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ROLE OF NEWLY SYNTHESIZED CYCLOOXYGENASES IN ASPIRIN RESPONSIVENESS IN THE HUMAN MEGAKARYOBLASTIC CELL LINE MEG01

by
ADHAM KASSEM FAKIH

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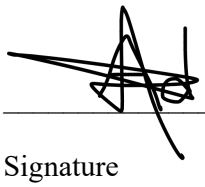

January 5, 2022

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ABSTRACT
OF THE THESIS OF

Adham Kassem Fakih for Master of Science
Major: Biochemistry

Title: Role of Newly Synthesized Cyclooxygenases in Aspirin Responsiveness in the Human Megakaryoblastic Cell Line MEG01

Background: Aspirin is one of the most commonly used drugs in cardiovascular medicine. It interferes with platelet aggregation by inhibiting cyclooxygenases in blood platelets and megakaryocytes. Low-dose aspirin has at least two distinct cellular targets that result in a long-lasting antiplatelet effect over a 24-hour dosing interval. The platelet (COX)-1 is the first target, whose blockage occurs pre-systemically in portal blood and is cumulative with repeated daily dosing. The second target is the megakaryocyte (MK) COX-1 and COX-2 isozymes, whose acetylation is dependent on systemic bioavailability and contributes to the long-lasting duration of thromboxane A_2 inhibition, as newly released platelets express acetylated COX isozymes derived from bone marrow progenitors for a significant portion of the 24-hour dosing cycle. Aspirin has been shown to have variability in its response among patients. However, the difference in the recovery of cyclooxygenase activity could be the consequence of a difference in the stability of cyclooxygenases or an enhanced rate of enzyme renewal. Because of the restricted accessibility of MKs *in vivo* in humans, and to study the effect of aspirin on MKs, the use of a megakaryocytic cell line is therefore of great importance.

Aims: In this project, we aim to detect the expression of several genes in the arachidonic acid metabolism pathway, investigate if aspirin affects their expression and if aspirin modifies the decay of these genes.

Methods: Meg01 cells were treated with 10 μM of ASA for 30 minutes, then plated in a 12 well plate for 24 or 72 hours. Cells were then centrifuged at 300g for 5 minutes followed by the addition of 200 μl trizol or qiazol to the pellet to extract RNA, then RT-PCR was performed. To investigate whether aspirin modifies the half-life of cyclooxygenases mRNA, the levels of gene expression were determined at different time points (0, 1, 3, 18, and 24 hours) by RT-PCR after the addition of actinomycin D, an inhibitor of the transcription machinery.

Results: The expression of several genes in MEG01 and CHRF-288 showed that both MEG01 and CHRF-288 express most of the genes essential for prostanoid synthesis and signaling. Investigating the effects of ASA on gene expression of MEG01, we found that treatment of cells for 24 hours resulted in a statistically significant decrease in

COX-2 gene (around 40% decrease ± 10%, $p<0.02$) compared to vehicle-treated MEG01. No significant change was observed for the other genes. Treatment of the cells for 72 hours resulted in a significant increase in the gene expression of TXAS (around 78% increase ± 27%, $p<0.05$) compared to vehicle-treated cells. Comparing ASA 24 hours and 72 hours cases, there was a significant increase in expression for most genes in the 72 hours treatment period. Investigating the effect of ASA on RNA stability of these genes, the results show that there was no modification on the mRNA of PTGS1, while there was a stabilization of mRNA at lower levels for COX-2 and PGES1. mRNA for TXAS decreased and gave half-life around 3 hours in the treatment group, while TXAR mRNA in the treatment group was stabilized at 100% preventing further increase in the expression. For EP1, EP2, and EP4 the mRNA was stabilized at lower values in the treatment group compared to the control group. However, in EP3 the half-life of the mRNA was around 6 hours in the presence of ASA compared to around 18 hours without ASA.

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ABBREVIATIONS

AA	Arachidonic Acid
ACD	Actinomycin D
ASA	Aspirin
CD	Cluster of differentiation
CFU-MK	Colony Forming Unit-Megakaryocytes
CHRF	Human Megakaryoblastic Cell Line
CMP	Common Myeloid Progenitor
COX	Cyclooxygenase
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
DMS	Demarcation Membrane System
DPs	Prostaglandin D_2 Receptors
EP1	Prostaglandin E_2 Receptor 1
EP2	Prostaglandin E_2 Receptor 2
EP3	Prostaglandin E_2 Receptor 3
EP4	Prostaglandin E_2 Receptor 4
FPs	Prostaglandin F Receptor
GATA-1	Globin Transcription Factor 1
GI	Gastrointestinal
HOX	Hydroperoxidase
HSC	Hematopoietic Stem Cell
IC50	Half Maximal Inhibitory Concentration
IL-1α	Interlukin-1 alfa

IMS	Invaginated Membrane System
IP	Prostaglandin I_2 Receptor
kDa	Kilodalton
MEG01	Human Megakaryoblastic Leukemia Cell Line
MEP	MK-Erythroid-Progenitor
mg	Milligram
µm	Micrometer
µmol/L	Micromoles/liter
MK	Megakaryocyte
ng/ml	nanogram/milliliter
ng/kg/min	nanogram/kilogram/minute
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PAS	Periodic Acid-Schiff
PD	Pharmacodynamic
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H-Synthase
PGI_2	Prostaglandin I_2
Ph1	Philadelphia Chromosome 1
PK	Pharmacokinetic

PPI	Protein Pump Inhibitor
PU.1	Transcription Factor Encode by SPI1 gene
P_2Y_{12}	Gi Receptor for Adenosine Diphosphate
ROS	Reactive Oxygen Species
Ser 516	Serine 516
Ser 529	Serine 529
TP	Thromboxane Receptor
t-PA	tissue-type Plasminogen Activator
$t_{1/2}$	Half-life
TX	Thromboxane
TXAS	Thromboxane A_2 Synthase
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
8-iso-$PGF_{2\alpha}$	8-iso-prostaglandin $F_{2\alpha}$

CHAPTER 1

INTRODUCTION

1.1. Aspirin

1.1.1. Variability in the Pharmacological Response to Aspirin

Aspirin (ASA) is one of the widely prescribed drugs in cardiovascular medicine. It interferes with platelet aggregation by inhibiting cyclooxygenases (COX) in blood platelets and megakaryocytes [1, 2]. Patients suffering from myocardial infarction, vascular mortality, and ischemic stroke are set to be treated by ASA as it is considered the gold standard treatment for such severe cases [3]. Despite its crucial function in therapy, some patients tend to have decreased response to standard low-dose aspirin given once daily [4].

Reduced response to standard low-dose ASA gradually results in higher levels of residual thromboxane A_2 (TXA_2) and thus platelet activation and aggregation [5]. Aspirin insensitivity, especially in high levels, could be related to or accompanied by an increased risk of cardiovascular incidents [6]. However, neither the response variability mechanism(s) nor its reversibility has been identified. A combination of cellular, molecular, clinical, and genetic properties influencing platelet function is likely to cause lower-than-expected ASA variability [7].

1.1.2. COX-1 and COX-2 the Targets of Aspirin

Despite their striking structural and functional similarities, COX-1 and COX-2 are encoded by separate genes, have diverse roles, and have been shown to selectively pair with different isoforms of prostaglandin (PG) H synthases [8]. COX-2 appears to

preferentially localize in the nuclear envelope [9-11], whereas COX-1 appears to be found in both the perinuclear zone and the cytoplasmic membrane system [9, 11, 12] (figure 1) [13].

COX-1 is well-known for its physiologic housekeeping duties, such as platelet production of proaggregatory TXA_2 and gastric mucosa production of cytoprotective PGE_2. COX-2, on the other hand, is almost undetectable at rest and is activated by cytokines, endotoxins, growth factors, or tumor promoters. Yet, there are numerous exceptions to this simplistic model of constitutive COX-1 and inducible COX-2 [8].

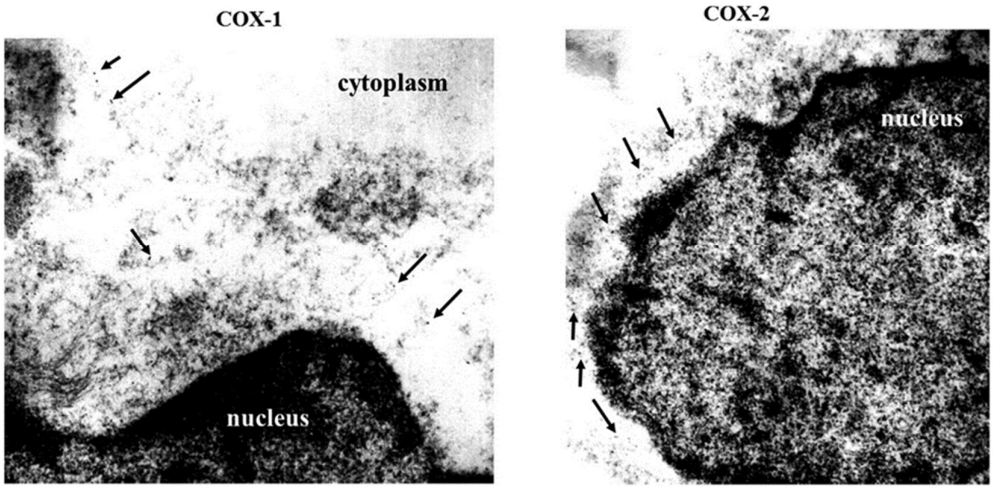


Figure 1. Immunogold Labeling of the COX Isozyme in Human Megakaryocytes

In platelets, COX-1 is expressed while COX-2 is not [8, 14]. Platelets produce TXA_2 only through COX-1. Both COX-2 and COX-1 are constitutively produced in mature human megakaryocytes, implying that both COX-isoforms are involved in prostanoid synthesis during human megakaryocytopoiesis [8, 15]. Because COX-1 is the most common COX isoform found in platelets, the anti-platelet activity of ASA is

mostly due to its suppression. COX-1 cannot be rapidly reproduced in platelets, hence COX-1 activity can only be restored through *de novo* platelet biogenesis [16].

1.1.3. Mechanism of Action of Aspirin

1.1.3.1. Site of Action and the Initial Steps of COX Inhibition

The ability of aspirin to permanently inactivate the cyclooxygenase (COX) activity of prostaglandin H-synthase (PGHS)-1 and -2 (also known as COX-1 and COX-2) is the best-understood mechanism of action [17-19]. These isozymes catalyze the conversion of arachidonic acid (AA) to PGH_2, the first committed step in prostanoid biosynthesis [1]. PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2 are all immediate precursors of PGH_2 (figure 2) [1]. COX-1 and COX-2 are homodimers of a monomeric unit of 72 kDa. An epidermal growth factor-like domain, a membrane-binding domain, and an enzymatic domain are all separate folding units in each dimer [19]. A peroxidase catalytic site and a distinct near site for COX activity are positioned at the apex of a narrow, hydrophobic channel within the enzymatic domain [1] (figure 3) [20].

