2) the same concentration of exogenous arachidonic acid was used for all experiments, 3) deuterated arachidonic acid was used to avoid confounding the results by contribution of prostanoids produced from endogenous arachidonic acid released during cell treatment, and 4) incubation time was the same for all cells. A detailed justification of these conditions is described in Supplemental Data. Washed platelets and activated adenocarcinoma cells were preincubated at 37 °C for 30 min with aspirin at different concentrations: 0, 10, 20, 30, 50 and 100 μ M. Then, 2.0 μ M [2H_8] AA was added and incubated for 15 min. The medium was harvested for GC/NICI/MS analysis.

Participant Urine Sample Procurement

This study was approved by the Vanderbilt University Institutional Review Board and registered on ClinicalTrials.gov (NCT 00761891). Participants provided written informed consent. Healthy non-smoker volunteers over the age of 18 with no chronic medical illness or medications were enrolled. Exclusion criteria included concurrent use of other antiplatelet drugs, NSAIDs or COX-2 inhibitors, history of coronary artery disease, uncontrolled hypertension (systolic blood pressure > 180 mmHg), significant GI bleeding, hematocrit < 35%, or platelet count < 100,000/ μ L. *Volunteers* received a blister pack containing a 2-week supply of a daily dose of aspirin 81 mg (McNeil Pharmaceuticals) administered in the evening. The importance of strict adherence to therapy was emphasized and participants were contacted by the research coordinator mid-study to assess and encourage continued compliance. A pill count was performed at the conclusion of the study. Urine was collected for 24 hours starting after the first morning void on the day before the baseline and again prior to the 2-week visit.

GC/NICI/MS Analysis of PGE₂ and TxB₂

PGE₂ and TxB₂ (the stable rearrangement product of TxA₂) were quantified by isotopic dilution by GC/NICI/MS monitoring selected ions as previously described (42). The signals for different molecules are: TxB₂, $m/z = 614$; and internal standards [²H₄] TxB₂, $m/z = 618$; [²H₄] PGE₂, $m/z = 528$. To account for the deuterium-protium exchange at the position C12 of [²H₈] PGE₂, the summation of the signals obtained at $m/z = 530, 531$ and 532 was performed (43).

Urinary Metabolite Analysis

PGE-M, the major PGE₂ urinary metabolite was quantified by isotopic dilution by LC/ESI/MS/MS as described previously (44). Detailed experimental protocol is described in Supplemental data. It should be noted that PGD₂ is metabolized by a mechanism analogous to PGE₂ and metabolites for this eicosanoid are detected using the same m/z transitions as PGE-M. The methoximated metabolites of PGE₂ and PGD₂ are chromatographically separated using this method (Supplemental Fig. S1).

PGI-M, the PGI₂ urinary metabolite, was quantified by isotopic dilution using GC/NICI/MS as described previously (45).

Calculation of platelet-derived PGE₂

To determine the effect of low dose aspirin on extra-platelet PGE₂ biosynthesis, it is necessary to account for its effect on the very small amount of platelet-derived PGE₂ production, assuming that this is inhibited almost completely by low dose aspirin. PGE₂ is produced by the platelet in an amount that is approximately 6.4% that of TxB₂ ((39), and Fig. 5). To estimate the fraction of PGE₂ that is derived from the platelet, the production rates for total PGE₂ and for platelet-derived PGE₂ in humans have been calculated. The production rates for PGE₂ in humans are 51 ng/mg creatinine in males and 22.7 ng/mg creatinine in females, where time is the amount of time in which 1 mg creatinine is excreted. This production rate is based on the determination that PGE-M represents 16% of infused tritium labelled PGE₂ (46), and on the amount of PGE-M/mg Cr measured in this study.

$$\text{Total PGE}_2 \text{ production rate ng/mg Cr} = [\text{PGE-M ng/mg Cr}] / 0.16$$

An estimate of platelet-derived PGE₂ is based on the fraction: PGE₂ produced by washed platelets/TxB₂ produced by washed platelets, and on the evidence that ~80% TxM is derived from platelets. Thus:

$$\text{Extra-platelet PGE}_2 \text{ production rate} = (\text{TxB}_2 \text{ production rate} \times 0.064) \times 0.8 / \text{PGE}_2 \text{ production rate}$$

where TxB₂ production rate is based on the determination that the normal excretion rate of the TxB₂ metabolite, 11-dehydrothromboxane B₂ (0.369 ng/mg Cr)(47), represents 10.3% of the production rate (48). Thus, platelet-derived PGE₂ constitutes 0.36% of total PGE-M in males and 0.8 % in females.

Cell Lysates and Western Blot Analysis

Adenocarcinoma cells lysates were obtained by washing cells with ice cold PBS containing a protease inhibitor (PI) cocktail and then scraping them off the well surface. Cells were pelleted by centrifugation at $300 \times g$ for 10 min at room temperature. Cells were resuspended in 100 μ l of PBS containing PI cocktail and protein were quantified using the BCA assay.

Proteins (80 μ g) were separated by LDS-PAGE (NuPAGE) and transferred on nitrocellulose membranes for analysis by Western blot. Nonspecific binding sites were blocked with TBST buffer (50 mM Tris, 150 mM NaCl and 0.2% (v/v) Tween-20, pH 7.5) containing 5% (w/v) non-fat dry milk for 1 h at room temperature. After blocking, membranes were incubated overnight at 4°C with 1:1000 dilution of appropriate primary antibody: COX-2 goat polyclonal IgG (sc-1747, Santa Cruz), COX-1 (sc-1752, Santa Cruz) and GADPH goat polyclonal IgG (AM4300, Ambion). Bands of interest were detected using horseradish peroxidase conjugated donkey anti-goat IgG polyclonal antibodies (1:5000 dilution; sc-2020, Santa Cruz) and enhanced chemiluminescence kit. Quantification of signal in western blots was performed using Image J downloaded from NIH website.

A549 and Activated Platelets Studies

A549 cells were grown to 90% confluence and starved overnight in serum free DMEM. Two batches of washed platelets were prepared as follow. Whole blood was incubated with 100 μ M aspirin (aspirin-treated) or not (no aspirin) for 40 min at room temperature. Washed platelets were then prepared as described separately from each batch as described above. Washed platelets (resuspended concentration of 600,000 platelets/ μ l) were activated with 50 μ M ADP for 2 min at 37°C prior to addition to A-549 cells at a ratio of about 180 platelets per adenocarcinoma cell. Washed platelets were activated by ADP because we found that 1) activating platelets with ADP yielded much less variability than without activation, and 2) ADP does not directly activate A549 cells. Cell and platelet mixture were then incubated at 37°C for 2 hours with no addition of exogenous arachidonic acid. The medium was harvested for analysis of PGE₂ by GC/NICI/MS as described above. The cells were then scraped from the wells, lysates obtained and protein quantified using BSA assay. Western blots were performed for COX-1, COX-2 and GADPH proteins as described above. Each experiment was performed in triplicates and a total of 3 experiments were performed (n = 9).

To confirm that the reduction in PGE₂ production was mediated by the effect of aspirin to inhibit platelet COX-1 and not due to direct inhibition of COX-2 expressed by A549 cells by residual aspirin present in washed platelets, we performed an additional control experiment in which A549 cells were incubated with IL-1 β overnight to induce COX-2 expression. A549 cells were then incubated for 2 h at 37°C with either PBS buffer, the supernatant from washed platelets or supernatant from washed platelets prepared from aspirin-treated whole blood. COX-2 catalytic activity in the platelet/A549 cell aggregates was assessed by measuring [²H₈]-PGE₂ production for 15 minutes following the addition of [²H₈]-AA.

Fluorescence Microscopy

The ability of aspirin to block COX-2 active site has been further demonstrated using a fluorophore conjugated to indomethacin as previously described (49). In short, A549 cells were grown to 90% confluence and expression of COX-2 was induced as described above. Cells were incubated in 2.0 ml of a 1:1 dilution of HBSS and modified Tyrode's buffer (20 mM HEPES, 10 mM glucose, 150 mM NaCl, 6 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, pH 7.4) containing 50 nM of the fluorescent probe, fluorocoxib A, for 30 min at 37°C. The cells were then washed briefly three times with PBS and a washout was performed by incubating the cells in HBSS/Tyrode's solution for 30 min at 37°C. Following the washout period, the cells were imaged in 2.0 mL fresh HBSS/Tyrode's on a Zeiss Axiovert 25 Microscope with the propidium iodide filter (0.5–1.0 s exposure, gain of 2). All treatments were performed in duplicate dishes in at least three separate experiments (n = 6). To block the COX-2 active site, the cells were preincubated with 10 µM NS-398 for 30 min at 37°C prior to the addition of fluorocoxib A. To test the ability of aspirin to block the COX-2 active site, the cells were pre-incubated with 100 µM aspirin for 1 hour at 37°C prior to the addition of fluorocoxib A.

Measurement of Diastolic Calcium

A549 cells were incubated for 2 hours at 37°C with washed platelets which were preincubated with ADP (50 µM) or aspirin (100 µM) or vehicle for 5 min. A549 cells were incubated with 2 µM Fura-2 acetoxymethyl ester (Fura-2 AM) for 8 min at room temperature to load the calcium indicator in the cytosol. A549 cells were washed twice for 10 min with Tyrode's solution containing 250 µM probenecid to retain the indicator in the cytosol. A minimum of 30 min was allowed for de-esterification before the cells were imaged. All the experiments were conducted in Tyrode's solution containing the following: 1 mM CaCl₂, 134 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH. The final concentration of Ca²⁺ was 1 mM. Baseline of Fura-2 fluorescence ratio were used as intracellular Ca²⁺ levels. Data were analyzed using commercially available data analysis software (IonWizard™, IonOptix, Milton, MA).

Statistical Analysis

Statistical analysis was performed using Prism 4 from GraphPad Software, Inc (San Diego, CA). Paired analyses with Wilcoxon signed ranked test were used to determine significant differences between PGI-M and PGE-M values before and after aspirin therapy. The significance of differences between PGE₂ and COX-2 production in A549 cells that were untreated, treated with activated platelets and treated with activated platelets preincubated with aspirin, was determined using one-way ANOVA with Tuckey's multiple comparisons test. Differences among the groups in diastolic calcium measurements were analyzed using one-way ANOVA. P<0.05 was considered statistically significant.

Results

Low dose aspirin inhibits non-platelet biosynthesis of prostaglandins

The effect of aspirin 81 mg daily for 2 weeks on urinary metabolites of PGE₂ (PGE-M) and prostacyclin (PGI-M) was quantified in healthy volunteers who had no medical history of chronic inflammatory conditions that could predispose them to elevated prostaglandin levels. Detailed medication history was reviewed prior to enrollment to ensure that those did not interfere with the investigation. Demographics of study population are described in Table 1. Volunteers were given two weeks of aspirin 81 mg daily, and the urinary metabolites of PGE₂ (11 α -hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid) was measured using LC/MS/MS. Because the average PGE-M excretion is greater in males (8.2 ng/mg creatinine) than in females (3.6 ng/mg creatinine), the effect of aspirin was determined separately by gender. Aspirin 81 mg daily was sufficient to reduce urinary PGE-M levels in healthy females by 56% and in healthy males by 39%. When correction is made for the estimated small contribution of platelet-derived PGE₂ to total PGE₂ biosynthesis (see Methods), the reduction in extra-platelet PGE₂ production by aspirin is 55.4% in females and 38.6% in males (Fig. 1). The same aspirin dose also inhibited urinary PGI-M by 37% ($p < 0.0001$, $n = 54$) (Supplemental Fig. S2). Serum thromboxane B₂ levels were reduced by 96.6%. These results demonstrate that low dose aspirin can inhibit non-platelet prostaglandin production.

Aspirin is a potent inhibitor of PGE₂ biosynthesis in adenocarcinoma cells

To examine the effect of aspirin on PGE₂ biosynthesis in lung adenocarcinoma cells, we tested a range of concentrations of aspirin on PGE₂ biosynthesis in the non-small cell lung adenocarcinoma cell lines, H2122, HCC827 and A549. As shown in figure 2A, all three adenocarcinoma cell lines express COX-2 after overnight stimulation with IL-1 β , as well as various levels of COX-1. We compared the effect of the same concentrations of aspirin on COX-1 derived biosynthesis of thromboxane A₂ in human washed platelets. Platelets were utilized as a comparator in this *in vitro* study, because the dose range designated as “low dose aspirin” in humans is defined as the lowest dose of aspirin that nearly completely inhibits platelet COX-1.

In these experiments, the same concentration of isotopically labelled arachidonic acid (2 μ M) was added to all cells as substrate and the correspondingly labelled product analyzed. In all three cell lines, PGE₂ biosynthesis was markedly inhibited by aspirin (IC₅₀s are 22.5 \pm 3.2 μ M, 7.2 \pm 1.1 μ M, and 15.0 \pm 1.3 μ M for HCC827, A549 and H2122 cells, respectively). By comparison, the IC₅₀ for aspirin inhibition of platelet TxB₂ biosynthesis assayed in the same conditions was 19.8 \pm 1.5 μ M (31).

Based on this evidence that aspirin potently inhibits PGE₂ biosynthesis in lung adenocarcinoma cells, its effect as a COX inhibitor in colon cancer cells also was determined (Figure 2C). The IC₅₀ for inhibition of PGE₂ biosynthesis in the colon cancer cells, HCA-7 and HT-29, was 3.9 \pm 0.7 μ M and 8.9 \pm 1.2 respectively. This demonstrates that aspirin is more potent as an inhibitor of COX in cells from colon adenocarcinomas than in the platelet ($p < 0.0001$). Thus, the catalytic activity of the cyclooxygenases in this group

of lung and colon adenocarcinoma cells is inhibited by aspirin to the same or greater extent than COX-1 in the platelet.

We have previously demonstrated that aspirin equally inhibits both COX isoforms (31). Interestingly our results show that each of the five adenocarcinoma cells express various levels of the two isoforms. A549 cells, for example, mainly express COX-2, whereas HT 29 mainly express COX-1. Our results show that aspirin effectively inhibits prostanoid synthesis in all these cell lines irrespective of the isoforms expressed demonstrating that it is not selective for platelet COX-1. To further confirm that aspirin is not isoform specific, we measured prostanoid production before and after preincubation with the COX-2 specific inhibitor valdecoxib. As shown in supplemental figure S4, 100 nM valdecoxib only inhibited 60% of PGE₂ synthesis in HCA-7, a concentration that fully inhibited PGE₂ synthesis in A549 and H2122. The same concentration of valdecoxib had no effect on TxB₂ synthesis by washed platelets (Supp. Fig. S4).

Aspirin blocks the COX-2 binding site in the COX-2 expressing A549 cell line

The experiments above examined aspirin's inhibition of PGE₂ biosynthesis in adenocarcinoma cells. To further support the hypothesis that aspirin can inhibit PGE₂ production by blocking the COX-2 active site, we analyzed the effectiveness of aspirin at preventing the binding of a COX-2-specific fluorescent probe (49) using fluorescence microscopy. A549 cells were chosen because COX-1 expression is negligible (8 %) compared to that of COX-2 when activated with IL-1 β (Fig. 2A and Sup. Fig. S3). A rhodamine conjugated-indomethacin probe, fluorocoxib A (49), was used to label the active site of COX-2 (Fig. 3). As expected, the probe did not label the non-activated A549 that are devoid of COX-2 (Fig. 3B) but strongly labelled IL-1 β -activated cells (Fig. 3D). Importantly, aspirin effectively blocked COX-2 labeling by fluorocoxib A (Fig. 3F), providing direct evidence that aspirin blocks the COX-2 active site. These results support the concept that inhibition of PGE₂ production by aspirin in A549 cells (Fig. 2) is caused by COX-2 inhibition and not by cyclooxygenases-independent effects of aspirin.

The interaction of platelets with lung adenocarcinoma cells induces PGE₂ biosynthesis via a calcium-mediated mechanism

The effect of the interaction of platelets with adenocarcinoma cells on PGE₂ production by COX-2 was evaluated in A549 cells because they produce low levels of PGE₂ at baseline (Fig. 4). Washed platelets from a normal volunteer were activated with ADP and subsequently incubated with A549 cells, and COX-2 expression and PGE₂ production were measured. Both COX-2 expression and PGE₂ production were significantly increased (Fig. 4). We then determined whether inactivation of platelet COX-1 by aspirin would prevent COX-2 expression and/or PGE₂ production by the platelet-A549 cell aggregates. We first incubated whole blood with 100 μ M aspirin for 40 minutes and then prepared washed platelets from the aspirin-treated blood. This was done to ensure that all residual aspirin was removed from the platelet preparation and that no free aspirin was available for direct inhibition of A549's COX-2. Platelets derived from aspirin-treated blood were activated using ADP and subsequently incubated with A549 cells. COX-2 protein expression in A549 cells was still upregulated by aspirin-inhibited platelets (Fig. 4A), but PGE₂ production was

significantly inhibited (Fig. 4B), suggesting that PGE₂ synthesis is induced by activated platelets by a mechanism independent of the level of expression of COX-2.