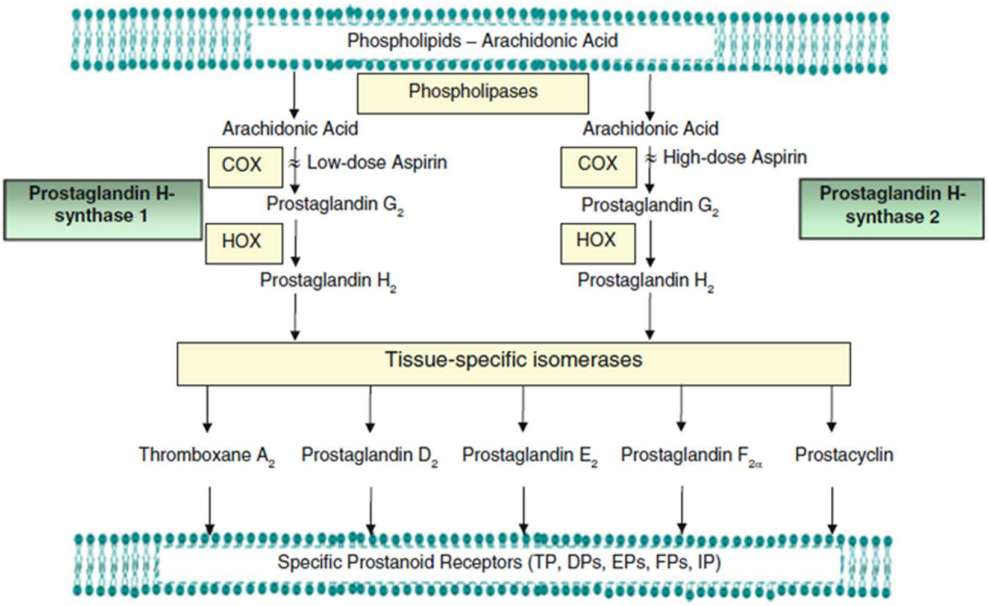


Figure 2. Arachidonic Acid Metabolism and Mechanism of Action of Aspirin

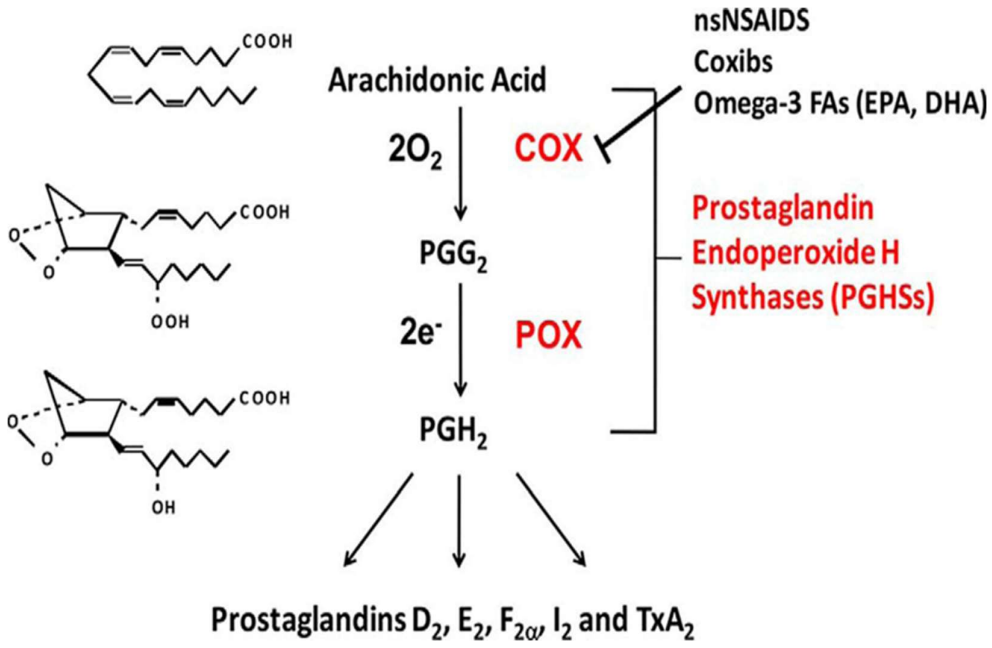


Figure 3. COX Mechanism of Action and its Inhibition by Aspirin

Aspirin permanently inactivates COX activity by blocking the COX channel as a result of acetylation of a strategically located serine residue (Ser 529 in human COX-1, Ser 516 in human COX-2) that prevents substrate access to the enzyme's catalytic site [21]. The modified serine side chain is stabilized against hydrolysis by the COX channel's hydrophobic environment. Low doses of aspirin given once daily can be used to inhibit COX-1-dependent platelet function. On the other hand, inhibition of COX-2-dependent pathophysiological processes (such as hyperalgesia and inflammation) necessitates higher aspirin doses (most likely because acetylation is determined by the oxidative state of the enzyme and is restricted in cells with high peroxide tone) [22], and a significantly shorter dosing interval as nucleated cells rapidly resynthesize the enzyme [1].

1.1.3.2. Arachidonic Acid Derivatives are Affected by COX Inhibition

PGH_2 is primarily processed by human platelets and vascular endothelial cells to produce TXA_2 and PGI_2, respectively [19]. TXA_2 promotes platelet aggregation and vasoconstriction, whereas PGI2 promotes platelet aggregation and vasodilation (figure 4) [13]. While TXA_2 is primarily a COX-1-derived product (mostly from platelets, where platelets can also produce PGE_2) and thus highly susceptible to aspirin inhibition, vascular PGI_2 can originate from both COX-1 and, to a greater extent, COX-2, even under physiological conditions [23]. COX-1-dependent PGI_2 production is transient and sensitive to aspirin inhibition in response to agonist stimulation, such as bradykinin [24]. COX-2-mediated PGI_2 production occurs over time in response to laminar shear stress [25] and is unaffected by standard aspirin antiplatelet doses. This could explain why, despite transient suppression of COX-1-dependent PGI_2 release [24], there is still

significant residual PGI_2 biosynthesis *in vivo* at daily doses of aspirin in the 30–100 mg range [26].

Higher doses of aspirin do not appear to be sufficient to initiate or predispose to thrombosis when PGI_2 formation is suppressed more profoundly. PGI_2 appears to be an important antithrombotic autacoid, according to two lines of evidence. The first is the finding that mice lacking the PGI_2 receptor were more susceptible to thrombosis in an experimental setting [27]. The second is the identification of an increased risk of myocardial infarction associated with COX-2 inhibitors [28], which supports the idea that PGI_2 is a key mechanism of thromboresistance when platelet TXA_2 biosynthesis is inadequately inhibited [29].

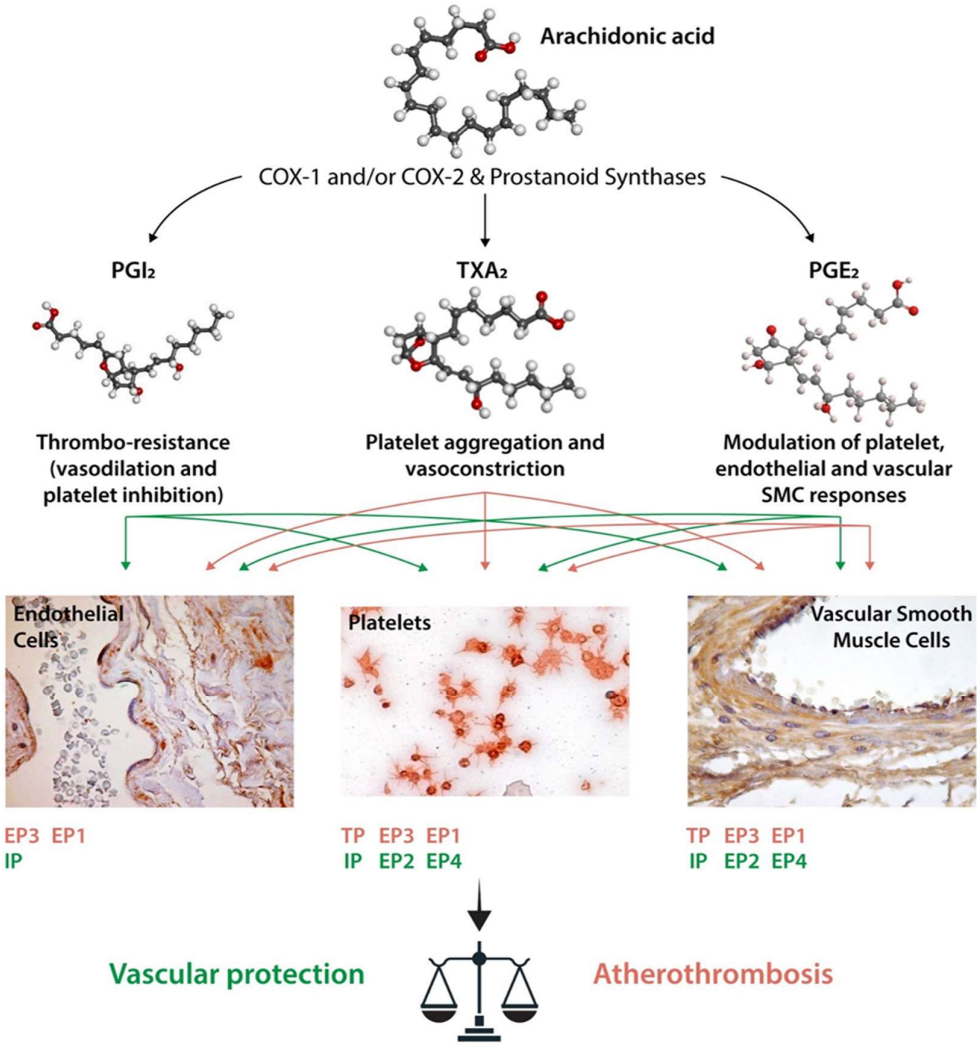


Figure 4. The Essential Role of Arachidonic Acid Derivatives

1.1.4. Pharmacokinetics and Pharmacodynamics of Aspirin

1.1.4.1. From Metabolism to Peak Activity

In the stomach and upper intestine, aspirin is absorbed quickly [30]. Aspirin reaches peak plasma levels 30–40 minutes after ingestion, and TXA_2-dependent platelet function is inhibited by 1 hour. Enteric-coated aspirin, on the other hand, can take up to 3–4 hours to reach peak plasma levels after administration. Over a wide range of doses,

the oral bioavailability of regular aspirin tablets is around 40–50 percent [31] (figure 5) [32]. Some enteric-coated tablets [33] and sustained-release, microencapsulated preparations [31] have been found to have lower bioavailability [33].

Platelet inhibition may be inadequate in heavier subjects due to poor absorption from the higher pH environment of the small intestine and lower bioavailability of some enteric-coated preparations [33]. To achieve selective inhibition of platelet TXA_2 production without suppressing systemic PGI_2 synthesis, a controlled-release formulation with negligible systemic bioavailability was developed [24]. This was used successfully in the Thrombosis Prevention Trial, but it is unclear whether the controlled-release formulation has any advantages over plain aspirin [1].

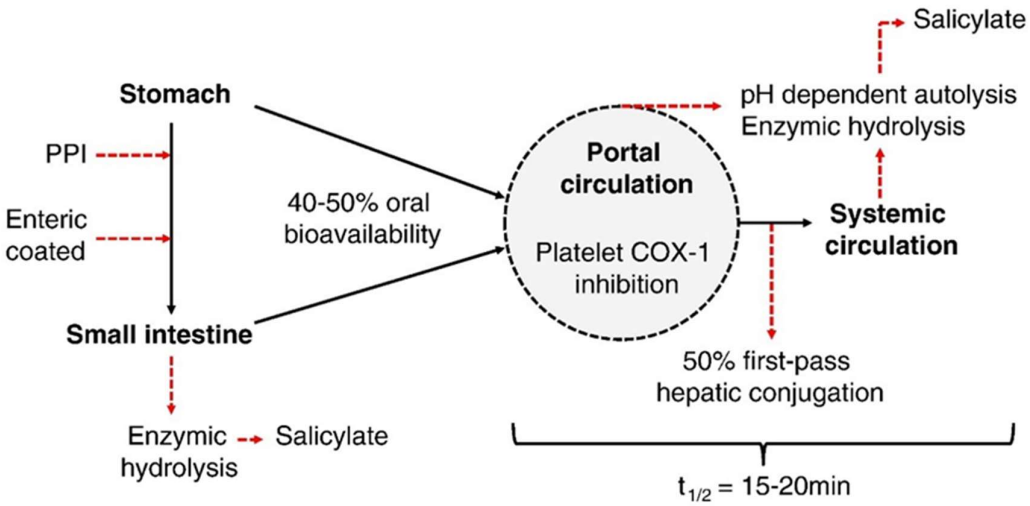


Figure 5. Aspirin Metabolism

1.1.4.2. Half-Life of Aspirin and the Recovery of TXA_2 Biosynthesis

In human circulation, the half-life of decay of aspirin plasma concentration is between 15 and 20 minutes [30]. Regardless of this rapid clearance, and since aspirin irreversibly inactivates COX-1 [17, 18], its inhibitory impact lasts for the life of the platelet [34]. Aspirin also acetylates the enzyme in megakaryocytes before the release of

new platelets into the circulation [35]. Regarding the human platelets, they have an average life span that ranges from 8 to 10 days. As a result, approximately 10–12 percent of circulating platelets are replaced every 24 hours [1]. Low-dose aspirin has at least two distinct cellular targets that result in a long-lasting antiplatelet effect over a 24-hour dosing interval. The platelet (COX)-1 is the first target [31], whose blockage occurs pre-systemically in portal blood and is cumulative with repeated daily dosing [36]. The second target is the megakaryocyte (MK) COX-1 and COX-2 isozymes [8], whose acetylation is dependent on systemic bioavailability and contributes to the long-lasting duration of TXA_2 inhibition, as newly released platelets express acetylated COX isozymes derived from bone marrow progenitors for a significant portion of the 24-hour dosing cycle. Reduced systemic bioavailability of aspirin as seen in obesity, for example, and abnormal megakaryopoiesis, as seen in essential thrombocythemia and other disease states, may limit the duration of platelet COX-1 suppression and necessitate a shorter dosing interval [37].

The recovery of TXA_2 biosynthesis *in vivo* after aspirin withdrawal is slightly faster than predicted by platelet turnover [26], possibly due to the nonlinear relationship between platelet COX-1 inhibition and inhibition of TXA_2 biosynthesis *in vivo*, explained by the pharmacological inhibition of serum TXB_2 *ex vivo* that gave a nonlinear relationship compared with the percentage of inhibition of urinary 11-dehydro-TXB_2 excretion *in vivo* [38, 39] (figure 6) [1]. Because complete suppression of TXA_2-dependent platelet function necessitates a >97% inhibition of COX-1 activity [39], even a minor recovery of this activity—as seen 2–3 days after aspirin withdrawal—can maintain a full aggregatory response [1].

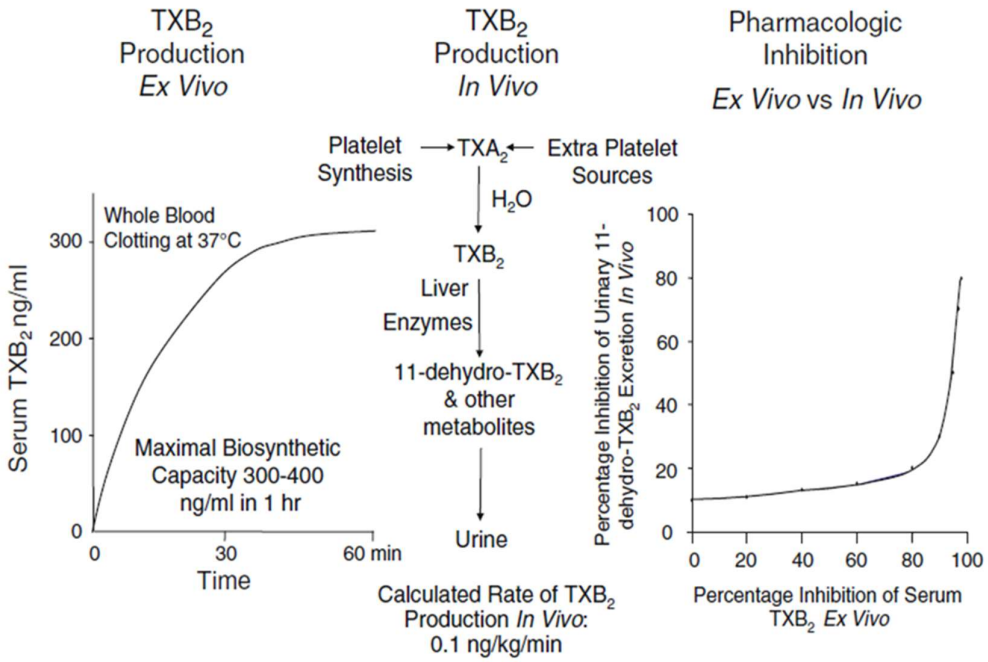


Figure 6. Platelet COX-1 Activity and Inhibition of TXA_2 Biosynthesis *In Vivo*

1.1.5. Antithrombotic Effects of Aspirin

Aspirin's main antithrombotic activity is to acetylate cyclooxygenase-1 (COX-1) in platelets, inhibiting TXA_2 formation. Platelet aggregation is hampered by decreased synthesis of this lipid platelet agonist [40]. Reduced thrombin generation and changes in fibrin structure, such as enhanced clot permeability, are potential side effects of aspirin. Reduced thrombin generation is likely related to poor platelet function or decreased tissue factor expression, whereas acetylation of fibrinogen causes alterations in the fibrin structure, which can lead to faster fibrin clot dissolution [40]. Increased tissue-type plasminogen activator (t-PA) release from endothelial cells could be another profibrinolytic effect of aspirin (figure 7) [40].

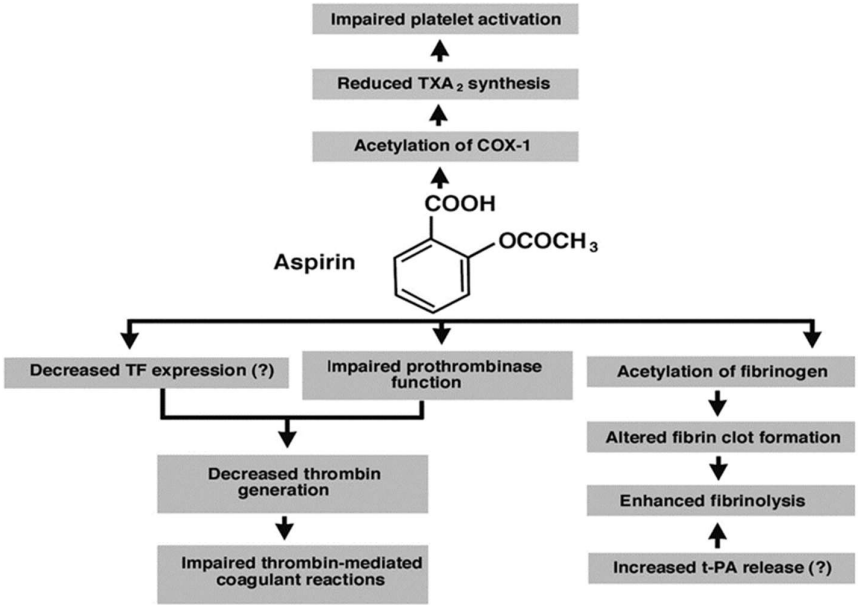


Figure 7. Aspirin's Antithrombotic Effects

Several issues concerning aspirin's antithrombotic effects have been debated over the last 20 years. The following are some of them: the ideal aspirin dosage to maximize clinical efficacy while minimizing gastrointestinal (GI) toxicity; the idea that part of aspirin's antithrombotic effect is unrelated to platelet TXA_2 inhibition; and the chance that some patients will be “resistant” to the drug's antiplatelet effects [41].

1.1.6. The Optimal Dose of Aspirin

Aspirin inhibits platelet COX-1 activity in a dose-dependent manner, with a maximum impact of 100 mg given as a single dose or 30 mg administered daily via cumulative inactivation of the drug target [36, 42]. Randomized placebo-controlled tests show that aspirin, when used for long periods at dose ranges of 50 to 100 mg once a day, is an effective antithrombotic agent and suggests that it is effective at doses of 30 mg once daily [43]. In patients with unstable angina and chronic stable angina, aspirin

at a dose of 75 mg once daily was demonstrated to reduce the risk of acute myocardial infarction or mortality, along with lowering the risk of postoperative stroke following carotid endarterectomy and preventing stroke in patients with transient cerebral ischemia [41, 43]. Despite a higher platelet count, aspirin 100 mg daily was effective in preventing thrombotic complications in patients with polycythemia vera [44]. Nevertheless, the same antiplatelet regimen was not found to be effective in prohibiting major vascular complications in patients with diabetes [45, 46] and asymptomatic atherosclerosis [47], possibly due to an accelerated platelet turnover, correlated with these vascular disorders that may limit the duration of the antiplatelet effect of low-dose aspirin given once daily [48]. The use of low-dose aspirin was recommended because of the saturability of its antiplatelet effect at low doses (figure 8) [16], as well as the lack of a dose-response relationship in assessing its antithrombotic effects and the dose dependence of its side effects. Knowing that prostaglandins synthesized from COX-1 can enhance the expression of COX-2 that is considered inducible (figure 8) and their inhibition will decrease COX-2 enhancement [16]. The lowest effective dose of aspirin (50–100 mg daily for long-term treatment) is currently the most acceptable strategy for maximizing efficacy while reducing toxicity [43].

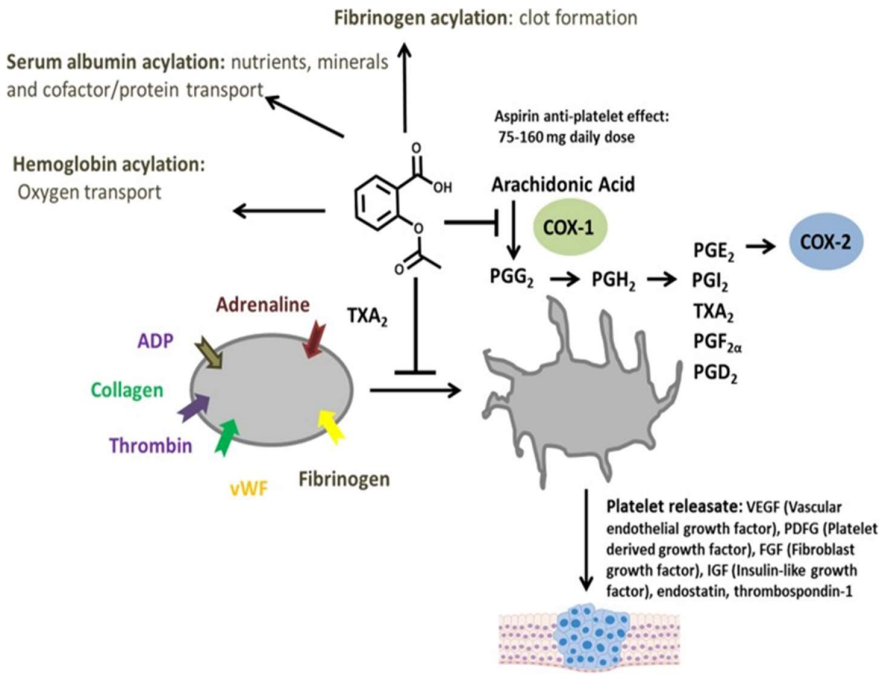


Figure 8. Aspirin Inhibits the Platelet Activation Mechanism

1.2. Aspirin Effects Unrelated to TXA_2 Suppression

Aspirin has been shown to have impacts on hemostasis and thrombosis that are unrelated to its capacity to inactivate platelet COX-1. These impacts could include dose-dependent inhibition of platelet function, stimulation of fibrinolysis, and inhibition of plasma coagulation [41]. In contrast to aspirin's well-known and saturable inhibition of COX-1, the presumed mechanisms underlying aspirin's non-prostaglandin effects on hemostasis and thrombosis are dose-dependent and poorly understood. Furthermore, all evidence pointing to aspirin's dose-dependent effects is indirect, with individual randomized clinical trials failing to show a dose-effect [49-53]. This lack of a dosage impact is associated with the aspirin action on platelet COX-1 being saturable [36]. Nanomolar concentrations of aspirin, for example, completely block PG synthesis

within 20 minutes of exposure in studies with purified COX-1 and isolated platelets [17, 18]. Because of its saturability, higher concentrations and longer exposures will not affect aspirin's inhibitory effect on PG synthesis. In clinical trials with aspirin as an antithrombotic agent, the same feature (maximal effect at low doses, no dose effect) is observed [43].

Thereby, the consistency of dose requirements and the saturability of aspirin's effects in acetylating the platelet enzyme [34], suppressing TXA_2 production [36, 42], and hindering atherothrombotic complications [43, 52] provide the strongest evidence that aspirin prevents thrombosis by inhibiting TXA_2 production. As a result, any possible impacts of aspirin on other markers of arterial thrombosis are likely to be far less substantial than the inhibition of platelet COX-1 activity [41].

1.3. Aspirin Resistance

1.3.1. Identification and Usage of the Term "Aspirin Resistance"

The term "aspirin resistance" was used for the first time in 1994, but no consensus has yet been reached on the definition, standardized assay, clinical effects, or its underlying mechanisms. Aspirin resistance has been identified based on a clinical and functional basis, ranging from treatment failure to low response regarding non-standardized platelet functional assays [2]. Recently, the idea of heterogeneity in response to ASA has emerged and properly described the unexplained ASA resistance term due to pathophysiological mechanisms and based on pharmacokinetics and/or pharmacodynamics of the drug that has developed [2].

Aspirin Resistance has been used to characterize a variety of occurrences, including aspirin inability to (1) protect people from thrombotic problems, (2) prolong

bleeding time, (3) diminish TXA_2 production, or (4) generate a typical effect on *in vitro* platelet function tests [54]. The fact or report or observation that certain individuals may have repeated vascular incidents despite taking aspirin should be referred to as "treatment failure" instead of "aspirin resistance". Treatment failure is a regular occurrence that can happen with any medicine (e.g., antihypertensive or lipid-lowering medications). Given the complex character of atherothrombosis, it is expected that only a portion (typically one-quarter to one-third) of all vascular problems is eliminated by a single preventative measure [55].

A variable proportion (up to one-fourth) of aspirin-treated individuals with cerebrovascular, coronary, or peripheral artery illness only accomplish partial inhibition of platelet aggregation at first testing, but others (up to one-third) appear to acquire “resistance” to aspirin over time, even with increased doses [41].

1.3.2. Limitations of the Studies that Claim the Presence of Aspirin Resistance

Nevertheless, most of these investigations had the following main flaws: (1) there was no biochemical or observed proof of patient compliance with the prescribed therapy; (2) one measurement was done for each specific test; (3) The assay intra- and inter-subject variability over time were rarely documented; (4) The criteria for defining the normal versus "aspirin resistant" range, as well as the assay settings, varied between investigations; (5) the daily aspirin dosage was varied, ranging from 75 to 1,300 mg; and (6) there was no proper control in any of these investigations [41].

In research looking at aspirin "unresponsiveness," a lack of biochemical measurement of compliance is a serious problem. In a trial of 190 patients with a history of myocardial infarction, researchers evaluated arachidonate-induced platelet

aggregation in individuals while on their regular aspirin therapy, after 7 days of withdrawal, and 24 hours after a single observed consumption of 325 mg aspirin [56]. Though 9 % of patients who claimed to have taken their normal medication failed to show platelet aggregation inhibition, then after an observed dose, this rate reduced to <1% (1 patient out of 190) [56]. Moreover, 12 hours before testing, this only patient admitted to taking NSAIDs. These findings are consistent with a study that found that following a 325 mg aspirin dose, the mean of arachidonic acid-induced platelet aggregation in previously resistant patients decreased below 20% (the standard level to designate “resistance”) [57]. As a result, questionnaires cannot be used to assess compliance to any medication, including aspirin, and studies that do not use salicylate measurements or serum TXB_2 have a significant, inherent bias that makes it difficult to interpret the data. Additionally, the few studies that explicitly compared several functional assays found no substantial agreement between tests, implying that aspirin nonresponsiveness is extremely test-specific [39].