To confirm that the reduction in PGE₂ production was mediated by the effect of aspirin to inhibit platelet COX-1 and not due to direct inhibition of COX-2 expressed by A549 cells by residual aspirin present in washed platelets, we performed an additional control experiment in which COX-2 catalytic activity was measured in IL-1 β -activated A549 cells incubated with either PBS buffer, the supernatant from washed platelets or supernatant from washed platelets prepared from aspirin-treated whole blood. PGE₂ production was no different in all three groups (3.5 ± 0.2 , 3.3 ± 0.2 , and 3.3 ± 0.3 ng/ml, respectively; n=3). These results demonstrate that there is insufficient residual aspirin present in aspirin-treated platelets washed as described in our protocol to directly inhibit COX-2-mediated PGE₂ biosynthesis by A549 cells.

Consideration was given to the finding that platelet COX-1 inhibition by aspirin inhibits PGE₂ production without affecting COX-2 expression (Fig. 4). This could occur only if the increase in PGE₂ elicited by the interaction of the A549 cells with platelets resulted from release of the COX substrate, arachidonic acid, from phospholipids by a phospholipase. In A549 cells, the phospholipase is cytosolic phospholipase A₂ (cPLA₂ or Group IVA PLA₂) (50, 51). cPLA₂ is activated by an increase in cytosolic calcium (52, 53), which binds to the C2 domain of the enzyme and increases its hydrophobicity, thereby initiating penetration of the lipid bilayer. In A549 cells, ionophore-induced increases in cytosolic calcium have been shown to increase production of PGE₂ in a cPLA₂ dependent manner (50). Accordingly, we measured cytosolic calcium in A549 cells in response to incubation with ADP-activated washed platelets inhibited or not by aspirin. Cytosolic calcium levels were quantified for the following conditions: A549 cells incubated with (a) vehicle, (b) incubated with ADP-activated platelets and (c) incubated with aspirin-pretreated ADP-activated platelets. Cytosolic calcium (expressed as fluorescence ratios) was increased by 21.9 % following incubation of A549 cells with ADP-activated platelets (Fig. 4C, n = 20) but this effect was abrogated when the platelets had been pre-incubated with aspirin (7.3 % increase, n = 10). These data indicate that platelets inhibited by aspirin are unable to trigger the mobilization of cytosolic calcium necessary for initiating PGE₂ synthesis by A549 cells. Interestingly, this calcium signal does not appear to be necessary for COX-2 induction.

The cellular origin of the increased PGE₂ in the platelet/A549 cell aggregates was investigated by employing the COX-2 inhibitor, NS-398, at 100 nM. We first demonstrated that this concentration of NS-398 that does not block the production of PGE₂ or thromboxane B₂ (TxB₂) in washed platelets (Fig. 5B and Supplemental Figure S5) but fully blocks PGE₂ production by IL-1 β -activated A549 (Supplemental Figure S4). When washed platelets were incubated together with A549 cells, the increased PGE₂ production was reduced by NS-398 by only 48%, indicating that approximately half of the increase in PGE₂ in the platelet/A549 aggregates was derived from the platelets, either by increased synthesis of PGE₂ in the platelet and/or intercellular transfer of PGH₂ from the platelet to the A549 cells. Thus, when platelets adhere to the cancer cells, both the platelets and the cancer cells are activated and both contribute to the increased biosynthesis of PGE₂.

Also, 31% of the increase in TxB₂ that resulted from exposure of platelets to A549 cells was blocked by NS-398 (Fig. 5B). Because no TxB₂ is formed by A549 cells in the absence of platelets, it may be inferred that intercellular transport of PGH₂ from A549 cells to the platelet contributes to the increase in TxB₂ that results from the interaction of platelets with A549 cells.

Discussion

We have demonstrated that aspirin 81 mg daily for 2 weeks inhibits the biosynthesis of PGE₂ in humans by an average of 45%. Thus, even at this low dose, extra-platelet COX is inhibited significantly. To place this in the context of the effect of the non-steroidal anti-inflammatory drugs, our group found previously that ibuprofen 800 mg 4 times daily reduced PGE₂ production by 63% (44).

Metastasis is facilitated by cyclooxygenase-derived PGE₂ (54–57). PGE₂ enhances the metastatic process via multiple mechanisms that in combination yield a strong pro-metastatic effect. The effects of PGE₂ that promote metastasis include upregulation of the cell adhesion receptor, CD44 (56, 58, 59), induction of a transition from an epithelial to a mesenchymal phenotype (16, 20, 60), activation of matrix metalloproteinases (23, 54, 61), enhanced tumor cell migration (62), increased angiogenesis (63), and disruption of the immune surveillance of tumor cells (24, 26, 64–66).

Prostacyclin biosynthesis also reflects oxygenation of arachidonic acid by extra-platelet COX. Previous studies with small sample size (67, 68) suggested that low dose aspirin also can inhibit prostacyclin biosynthesis. Here we clearly demonstrate a 37% reduction in prostacyclin biosynthesis by aspirin 81 mg daily.

The potency of low dose aspirin as an inhibitor of COX-1 in platelets is one of the factors contributing to its anti-platelet efficacy even when administered at low doses. We found that aspirin inhibited COX isoforms expressed in lung adenocarcinoma cells as potently as its inhibition of platelet COX-1.

Inhibition of both COX isoforms expressed in cells from adenocarcinomas of the colon by aspirin is significantly greater than inhibition of platelet COX-1. Hepatic metastases from colon cancers derive from circulating tumor cells that lodge in the liver where they are exposed to the same high concentrations of aspirin that are so effective in inhibiting platelet COX-1 (Fig. 6), and are exposed to portal vein concentrations of aspirin constantly whereas circulating platelets are in the portal circulation only about 20–25% of the time. Our demonstration of the potency of aspirin in colon cancer cells, therefore, supports a hypothesis that low dose aspirin produces a high degree of inhibition of both COX isoforms in tumor cells from colon cancers that are sequestered in the hepatic portal vasculature. Given the evidence that PGE₂ is pro-metastatic, substantial inhibition of the biosynthesis of PGE₂ in tumor cells by low dose aspirin could be hypothesized to contribute to the anti-metastatic effect in cancers with venous drainage into the portal circulation. Indeed, the examination of metastasis in the meta-analysis of randomized controlled clinical trials of aspirin demonstrated exceptional prevention of metastasis from colon cancer. During the

trials while randomized aspirin was being administered, if patients had no clinical evidence of metastasis at the time of diagnosis of colon cancers, the hazard ratio for later metastasis was 0.13 (95% CI 0.03–0.56, p=0.007) (7). For adenocarcinomas other than those of the colon, the hazard ratio was 0.47. The profound inhibition of metastasis from colon adenocarcinomas by low dose aspirin is consistent with its marked inhibition of the biosynthesis of PGE₂ by COX-2 in the circulating tumor cells acting in concert with inhibition of platelet COX-1.

Circulating tumor cells from cancers with venous drainage into the systemic circulation would be exposed to lower concentrations of aspirin than cells released from colon adenocarcinomas. Consequently, less than complete inhibition of COX in these cells by low dose aspirin would be expected, as exemplified by the 45% reduction in total body PGE₂ biosynthesis. The potential biological importance of a partial reduction in PGE₂ biosynthesis is contingent on whether the effect of PGE₂ on its receptor approximates a log-linear function of the concentration of the agonist. The explosive release of TxA₂ within seconds after platelet activation (69) produces concentrations of this agonist that are supramaximal relative to receptor response. In contrast to the platelet, PGE₂ release progresses slowly from tumor cells (70). Inhibition of PGE₂ biosynthesis by ibuprofen in humans by only 63% (44) occurs at doses that are associated with both efficacy and gastrointestinal bleeding, and an effect of aspirin on tumor volume in NNK-treated mice is observed even at doses inhibiting PGE₂ levels by less than 50%. Accordingly, it is highly likely that a partial inhibition of PGE₂ biosynthesis by aspirin would reduce receptor-mediated responses, and importantly, could be additive with the anti-platelet effects of aspirin on metastasis. However, adding an intervention that achieves a more complete and selective inhibition of PGE₂ biosynthesis or action, e.g. an mPGES-1 inhibitor or an EP antagonist, conceivably could produce an anti-metastatic effect in non-colonic cancers that is superior to aspirin alone.