1.3.3. The Contribution of NSAIDs to Aspirin Resistance

Individual pharmacokinetic (PK) properties of currently available antiplatelet medications may explain the transport of varying amounts of the active moiety of the drug to its site(s) of action in different individuals, and so give a PK basis for interindividual variability in pharmacological response [58]. Limited bioavailability of intact aspirin or thiol-containing active metabolites of thienopyridines due to galenic or genetic factors may be enough to explain a diminished antiplatelet action in some patients without arguing "resistance" of the drug target [58].

Concomitant use of various commonly available NSAIDs, like ibuprofen, may decrease the antiplatelet effects of low-dose aspirin, contributing to several reports of so-called aspirin "resistance" [59]. This is because of competition for a shared docking site (arginine-120) within the COX 1 channel, which aspirin must bind to selectively acetylate Ser-529 [21]. This interaction has also been found with naproxen and low-dose aspirin [60, 61], but not with diclofenac the non-selective COX-2 and COX-1 inhibitor [59] and there was no interaction with rofecoxib [59], celecoxib [62], which are all COX-2 inhibitors with moderate to high selectivity [63]. Although the clinical ramifications of this interaction between aspirin and several classic NSAIDs are unknown, it may account for the infrequent discovery of less-than-complete inhibition of platelet COX-1 activity in older patients with concomitant cardiovascular and osteoarthritic illnesses [1].

1.3.4. Classification of Patients as Resistant or Non-Responders to Aspirin

According to a PD analysis of the variability in response to aspirin and P_2Y_{12} blockers, there appears to be no solid evidence to support the practice of phenotyping patients as “resistant” or “non-responders” to these drugs based on a single platelet function measurement taken at a variable time point after dosing and using a largely arbitrary response threshold [58]. Furthermore, until the major reason causing a patient's recurring discovery of less-than-expected platelet function inhibition at a defined time point (e.g., 24 hours after an observed dose) is identified, modifying the patient’s antiplatelet medication will be simply empirical [1].

According to several research studies, both primary and secondary nonadherence increases the risk of cardiovascular events and mortality [64]. Also, the

use of greater dosages of aspirin, clopidogrel, or both in the early stages of acute coronary syndromes has not been proved to be effective [53]. To further understand the diverse drivers of interindividual variability in response to aspirin or P_2Y_{12} blockers, researchers have proposed that the term "resistance" be dropped [58, 65, 66]. Several elements that contribute to aspirin resistance could be described systematically, beginning with the drug's prescription and finishing at the platelet level (figure 9) [32].

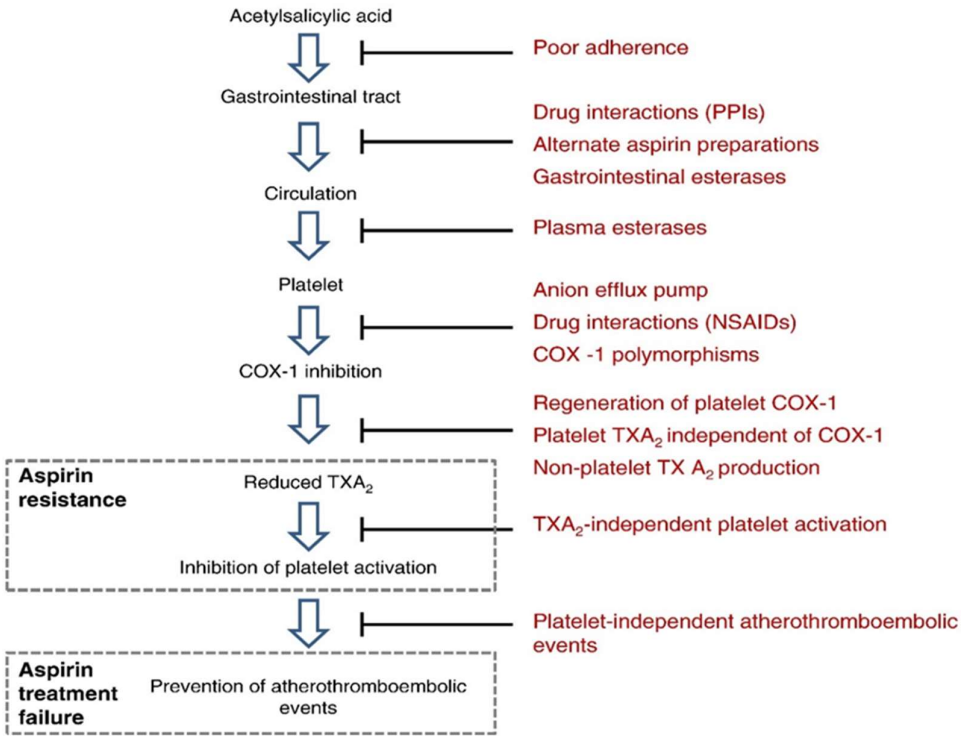


Figure 9. Factors Contributing to Aspirin Resistance

1.4. Predictors of Aspirin Responsiveness Correlated with Resistance Possibility

Because the effect of low-dose Aspirin depends on platelet turnover, ASA sensitivity of platelet, and megakaryocyte COX, the adequacy of the treatment regimen can differ in various disease states [5]. Increased platelet turnover by megakaryocytes in response to physiological stress has been shown to play a significant role. Between 12

and 24 hours after dosing, a progressive increase in the TXA_2 generation occurred as short-lived ASA was unable to acetylate new platelets released at a rate higher than the average of MKs during the 24-hour dosing period [2, 32, 40, 67]. Other potential causes might be associated with increased TX biosynthesis by pathways that are not blocked by low-dose aspirin (non-platelet sources) [32, 67]. These might include, for example, COX-2 synthesis of TX in macrophages, monocytes, and vascular endothelial cells [32, 68]. Cellular and biological factors involved could be related to insufficient COX-1 suppression or enhanced regeneration, or overexpression of COX-2 in the induced platelets [32, 68]. TXA_2 receptors can be activated by TXA_2 and by isoprostanes (produced by oxidative stress) both reflecting aspirin-insensitive mechanisms of TP activation [32, 67, 69, 70] (figure 10) [70]. Another contributor is vascular inflammation, which could increase the expression of CD40 ligand in the platelet membrane, increase activation of the platelet, and platelet-mediated involvement in vascular inflammation [67, 69].

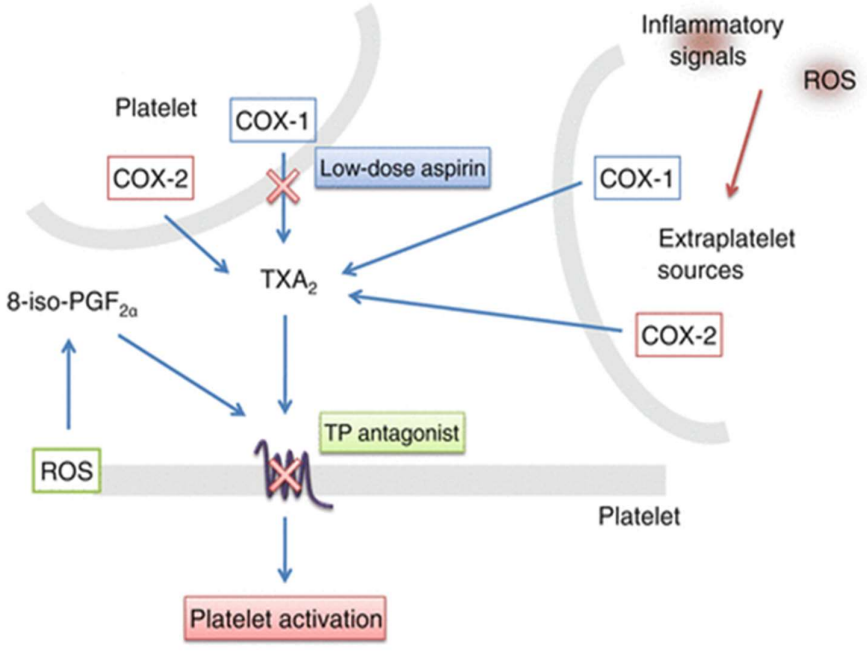


Figure 10. TP Antagonism and the Factors Contributing to Aspirin Resistance

1.5. Megakaryocytes, an Important Target of Aspirin

1.5.1. Overview of Megakaryocytes and Megakaryopoiesis Process

Megakaryocytes (MKs) are rare myeloid cells found primarily in bone marrow but also in the lung and peripheral blood [71]. MKs are the largest (50–100 μm) and rarest cells in the bone marrow, accounting for less than 0.01% of all nucleated bone marrow cells [72]. Megakaryopoiesis takes place in the fetal liver and yolk sac until the marrow cavities have grown large enough to allow blood cell growth [71]. MKs, like all other blood cells, are derived from hematopoietic stem cells (HSC) [73]. A hematopoietic progenitor cell differentiates into a huge polyploidy megakaryocyte through the process of megakaryopoiesis [74] (figure 11) [73]. Following the commitment of the multipotent stem cell to the MK lineage, the proliferation of the progenitors, and ultimate differentiation of MKs, MKs give rise to circulating platelets via a process known as thrombopoiesis [74].

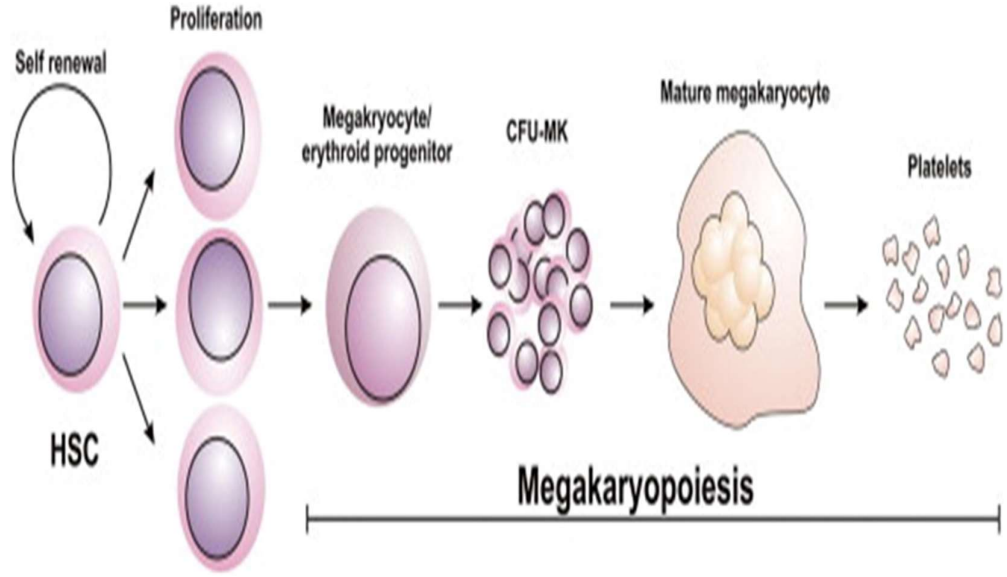


Figure 11. Overview of Megakaryopoiesis

The HSC produces the early common myeloid progenitor (CMP), which can be cloned as a multi-lineage colony-forming unit (granulocyte, erythrocyte, MK, and monocyte) [75]. CMP can differentiate into granulocyte/monocyte progenitor or common MK-erythroid progenitor (MEP) [75]. MEPs are bipotential precursors that can give rise to megakaryocytic and erythroid cells [73]. Molecular signals mediated by regulatory transcription factors orchestrate CMP differentiation. GATA-1, which drives MEP differentiation, and PU.1, which regulates granulocyte-monocyte precursors, are two transcription factors implicated in CMP differentiation. The initial event linked with differentiation restriction to erythroid and MK lineages is the downregulation of PU.1 expression in the CMP [73].

1.5.2. Megakaryocytes Maturation, Development, and Platelet Biogenesis

For platelet biogenesis, megakaryocytes modify their cytoplasm and membrane systems. Before a megakaryocyte can release platelets, it expands significantly to a diameter of about 100 μm and fills with high concentrations of ribosomes that aid in the creation of platelet-specific proteins. Multiple cycles of endomitosis mediate cellular expansion [71]. During endomitosis, chromosomes replicate and the nuclear envelope disintegrates. Despite the assembly of interconnected mitotic spindles, the typical mitotic cycle is arrested during anaphase B. Both telophase and cytokinesis are skipped because the spindles fail to separate. Within each megakaryocyte, nuclear envelope reformation results in a polyploid, multilobed nucleus with DNA contents varying from 4N to 128N [71, 72]. Megakaryocytes undergo extensive maturation in addition to DNA growth as internal membrane systems, granules, and organelles are built-in bulk during their development. In particular, the invaginated membrane system (IMS) forms an expansive and linked membranous network of cisternae and tubules [71] that is dispersed throughout the MK cytoplasm and continuous with the plasma membrane [72]. The IMS is largely used as a membrane reservoir for the development of proplatelets, which are the precursors to cytoplasmic extensions [72, 75]. Megakaryocytes, in addition to the DMS, increase the amount and density of granules when cytoplasmic expansion occurs, and form a dense tubular network with an open canalicular system for granule release [74].