Adherence of platelets to circulating tumor cells (12, 13, 71) is important to the process of implantation at the metastatic site (14, 15) via interaction of platelet P-selectin with P-selectin glycoprotein ligand-1 on cancer cells (71, 72). The phenotype of tumor cells is reprogrammed by their interaction with platelets to a more metastatic phenotype (20). Recently, Paola Patrignani and her colleagues Dovizio *et al.* (39) and Guillem-Lobat (16) demonstrated that tumor cell-platelet interaction upregulates COX-2 expression and PGE₂ release in the colon cancer cells, and provide convincing evidence that blockade of platelet COX-1 by aspirin inhibits the PGE₂ release, inhibits EMT and also abrogates metastasis to the lung. We now provide evidence that adherence of platelets to lung adenocarcinoma cells also leads to PGE₂ production, indicating that the phenomenon they describe in colon cancer cells is generalizable to other cell types. We further demonstrate in lung adenocarcinoma cells that the release of PGE₂ is linked to a platelet-induced increase in cytosolic calcium in the tumor cells. Treatment of platelets with aspirin prevents calcium mobilization in the tumor cells and PGE₂ release, but does not alter the expression of COX-2. We propose that interaction of activated platelets with lung adenocarcinoma cells leads to calcium-mediated activation of cPLA₂ (52, 53), which yields the COX-2 substrate, arachidonic acid, from membrane phospholipids. Inhibition of platelet activation with aspirin reduces calcium mobilization in the tumor cells, thereby inhibiting formation of PGE₂. The mechanism of thromboxane-dependent calcium mobilization in A549 remains to be characterized.

However, it has been recently demonstrated that A549 cells express the thromboxane receptor (TP)(73). Activation of TP is well known to trigger calcium mobilization via G_q activation, a mechanism that could possibly be involved in the effective PGE₂ production observed in our experiments.

In conclusion, low dose aspirin has a significant effect on extra-platelet cyclooxygenases. It inhibits systemic biosynthesis of PGE₂ in humans by 45%, while blocking platelet TxB₂ production by 96.6 %, and therefore is only relatively selective for the platelet. Aspirin inhibits COX catalytic activity and PGE₂ biosynthesis in lung and colon adenocarcinoma cells with a potency as great or greater than that for COX-1 in platelets.

These findings provide a basis for considering that even partial inhibition of tumor COX activity by low dose aspirin could reduce the biosynthesis of PGE₂ by these cells. In the context of the convincing body of data that PGE₂ facilitates metastasis, it can be hypothesized that a reduction in tumor cell PGE₂ could be additive with its anti-platelet effect, and thereby contribute to the reduction of mortality and metastasis from adenocarcinomas. Low dose aspirin is likely to produce a high degree of inhibition of COX in tumor cells arising from colon cancer that are sequestered in the portal circulation where they are exposed to the same high concentration of aspirin as the platelet. This forms the basis for a hypothesis that the remarkable prevention of metastasis from colon cancer by low dose aspirin results from its well recognized effect on platelet COX-1 combined with strong inhibition of PGE₂ biosynthesis in potentially metastatic tumor cells sequestered in the liver. A conclusion that PGE₂ contributes to colon cancer metastasis also implies that the metastatic potential of cancer cells that are not sequestered in the portal circulation could be further inhibited by combining aspirin with an inhibitor of PGE₂ production or action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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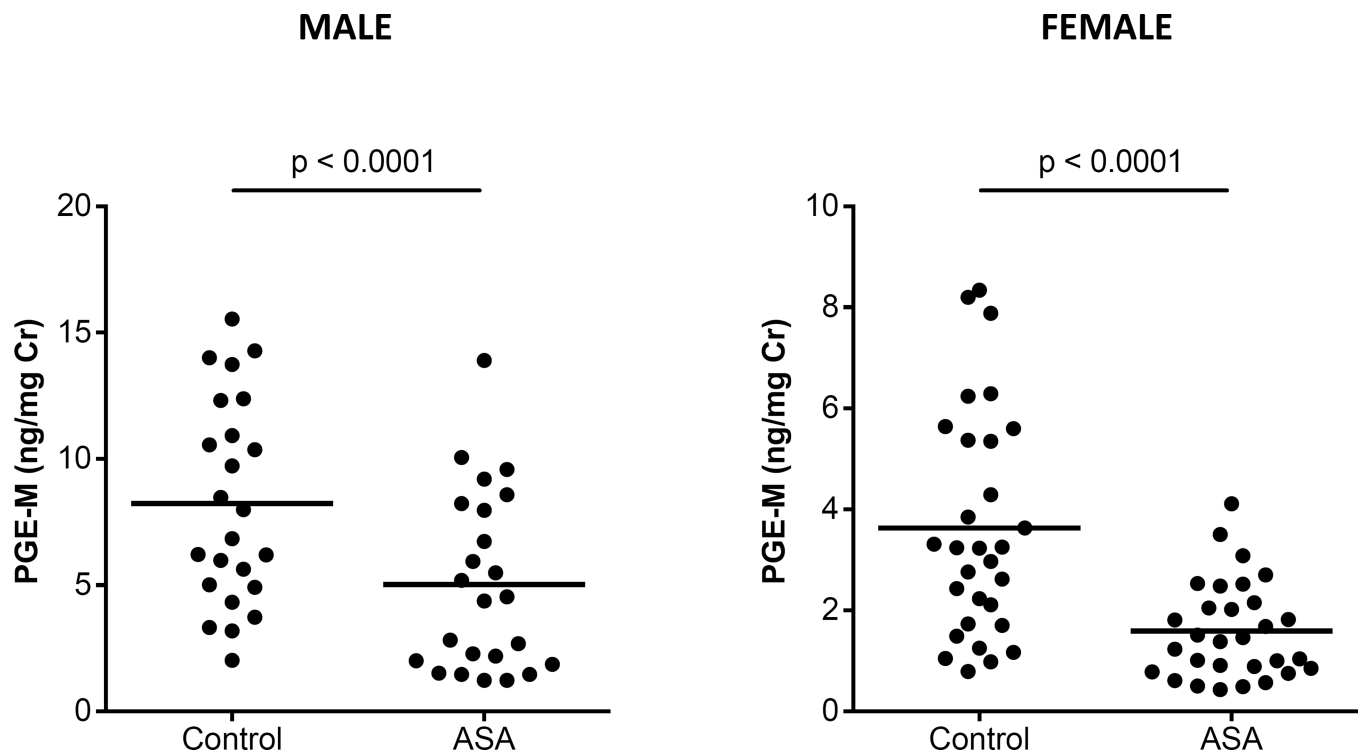
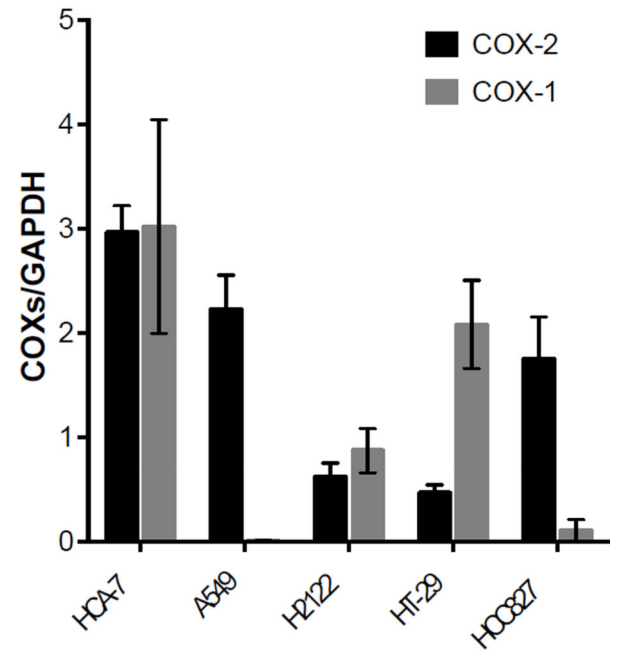
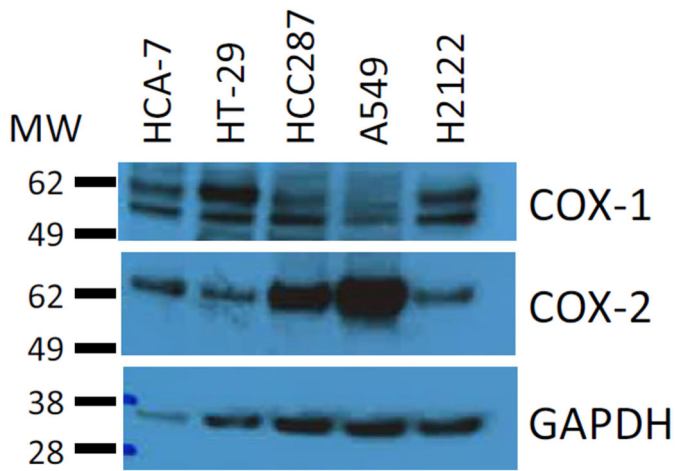