MKs are positioned less than 1 micron away from the marrow sinus wall, allowing newly generated platelets to enter the circulation, according to quantitative electron microscopic examination [71]. Despite its discovery over 120 years ago, many of the mechanisms involved in platelet biogenesis remain controversial. Recent

research, on the other hand, supports a modified platelet assembly flow model. The outflow and evagination of the mature megakaryocyte's large internal membrane system generate proplatelets, which are critical intermediate pseudopodial extensions [71]. Each of the 10–20 proplatelets that a megakaryocyte can extend begins as a blunt protrusion that elongates, thins, and branches repeatedly through time [72]. Proplatelets extend into sinusoidal gaps, where they detach and fragment selectively from proplatelet tips into individual platelets, producing 2000–5000 new platelets [75], and their nucleus is extruded and phagocytosed [72]. These nascent platelets have mitochondria and ribosomal RNA, as well as all of the other components required for platelet function in hemostasis, but no nuclear material [72]. MKs take around 5 days to complete polyploidization, mature and release platelets in humans and 2–3 days in rodents [76-78]. Human platelets last 7–10 days after being discharged into the bloodstream, whereas rodent platelets last 4–5 days [79-81]. Due to the scarcity of megakaryocytes in the bone marrow, the actual events behind platelet release *in vivo* have not been identified [71]. As a result of the limited accessibility of MKs *in vivo* in humans and the need to research their properties, the use of megakaryocytic cell lines is important.

1.6. Megakaryocytic Cell Lines

At least two human megakaryocytic cell lines were developed. Some of its features will be discussed in the below paragraphs.

1.6.1. CHRF Cell Line

A biopsy of a metastatic tumor in a 17-month-old infant with acute megakaryoblastic leukemia led to the discovery of CHRF-288. This line has been shown to produce a distinct collection of growth factors, some of which are generally linked with platelets. This cell line appears to have characteristics that are typical of megakaryocytes, notably an early Mk phenotype, and platelets, according to a variety of criteria [82]. Platelet peroxidase, platelet factor 4, platelet Ca2+ adenosine triphosphatase, glycoprotein llb-llla (CDw411, factor Vlll antigen, and the MY7 (CD13) and MY9 (CD33)) antigens are all expressed by the cells in the CHRF-288 cell line. These cells take around 33 hours to double [83].

1.6.2. MEG01 Cell Line

MEG01, a megakaryoblastic cell line derived from the bone marrow of a 55-year-old man with blast crisis of Philadelphia (Ph1) chromosome-positive chronic myelogenous leukemia, was developed in 1983 [84]. MEG01 cells are mononuclear and hyperdiploid, with CD41+, CD61+, CDw14+, and cytoplasmic factor VIII expression. The periodic acid-Schiff (PAS) reaction, alpha naphthyl acetate esterase, and acid phosphatase, all of which are known in MKs, stain positively in MEG01 [84]. With a doubling period of 36 to 48 hours, MEG01 cells developed in a single cell suspension where approximately half of the cells adhere to the cell culture flask with pseudopods extension [84]. This unique human megakaryoblastic cell line could be used to explore human megakaryopoiesis as well as the biosynthetic pathways of proteins specific to the megakaryocytic lineage [84].

1.7. Aspirin-Insensitive Thromboxane Biosynthesis

In vitro studies have shown that platelets treated with aspirin can regain their ability to generate TXA_2 when cultured with thrombin-stimulated endothelial cells [85]. Because the amount of endothelial COX-2 expression was proportionate to the amount of TXA_2 produced, it was hypothesized that COX-2-dependent PGH_2 could be used by the TXAS of aspirinated platelets to create TXA_2 [68]. Unlike platelets, which are permanently inactivated by aspirin, endothelial cells can quickly (2–4 h) regain the COX-2 activity after treatment with phorbol esters or interleukin (IL) 1α due to *de novo* COX-2 synthesis [85]. These *in vitro* findings imply that additional platelet TXA_2 production can be recovered after a single daily treatment of the short-acting aspirin via the activation of COX-2 in nucleated cells in response to a local inflammatory environment (figure 12) [68].

Therefore, COX-2 activation in plaque monocytes/macrophages or activated vascular cells may contribute to aspirin-insensitive TXA_2 biosynthesis in individuals with acute coronary syndromes by producing PGH_2 as a substrate for the same cell's TXAS or by giving PGH_2 to the TXAS of aspirinated platelets [68].

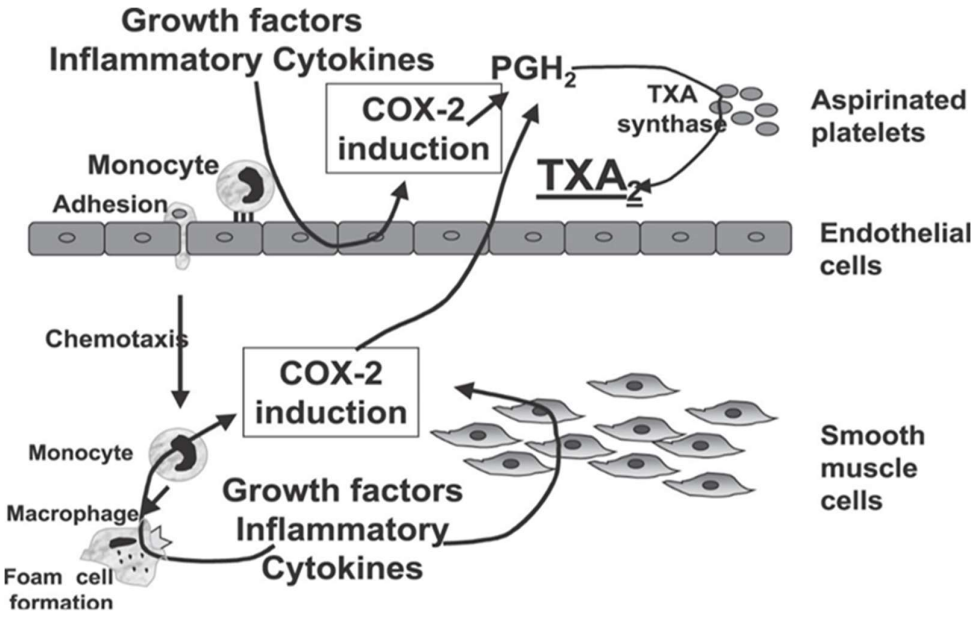


Figure 12. Platelet's Capacity to Re-Synthesize COX-2

1.8. Regeneration of Platelet COX-1

1.8.1. High Platelet Turnover

If platelet turnover is normal, COX-1 activity is restored at a rate of 10% daily after a single dosage of aspirin [86]. Platelet COX-1 renewal enhances the potential of the circulating pool of platelets to produce TXA_2 and therefore aggregate. This process is ongoing, and at the usual platelet lifespan of 10 days [87], a once-daily aspirin dose is sufficient to maintain optimal platelet TXA_2 inhibition [36]. This may not always be the case in situations where platelet turnover is significant [32].

High platelet turnover can be caused by a variety of factors, including aberrant megakaryocytopoiesis or higher peripheral consumption [32]. In essential thrombocythaemia, twice-daily 100 mg aspirin proved to be superior to once-daily 100 or 200 mg aspirin, with the number of immature platelets being the single predictor of

24-hour inhibition of TXA_2 production [88]. The number of immature platelets has been reported to correspond with a decline in aspirin efficacy in a group of patients with atherosclerosis [89], with twice-daily aspirin treatment appearing to improve platelet function testing in individuals with type 2 diabetes mellitus [90, 91].

1.8.2. De Novo Synthesis of COX-1

One of the basic tenets of aspirin's cardioprotective activity is the irreversible binding of aspirin to COX-1 and the inability of anucleate platelets to renew the enzyme [32]. However, it has become clear that platelets not only have the ability to synthesis *de novo* protein but also do so as part of their normal activity in response to external stimuli [92, 93]. *In vitro*, platelet COX-1 regeneration employing controlled protein transduction pathways has been seen in response to thrombin and fibrinogen stimulation [94].

The recently discovered ability of platelets to synthesize proteins *de novo* opens the door to identify new processes implicated in the varied response to aspirin. TXA_2 production was completely suppressed by aspirin in washed human platelets in vitro, but it recovered in response to thrombin and fibrinogen in a time-dependent manner (0.5 and 24 hours), with TXB_2 averaging 0.1 ± 0.03 and 3 ± 0.8 ng/mL [94]. In the presence of [10 μmol/L] arachidonic acid, it was 2 ± 0.7 and 25 ± 7 ng/mL, respectively, and it was inhibited by translational inhibitors, rapamycin, and phosphatidylinositol 3-kinase inhibitors [94]. The findings that COX-1 mRNA was readily found in resting platelets and that [^{35}S]-methionine was integrated into COX-1 protein following stimulation significantly support the possibility of *de novo* COX-1 production in platelets [94]. TXA_2 production happened via a controlled protein translation pathway in aspirin-

treated platelets activated with fibrinogen and thrombin [94]. Puromycin, which promotes the premature release of nascent polypeptide chains, cycloheximide, which hinders the translocation reaction on ribosomes, and rapamycin, a bacterially derived immunosuppressant that suppresses the translation of a specific group of mRNAs, all prevented this TXA_2 biosynthesis [94]. On the other hand, Actinomycin D, a transcriptional inhibitor, did not affect this prostanoid's production. Eventually, aspirin-treated platelets regained their ability to synthesize TXA_2 by a signal-dependent *de novo* protein synthesis [94].

New models studying the variability in the response to low-dose aspirin suggest that aspirin inhibits peripheral platelets and bone-marrow megakaryocytes (MKs) under normal situations, resulting in a generally consistent suppression of platelet COX activity during a 24-hour dosage interval. When platelet turnover is high, the short-acting aspirin appears unable to acetylate new platelets released from MKs over the 24-hour dosage interval, resulting in a gradual rise in TXA_2 synthesis between 12 and 24 hours (figure 13) [2].

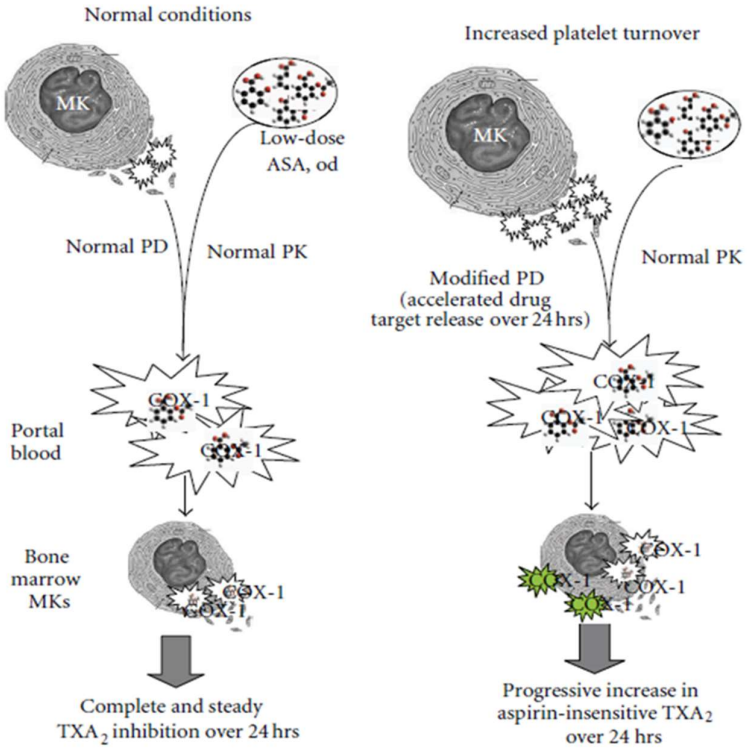


Figure 13. Models to Varied Reactivity to Once-Daily Low Dose Aspirin

CHAPTER 2

AIM OF THE STUDY

Given that the human megakaryoblast cell lines CHRF-288 and MEG01 both express COX-1 and COX-2, and that thromboxane and prostaglandin levels within those cell lines can be examined, investigating the recovery of cyclooxygenase activity after aspirin treatment is appropriate.

Since aspirin inhibits irreversibly COXes, the recovery of COX activities after aspirin treatment may depend on the gene expression of COXes. The kinetics and features of COX activity recovery in MKs are under investigation. The effect of ASA pretreatment on the RNA stability of the COXes genes and the different enzymes of the COX metabolic pathway may play a role in the recovery of the COX activity.

2.1. Hypothesis

Could aspirin modify the decay of some important genes in the arachidonic acid metabolism pathway?

Aims of this study were to:

- Aim 1: Detect the level of expression of the COX prostanoid genes in the human megakaryoblast cell lines
- Aim 2: Investigate the effect of ASA on the expression of these genes at different time points
- Aim 3: Investigate if ASA affects the stability of mRNA genes of cyclooxygenases in megakaryocytes

CHAPTER 3

MATERIALS AND METHODS

3.1. Cell Culture

Megakaryoblastic cell lines are obtained from ATCC: CHRF-288-11 [95] and MEG01 [84]. These cells will be cultured in RPMI media supplemented with 20% and 10% FBS respectively, 1% HEPES, 1% Non-essential amino acid, 1% penicillin, and 1% streptomycin in a 5% CO2 humidified incubator at 37°C.

3.2. Cell Treatment with Aspirin

Count the MEG01 cells then resuspend in 2% FBS media. After that, cells will be treated in the presence and absence of 10 µM of ASA for 30 min, followed by centrifugation at 300g for 5 min at 4°C. The cells will be washed with PBS as cells are diluted to get 500,000 cells/ml, then they are plated in a 12 well plate. At each time interval (24 and 72 hours) cells will be removed and centrifuged at 300 g at 4°C for 5 min. Finally, we add to the remained pellet 200 µl trizol or qiazol and store it at -20°C for the RNA experiment (figure 14).

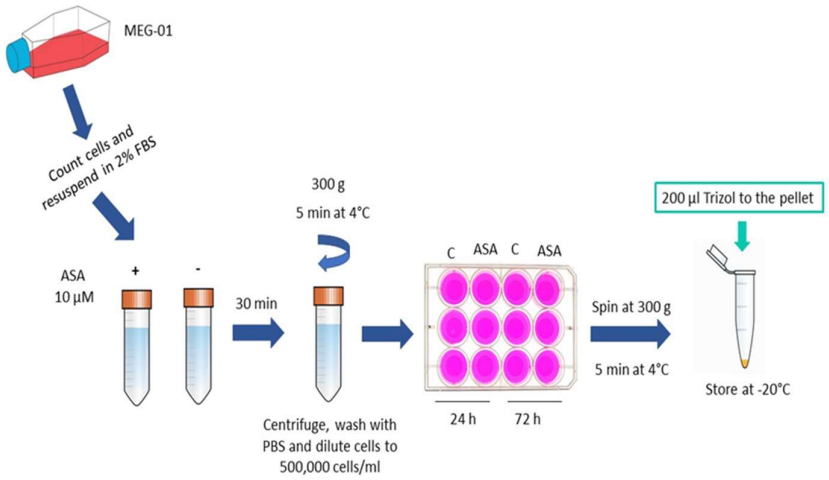


Figure 14. Scheme of Cell Treatment with Aspirin

3.3. RNA Stability for COX Isoforms

The RNA stability of COX isoforms in the presence and absence of ASA will be determined using the inhibitor of transcription, actinomycin D. Cells will be treated in the absence or presence of aspirin for 30 min, then centrifuged at 300g for 5 min at 4°C, followed by washing with PBS and plating at 500,000 cells/ml in 12 well plates for 24 hours. Next, 4 µM of actinomycin D will be added to stop transcription, where at each time point we take the cells, centrifuge, and add trizol or qiazol to the pellet (figure 15). The level of COX-1 and COX-2 mRNA will be measured at different time points (0, 1, 3, 18, and 24 hours) by RT-PCR and expressed as a percentage of day 0. The half-life of the mRNA will be determined.

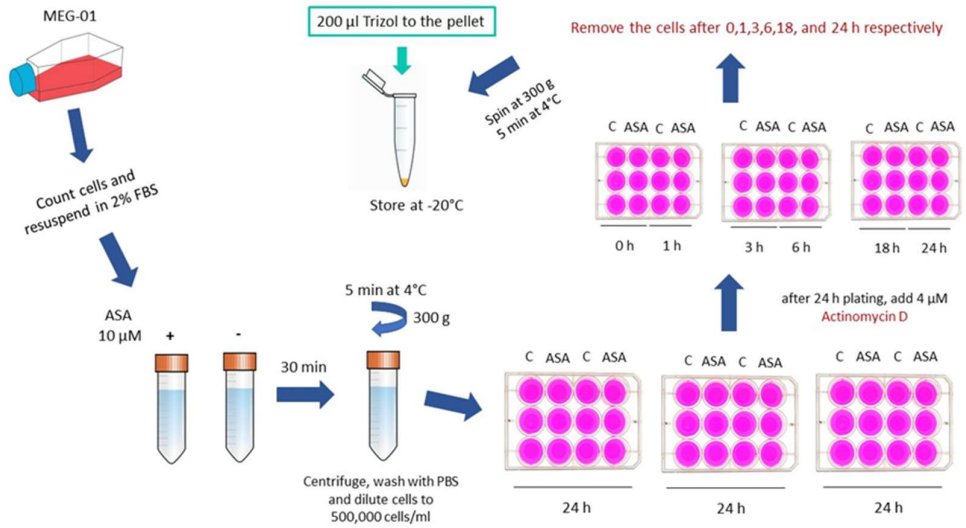


Figure 15. Scheme of RNA Stability Experiment

3.4. Gene Expression Measurement

3.4.1. RNA Extraction

RNA was extracted using 200 µl QIAzol reagent (QIAGEN, 79306) and quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific) after resuspension in 22 µl of RNase, DNase free water. Ratio 260/280 was from 1.8-2.

3.4.2. Reverse Transcription

2 µg of the total RNA was reverse transcribed into cDNA in a final volume of 20 µl according to the manufacturer's instructions (Thermo Fischer Scientifics, reference: 4368813), and the reaction was done in the T100 thermal cycler machine

(Bio-Rad Laboratories, California, USA) as follows: 10 min at 25°C, 2 hours at 37°C followed by 5 min at 85°C and ends at 4°C.

3.4.3. Quantitative Real-Time PCR

qRT-PCR will be carried out on the CFX384 system (Bio-Rad Laboratories, California, USA) using Absolute Blue QPCR Mix, SYBR Green (Thermo Fisher Scientific). Gene expression of many genes of the arachidonic metabolism pathway will be analyzed in addition to COX-1 and COX-2. These include PGE_2 receptors, EP 1, 2, 3, 4, TXA_2 receptor, TX synthase, PGES1, and c-PGES. Primers were tested before according to Table 1 and Table 2.

Following this protocol: 95°C for 3 minutes, 95°C for 15 seconds, 60°C for 30 seconds then plate read, GOTO 2 (55 more times), Melt Curve 65.0°C to 100.0°C: Increment 0.5°C 0:05 then plate read. Results were calculated using the 2-□□Ct method and normalized to the housekeeping gene 18S. Normalizing to GAPDH and Beta-actin was done only to the experiment that includes detection of genes without any treatment. ΔCt (normalization step) is calculated by subtracting the average Ct value of the housekeeping gene from the Ct value of each time point. ΔΔCt is obtained by subtracting ΔCt of each time point from the average ΔCt of the control group. Then, by computing $2^{-\Delta\Delta Ct}$, the ratio of gene expression will be obtained where it is multiplied by 100 to obtain the values in percentages.

3.5. Data Analysis

The data were analyzed by Unpaired t-test for comparing groups, and nonlinear regression analysis was done using one phase decay for stability experiment. These tests

were conducted using “GraphPad Prism” software. Values were considered significant for P-value < 0.05. Error bars on graphs represent the standard error mean for each sample.

Table 1. Characteristics of Used Human Primers

Gene	Sequence (5’ to 3’) F R
PTGS1 (COX-1, old)	TTCTTGCTGTTCCTGCTCCTG (21) GCATTGACAAACTCCCAGAAC (21)
PTGS2 (COX-2)	TGCTGGCAGGGTTGCTGGTGGTA (23) GGGCTTCAGCATAAAGCGTTTGCGG (25)
EP1 (old)	CCGCGCTGCCCATCTTCTCC (20) CAGGCTGGCCACGAACAGCA (20)
EP2 (old)	ACCTCATCCGCATGCACCGC (20) AGGTGGTCCGTCTCCTCCGC (20)
EP3 (old)	TGCTTCATCAGCACCGGGCG (20) GTCAGCGCCAAGAGCCCCAG (20)
EP4 (old)	AAGCGATTGGCGGGCCTCAC (20) TCTGGGTACTGCAGCCGCGA (20)
PGES1 (mPGES-1)	CCTGGTGATGAGCAGCCCGG (20) GGCAAAGGCCTTCTTCCGCAG (21)
Beta-actin	ATGGCCACGGCTGCTTCCAG (20) CCACAGGACTCCATGCCCAGG (21)
m-GAPDH	CGTCCCGTAGACAAAATGGTGAA (23) GCCGTGAGTGGAGTCATACTGGAACA (26)
PTGS1 (COX-1)	TCTTGCTGTTCCTGCTCCTG (20) AACAGGGATTCACTGGCGTG (20)

TXAS	TTTCTACCTGCAGAGCACGG (20) TGCTGATGTGGAGTACCATTTC (22)
TXAR	GAGGTCTCTGAAGGTGTGCC (20) CCGTCTCTCCTCCAGGGTAA (20)
m-18S	AACTTTCGATGGTAGTCGCCGT (22) TCCTTGGATGTGGTAGCCGTTT (22)
PGES-3 (c-PGES)	CCGGAGAGAAAAAGCGGAGT (20) AGAAGCAGGCTGCATTGTGA (20)
EP1 (new 2021)	TATCATGGTGGTGTCGTGCATC (22) TGTACACCCAAGGGTCCAGG (20)
EP2 (new 2021)	GCTCCTTGCCTTTCACGATTT (21) AGGATGGCAAAGACCCAAGG (20)
EP3 (new 2021)	CTTCGAAAGTTTTGCCAGATCAG (23) AAATGTCCAACTCCGTTCTTTCA (23)
EP4 (new 2021)	AACTCTGACCTCGGTGTCCA (20) CAAGGCTGGGTCTGTAGCG (19)

Table 2. Primers Testing Outcome

Gene	**CT value (55 cycles)**	**Dilution Ratio of cDNA (2 ug RNA)**
PTGS1 (COX-1, old)	18	1/2.5
PTGS2 (COX-2)	22	1/2.5
EP1 (old)	23	1/2.5
EP2 (old)	24	1/2.5
EP3 (old)	22	1/2.5
EP4 (old)	22	1/2.5
PGES1 (mPGES-1)	25	1/2.5
Beta-actin	15	1/2.5
GAPDH	14	1/2.5

PTGS1 (COX-1)	22	1/5
TXAS	23	1/5
TXAR	27	1/5
18S	7	1/5
18S	12	1/100
PGES-3 (c-PGES)	23	1/5
EP1 (new 2021)	27	1/10
EP2 (new 2021)	26	1/10
EP3 (new 2021)	25	1/10
EP4 (new 2021)	27	1/10

CHAPTER 4

RESULTS

4.1. Detection of Gene Expression in Megakaryocytes

We first investigated the level of expression of COX-1, COX-2, and other important genes in the arachidonic acid pathway. RT-PCR was performed, extracted from MEG01 or CHRF-288 (500,000). Dilution of the cDNA for these genes was done based on previous tables mentioned and was dependent on the level of expression. Since in RT-PCR, CT, which corresponds to the number of cycles to see significant amplification, we first determined the CT for each gene. Results show the CT of all genes for COXes, the PGES1 (mPGES-1) (Figure 16A), and the EP receptors (Figure 16B) was within an acceptable range. These results indicate the expression of these genes in the cells and define the required dilution for the cDNA to be used.

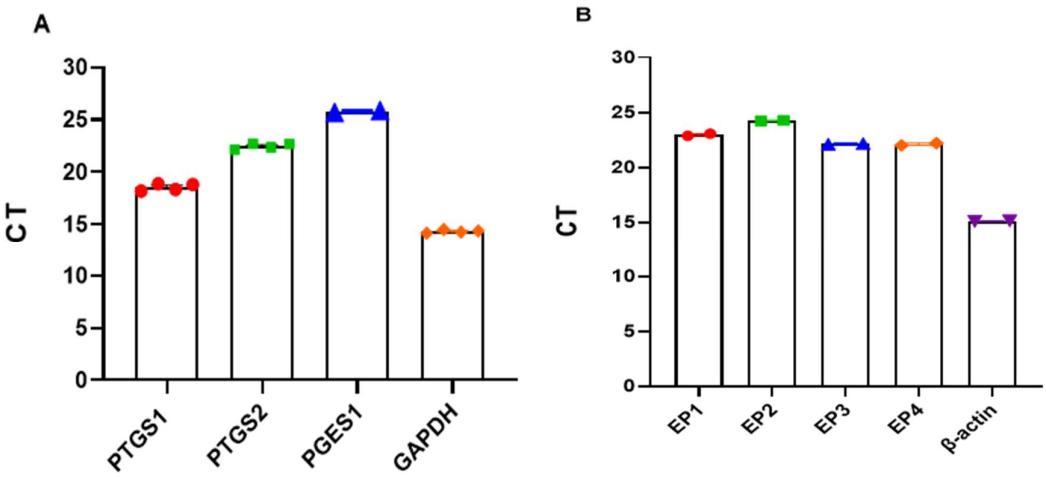


Figure 16. Detection of Different Genes of the Arachidonic Acid Pathway in MEG01 Cells. (A) Representation of CT values of PTGS1 and PTGS2 (COXes), microsomal prostaglandin E synthase-1 PGES1 (mPGES-1), and the housekeeping gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH). (B) Expression of EP receptors and the housekeeping gene beta-actin (β-actin). Data are expressed as Mean ± SEM. CT values for RT negative was 1.5- 2 times (not shown). The test was done on 4 different samples.