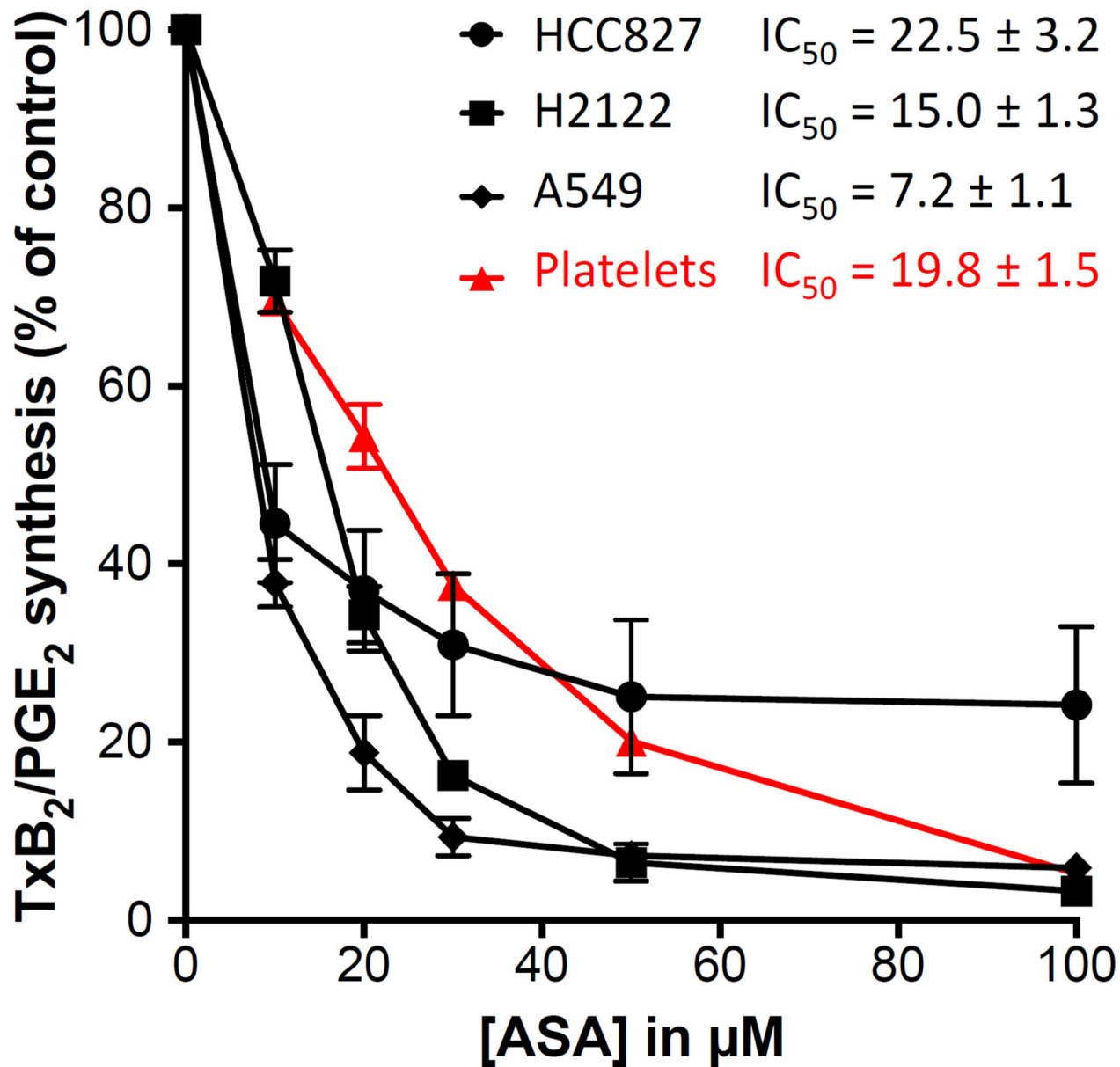
Figure 1.

Daily low dose aspirin inhibits prostaglandin E₂ biosynthesis in humans. 81 mg of aspirin was administered daily to healthy participants for 2 weeks. PGE-M, the urinary metabolite of prostaglandin E₂, was measured by LC/MS before and after aspirin therapy. Data is presented for males and females. Paired analysis comparisons were performed using Wilcoxon-ranked tests. Low dose aspirin inhibited the biosynthesis of PGE₂ by 45% ($p < 0.0001$, $n = 54$).

A.



B.



C.