Similar results were obtained in the megakaryocytic cell line, CHRF-288 (Figure 17). Thus our results confirm that these megakaryocytic cell lines express most of the genes essential for prostanoid synthesis and signaling.

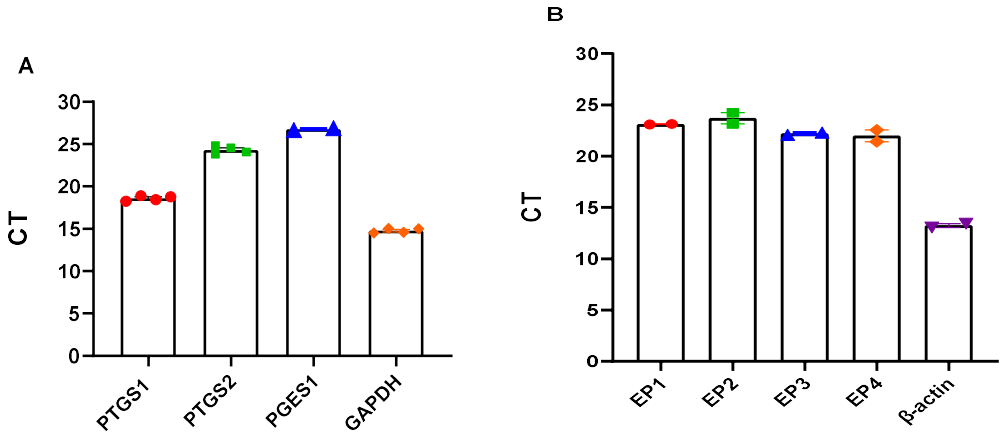


Figure 17. Detection of Different Genes of the Arachidonic Acid Pathway in CHRF-288. Data expressed as described in the legend for figure 16.

Next, since the COX-1 primers sets used in Figures 16 and 17 were not ideal because of a large amplified segment of DNA, we generated other more suitable COX-1 primers (PTGS1). As for the prostanoid receptors (EP1,2,3, and 4), since the primers of Figures 16 and 17 were designed within the same exon, we generated more suitable primers overlapping the exon-exon junction, thus avoiding the amplification of large

genomic contaminant cDNA. The newly designed EP receptors were used. Primers for thromboxane (TX) synthase and receptor (TP) and the cytosolic form of PGE_2 synthase were also designed and tested. Moreover, the housekeeping gene for 18S was selected to correct the expression of the different genes for the next experiments.

Figures 18A and 18B show the significant expression for the new COX-1 primers (PTGS1), and TXAS and TXAR in MEG01 and CHRF-288, respectively, and Figure 18C for the EP(1,2,3 and 4) and cPGES-3. We conclude that these new genes are well expressed in MEG01 and CHRF-288.

We selected to perform the next experiments on MEG01 cell lines, as their cells duplicated very well in cell culture, and can provide a model for biosynthetic mechanisms of proteins unique to the megakaryocytic lineage.

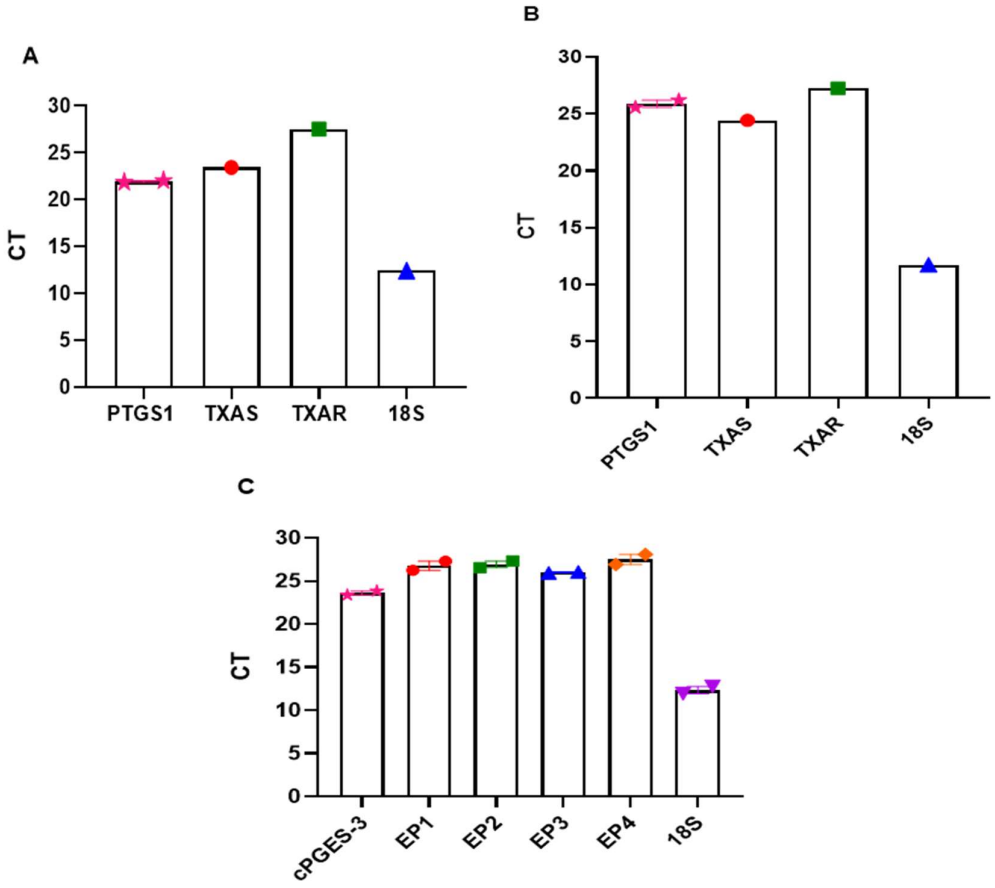


Figure 18. Detection of Different Genes of the Arachidonic Acid Pathway in MEG01 and CHRF-288 Upon Using the Newly Designed Primers. (A) Representation of CT values of PTGS1 (COX-1), thromboxane A2 synthase (TXAS), thromboxane A2 receptor (TXAR), and the housekeeping gene 18S in MEG01. (B) Representation of CT values PTGS1, TXAS, TXAR, and 18S in CHRF-288. (C) Expression of EP receptors, cytosolic prostaglandin E synthase-1 (cPGES-3), and the housekeeping gene 18S in MEG01. Data are expressed as Mean ± SEM. CT values for RT negative was 1.5- 2 times (not shown). The test was done on 3 different samples.

4.2. Effect of Aspirin on Gene Expression at 24 and 72 hours

Next, we examined the effect of ASA on the level of expression of these genes. MEG01 cells (500,000 cells/ml) were incubated with ASA for 30 minutes, then washed with PBS. Cells were plated for 24 and 72 hours, followed by extraction and RT-PCR. Then results were normalized to mouse 18S (1/100) with specific dilutions to each gene. As shown in Figure 19A, treatment of cells for 24 hours resulted in a statistically significant decrease in COX-2 gene (PTGS2) (around 40% decrease ± 10%, $p<0.02$) compared to vehicle-treated MEG01. No significant change was observed for the other genes.

Treatment of the cells for 72 hours with ASA resulted in a significant increase in the gene expression of TXAS (around 78% increase ± 27%, $p<0.05$) compared to vehicle-treated cells. No other gene expression was observed for the tested arachidonic acid pathway genes (Figure 19B).

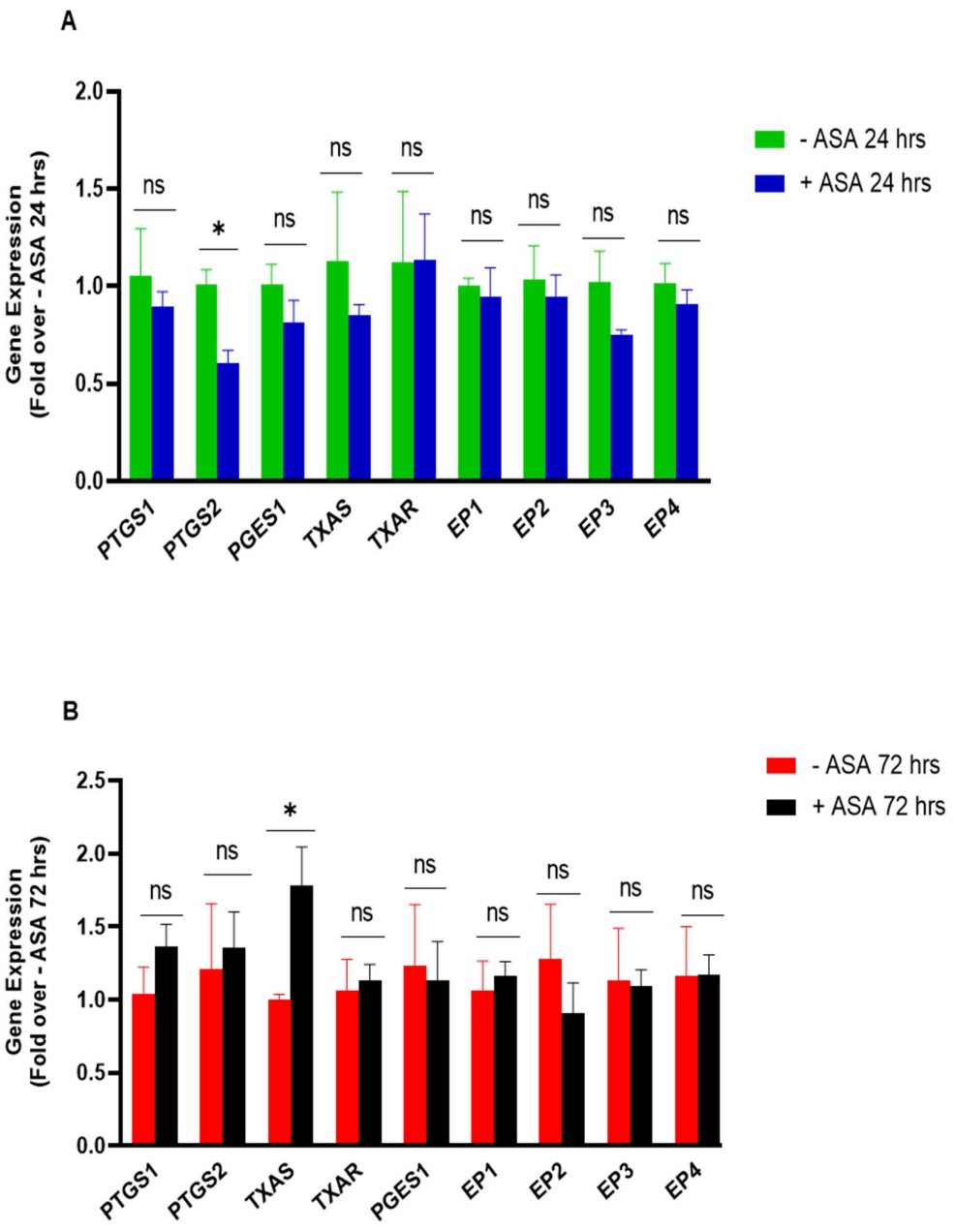


Figure 19. Aspirin Treatment didn't Affect the Expression of Most Genes in the Arachidonic Acid Pathway. MEG01 cells were treated with or without 10 µM ASA, and the level of expression was analyzed and normalized to 18S. (A) Gene expression compared between -ASA 24 h and + ASA 24 h. (B) Gene expression compared between - ASA 72 h and + ASA 72 h. Data are expressed as Mean ± SEM; ns, not significant; * $P < 0.05$ (unpaired t-test). The experiment was done in triplicates for the control (-ASA 24 and 72 hours) and the treatment (+ASA 24 and 72 hours treatment).

Furthermore, in the same experimental settings, the comparison of the expression of these genes between 24 and 72 hours, in the absence of ASA, revealed no significant changes in the expression of all tested genes (figure 20A). However when comparing the expression between 24 hours- and 72-hours- ASA treated cells, a significant increase in the gene expression of PTGS1, PTGS2, PGES1, TXAS, TXAR, and EP3 genes, No modification of gene expression was observed for EP1, EP2, and EP4 (figure 20B).