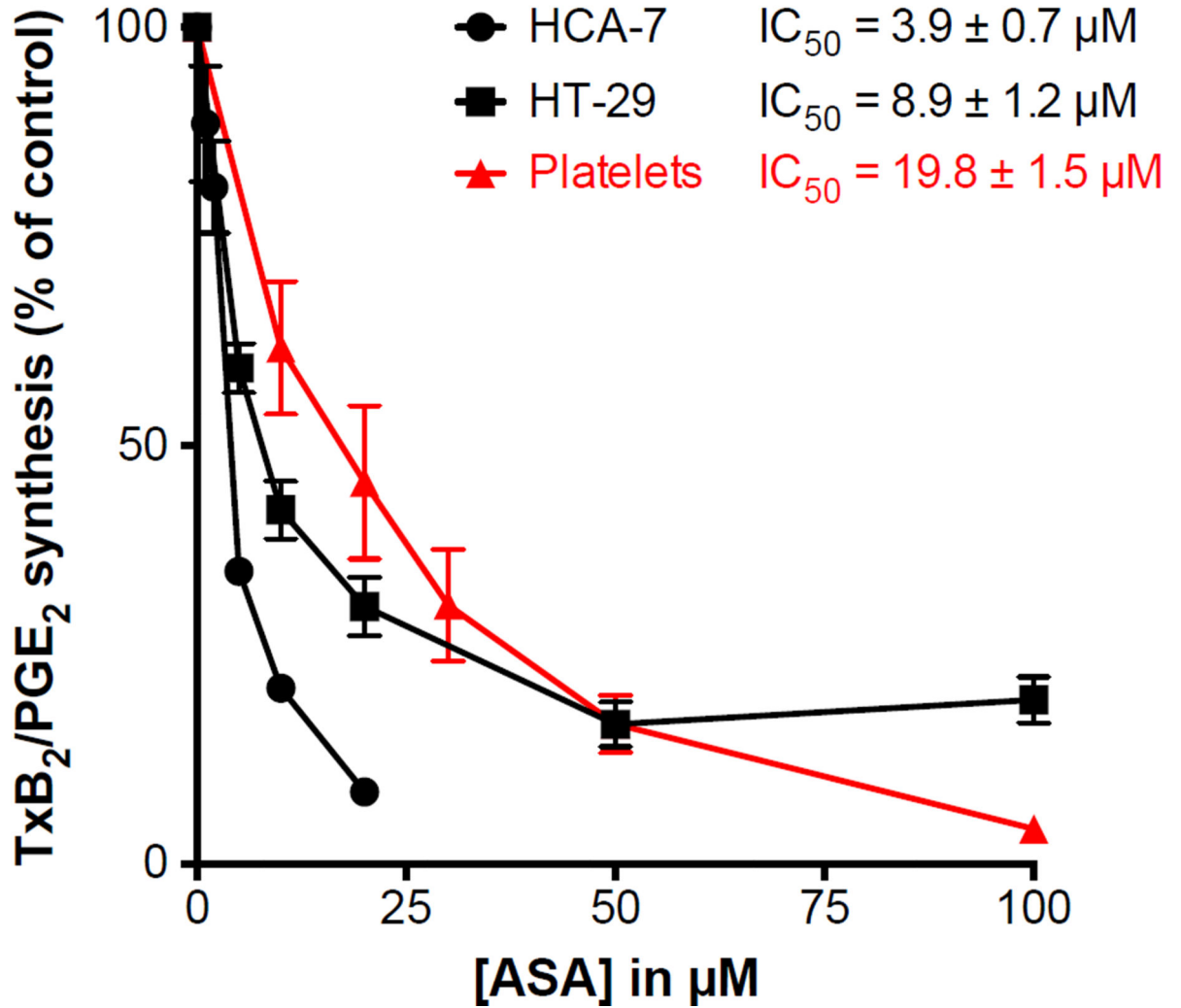


Figure 2. Aspirin inhibits COX-derived PGE₂ in adenocarcinoma cells as potently as it inhibits COX-1-dependent TxB₂ in washed platelets. Three lung and two colon adenocarcinoma cell lines were used to examine the potency of aspirin to inhibit PGE₂ synthesis. (A) Western blots analysis of COX-1 and COX-2 expression in H2122, HCC827, A549 and HT 29 cells after cell activation with IL-1β ng/ml. HCA=7 was not activated prior analysis. Protein expressions are expressed as a ratio against housekeeping protein GAPDH. n = 3 (B) Washed platelet biosynthesis of TxB₂ and lung adenocarcinoma cell line biosynthesis of PGE₂ were measured by GC/MS. (C) Washed platelet biosynthesis of TxB₂ and biosynthesis of PGE₂ in the colon adenocarcinoma cell lines, HCA-7 and HT-29, were measured by GC/MS. Aspirin IC₅₀ for the different cell types are indicated. Values represent means ± S.E.M. n = 6.

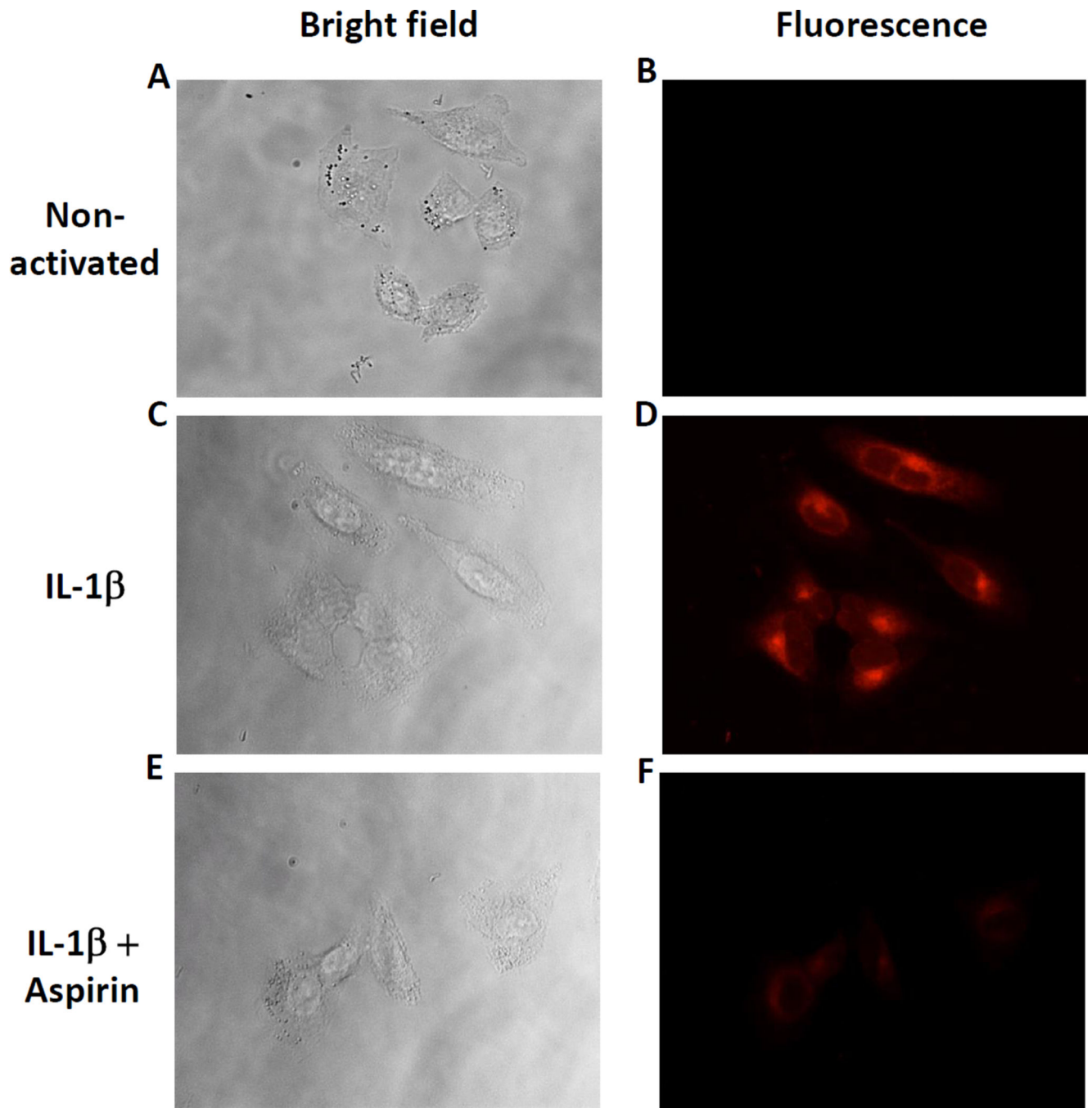
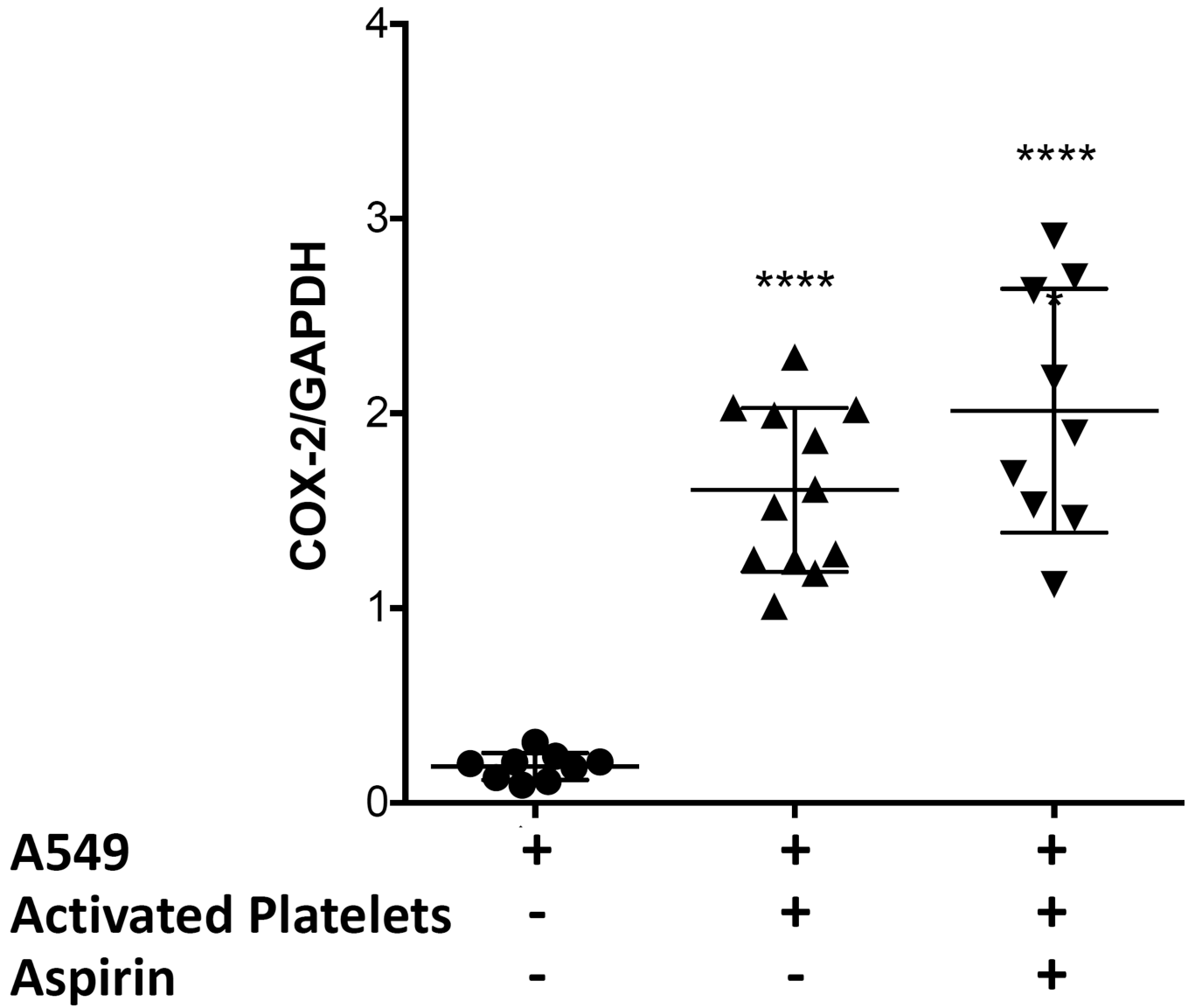
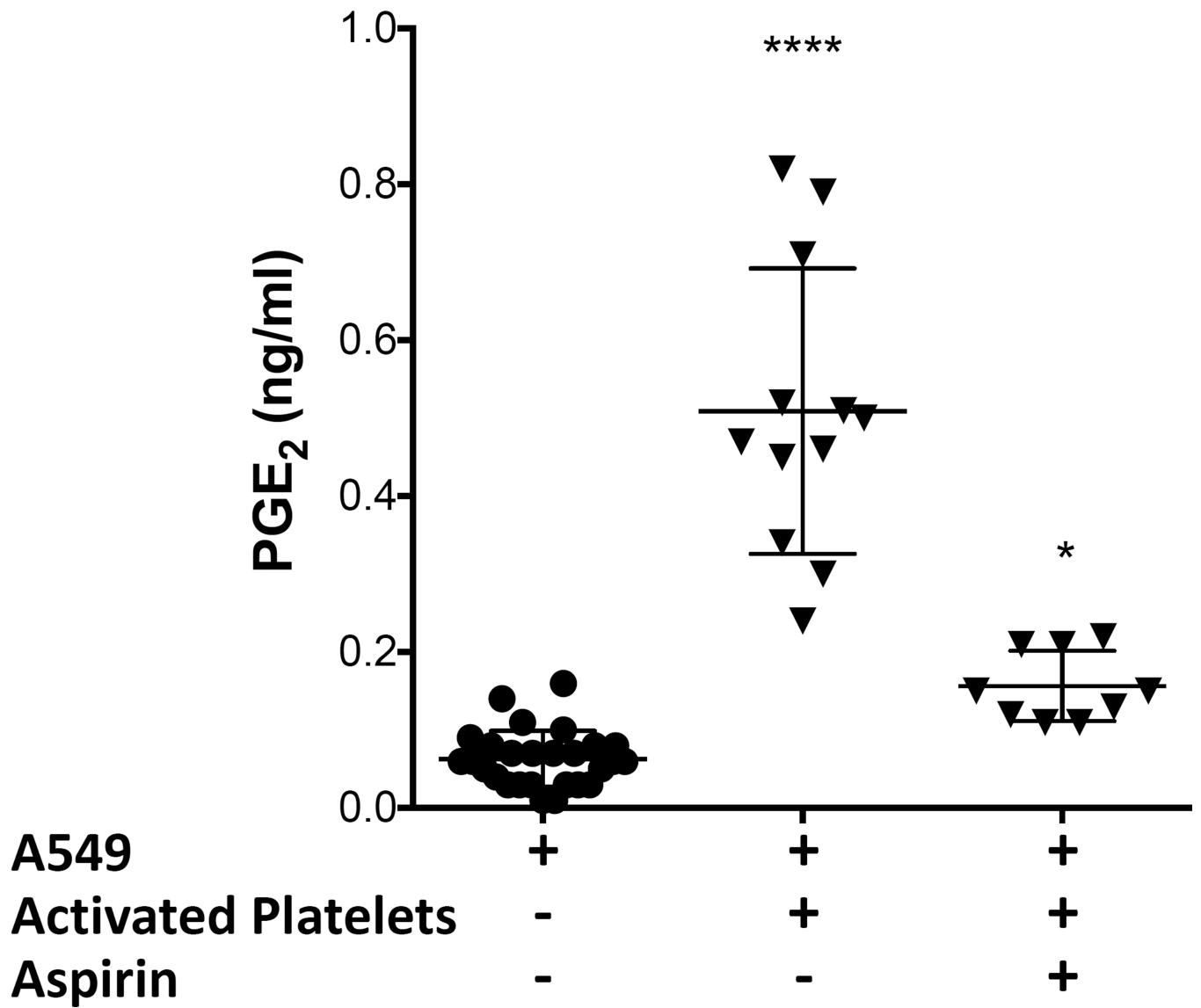


Figure 3. Aspirin inhibits COX-2 labeling by fluorocoxib A in A549 lung adenocarcinoma cells. (A) (C) and (E) Bright field images of A549 cells. (B) Non-activated A549 cells were treated with 200 nM of rhodamine-conjugated indomethacin (fluorocoxib A) for 30 min. (D) IL-1 β -activated A549 cells were treated with 200 nM of rhodamine-conjugated indomethacin (fluorocoxib A) for 30 min. (F) IL-1 β -activated A549 cells were pretreated with 100 μ M aspirin for 30 min before adding fluorocoxib A. Pretreatment with aspirin inhibited COX-2 associated fluorocoxib A fluorescence.

A.



B.



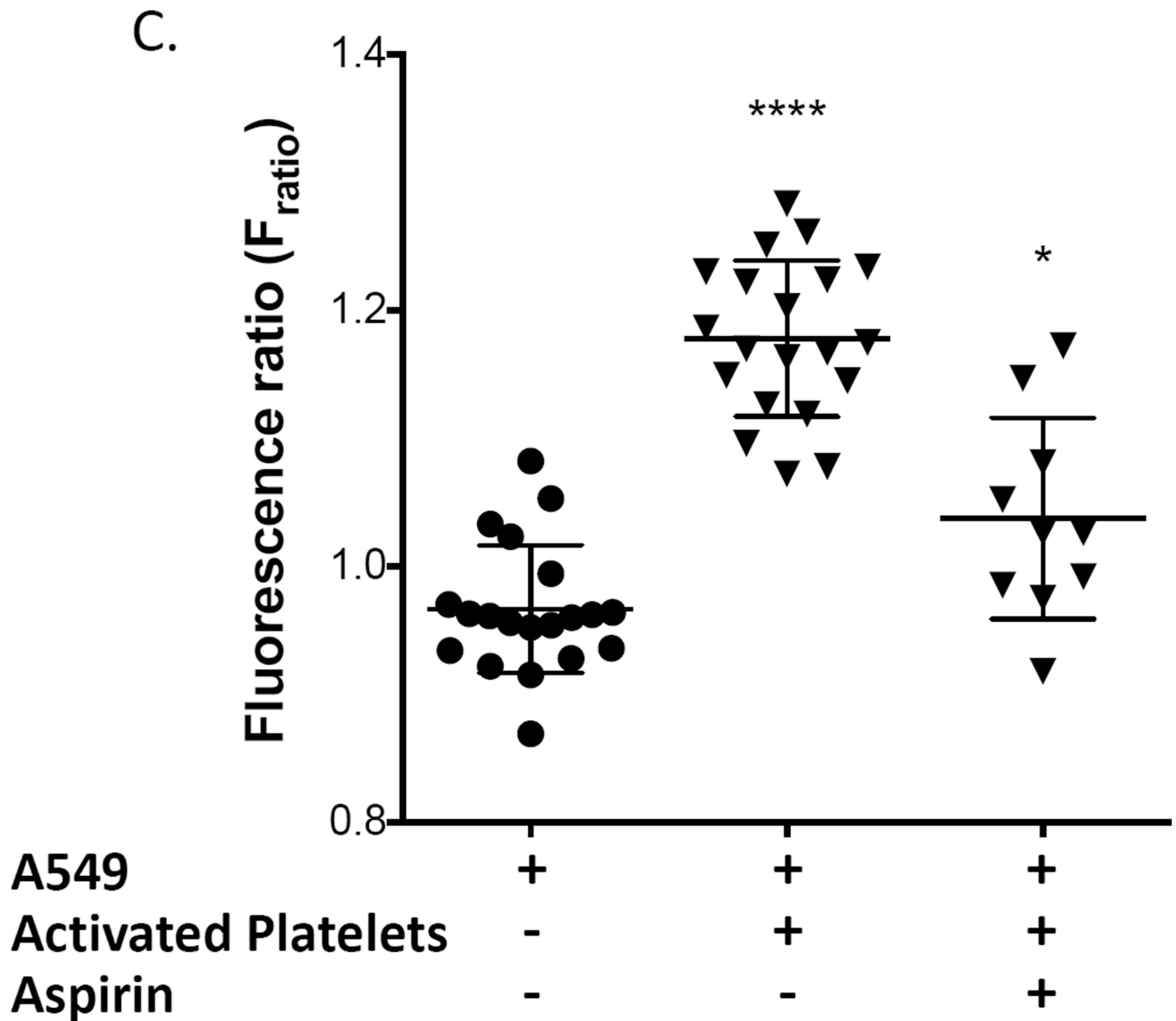


Figure 4.

Activated platelets induce COX-2 expression and PGE₂ production in lung adenocarcinoma cell line A549. Washed platelets were activated with 50 μM ADP and subsequently incubated with A549 cells. Incubation of A549 cells with activated platelets increased COX-2 protein expression (A), PGE₂ production (B), and intracellular calcium mobilization (C) from baseline. Pretreatment of blood with aspirin (100 μM) prior to isolation of washed platelets and their activation with ADP effectively blocked PGE₂ production by A549 cells (B) and intracellular calcium mobilization (C) but not COX-2 protein upregulation (B). (A) **** indicate p < 0.0001 versus A-549 alone, n = 9; (B) and (C) **** indicate p < 0.0001 versus all other conditions, * indicates p < 0.05 vs A-549 alone, n = 9.

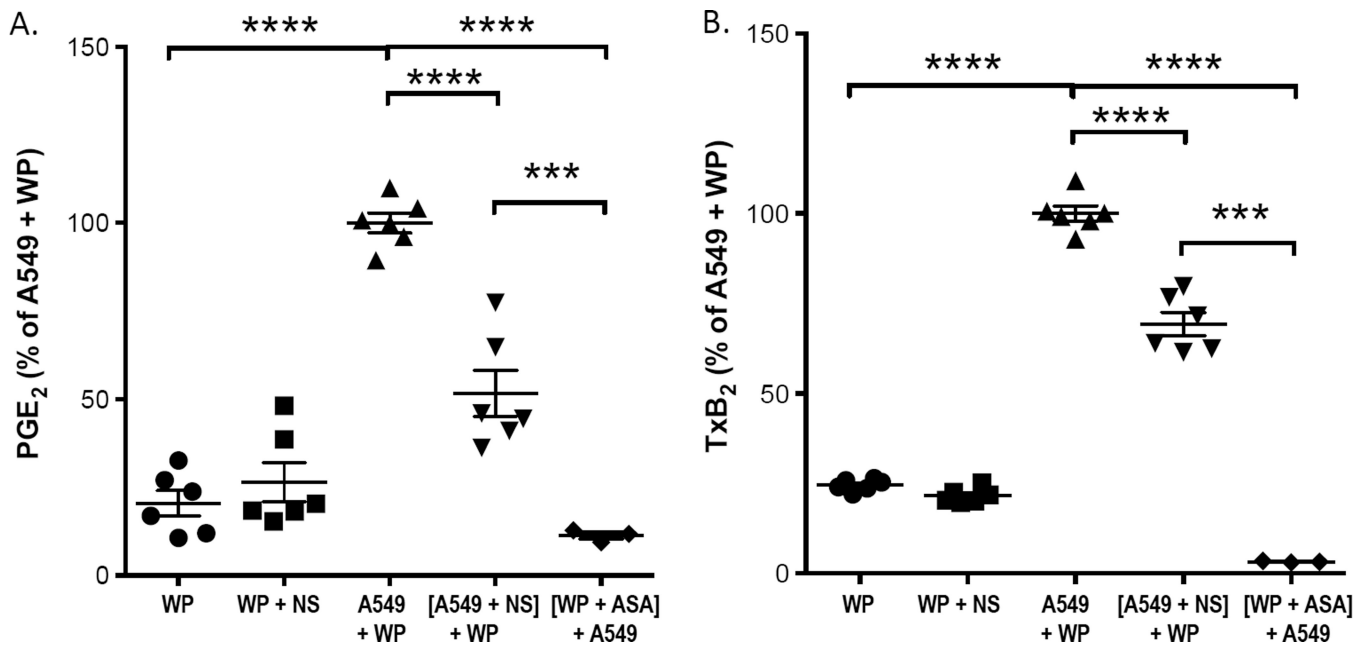


Figure 5.

Both platelet COX-1 and tumor cell COX-2 contribute to PGE₂ and TxB₂ production when platelets interact with the lung adenocarcinoma cells, A549. Washed platelets obtained from blood treated with aspirin ([WP + ASA]) or vehicle (WP) were activated with 50 μM ADP and subsequently incubated with A549 cells. PGE₂ production (**A**), and TxB₂ production (**B**) were measured by GC/NICI/MS. The COX-2 specific inhibitor NS-398 inhibited PGE₂ production by 48% and platelet TxB₂ production by 31%. NS-398 (100 nM) treatment of platelets in the absence of A549 cells did not inhibit TxB₂ production (WP + NS). **** indicates $p < 0.0001$ and *** indicates $p < 0.001$.

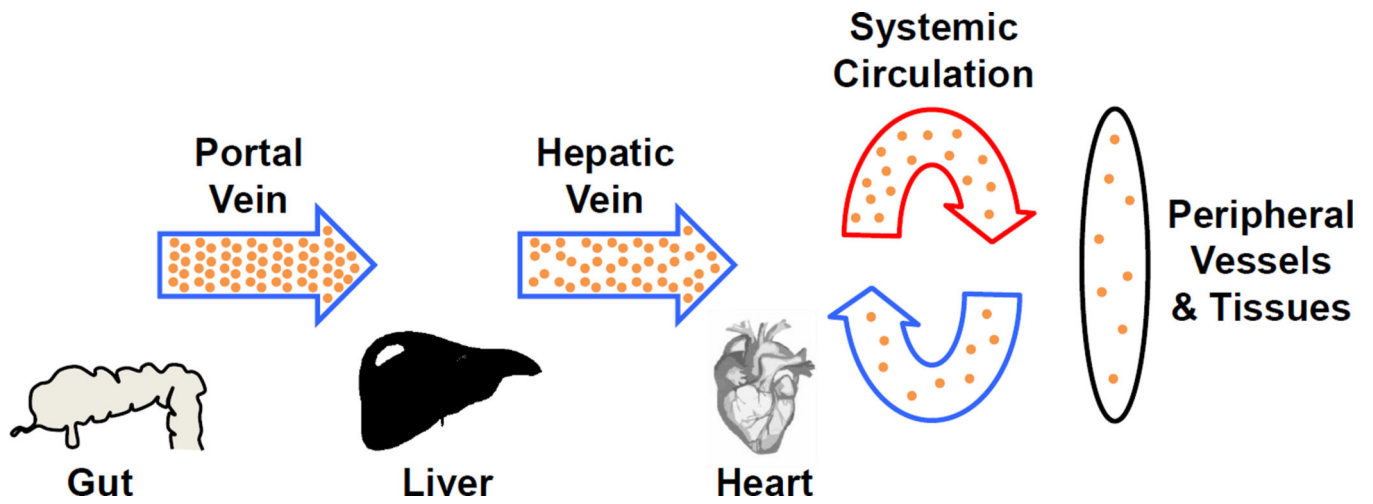


Figure 6. Aspirin concentration in the different circulation compartments. Aspirin concentration is represented by the density of the yellow dots. Aspirin concentration decreases as it travels through the liver and is further diluted in the systemic circulation. The highest plasma concentrations are in the portal vein and in the liver where colon cancer metastases will be exposed to aspirin.

Table 1

Demographics of Study Population.

	All Subjects (N=52)
Age (y)	68[61, 76]
Male sex	36 (69%)
Race – White	49 (94%)
Black	2 (4%)
Other	1 (2%)
Current or Former Smoker	26 (50%)
BMI (kg/m ²)	26.2[23.7, 29.2]
Platelet count (×10 ³ /mL)	184[168, 219]

Values are median [IQR] or counts (%). BMI = body mass index;

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