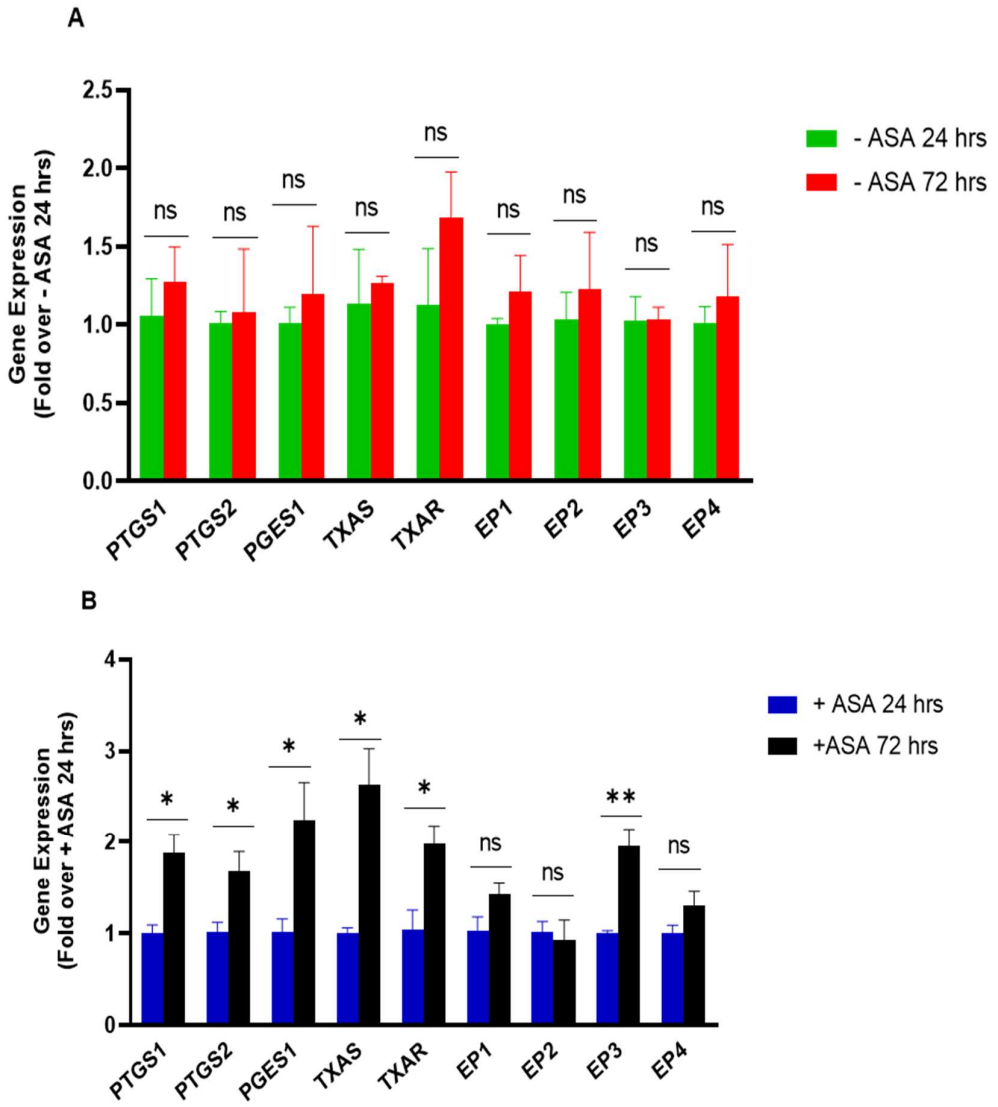


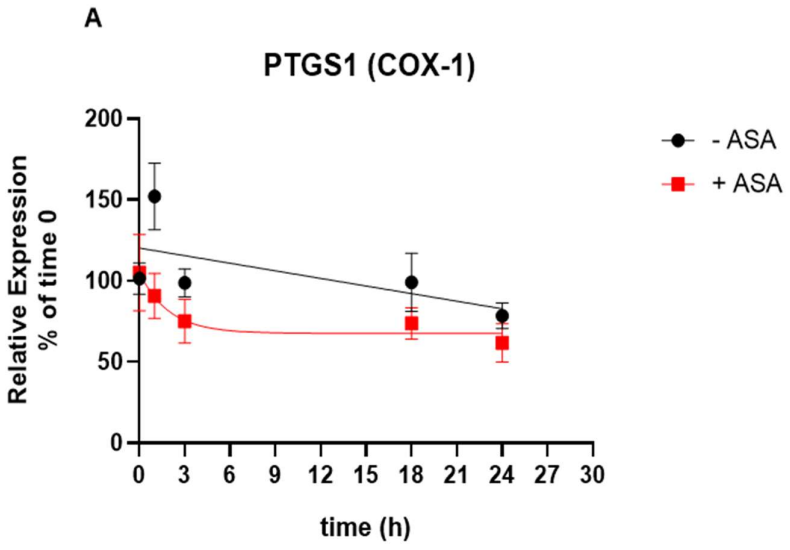
Figure 20. Aspirin Treatment Increased the Expression of Most Genes in the Arachidonic Acid Pathway. MEG01 cells were treated with or without 10 µM ASA, and the level of expression was analyzed and normalized to 18S. (A) Gene expression was compared between - ASA 24 h and - ASA 72 h. (B) Gene expression was compared between +ASA 24 h and +ASA 72 h. Data are expressed as Mean ± SEM; ns, not significant; * $P < 0.05$; ** $P < 0.01$ (unpaired t-test). The experiment was done in triplicates for the control (-ASA 24 and 72 hours) and the treatment (+ASA 24 and 72 hours treatment).

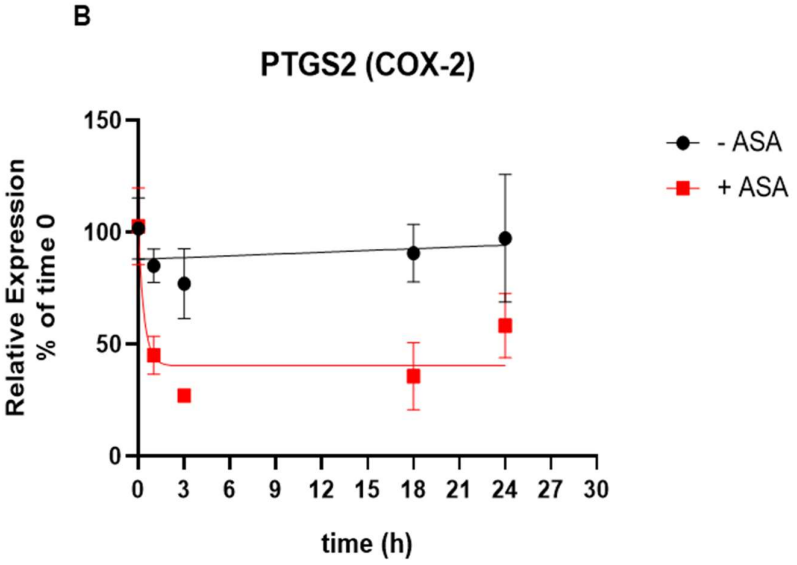
4.3. Effect of ASA on RNA Stability of COX Prostanoid Genes

Next, we examined the effect of ASA on the RNA stability of these genes. Actinomycin D, the inhibitor of transcription, was added to the cells to stop transcription and assess the level of mRNA for different genes with time to determine the half-life. Figures 21A, 21B, and 21C show the relative expression of PTGS1 (gene for COX-1), PTGS2 (gene for COX-2), and PTGES (gene for mPGES-1) expressed as a percentage of time zero, which is allocated a 100 %, respectively.

For PTGS1, the data indicate that both the control (-ASA) and the aspirin-treated group show a slight decrease in the expression over time, with the ASA group giving lower values compared to the control and stabilized around 68% (figure 21A). These results don't reflect a modification in the half-life of the mRNA.

For PTGS2 (for COX-2 protein), the untreated cells showed a sustained stabilization with 90% gene expression over time, while the ASA-treated group showed a decrease in the mRNA expression reaching a lower plateau at around 40% (figure 21B). For PGES1 (mPGES-1), ASA treatment decreased the expression that is then stabilized around 63% (figure 21C).




B
PTGS2 (COX-2)
Relative Expression
% of time 0
150
100
50
0
0 3 6 9 12 15 18 21 24 27 30
time (h)
- ASA
+ ASA

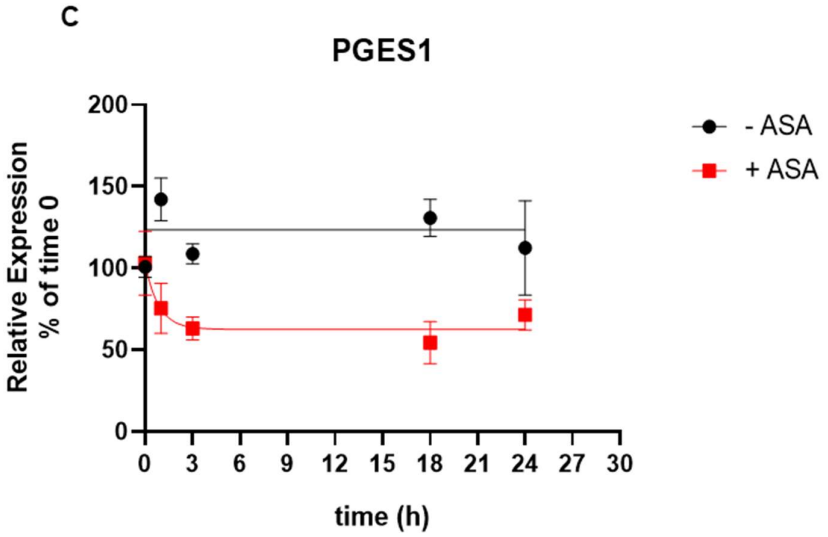
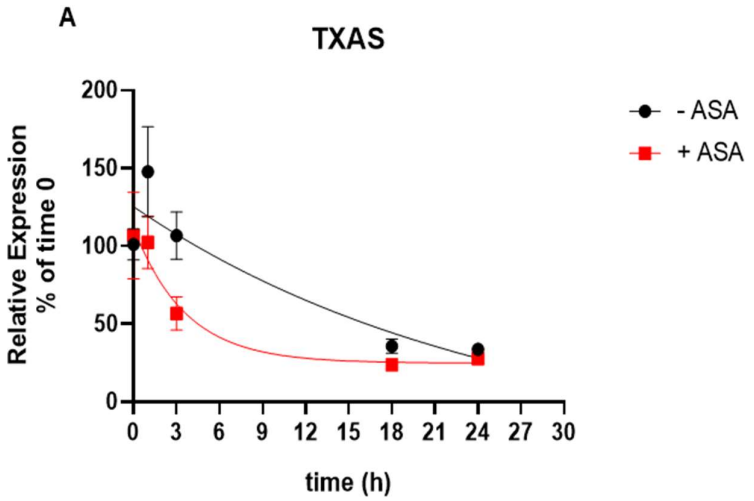

C
PGES1
Relative Expression
% of time 0
200
150
100
50
0
0 3 6 9 12 15 18 21 24 27 30
time (h)
- ASA
+ ASA

Figure 21. mRNA Decay of Several Genes of the Arachidonic Acid Pathway. MEG01 cells were treated with or without 10 µM ASA for 30 minutes, then washed by PBS and centrifuged then left for 24 hours incubation, then 4 µM actinomycin D was added. At each time point (0, 1, 3, 18, 24 hours), the cells were collected and the level of expression was analyzed and normalized to 18S. One-phase decay for (A) PTGS1 (COX-1); (B) PTGS2 (COX-2); (C) PGES1 (mPGES-1). Data are expressed as Mean ± SEM. Curves were done by GraphPad Prism using nonlinear regression. The experiment was done in triplicates for the control (-ASA) and the treatment (+ASA).

Figure 22A shows the relative expression of thromboxane (TX) synthase. For this gene, the expression is decreasing with time in both groups (control versus ASA treatment), so this might not indicate a direct effect of ASA on the stability of this gene despite having lower values in the treatment group compared to the control. The ASA group stabilized the expression at 25% between 18 and 24 hours. The calculated half-life shows a lower half-life of 3 hours in the presence of ASA compared to 18 hours in the absence of ASA.

Moreover, the thromboxane receptor (TP) was assessed in Figure 22B. The untreated cells show that the expression continues to increase slightly over time while aspirin treatment prevents the increase in the expression at different time points, thus stabilizing the expression at around 82%.



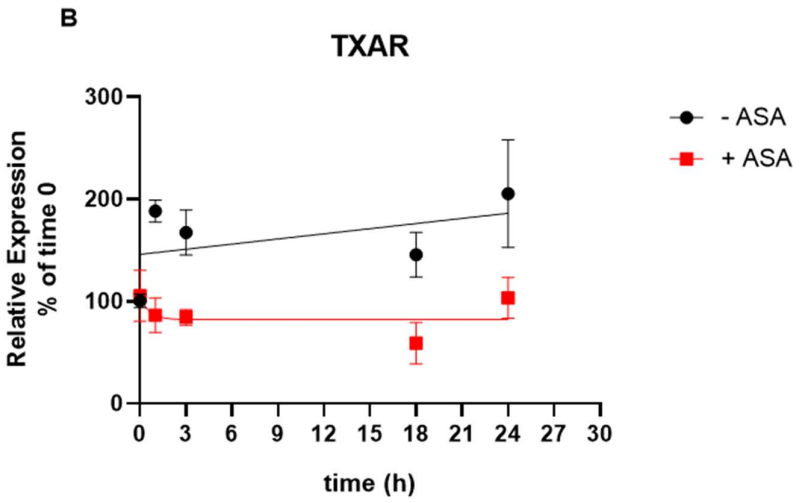


Figure 22. mRNA Decay of TXAS and TXAR. MEG01 cells were treated with or without ASA and the half-life of mRNA was determined and data expressed as described in the legend for figure 21.

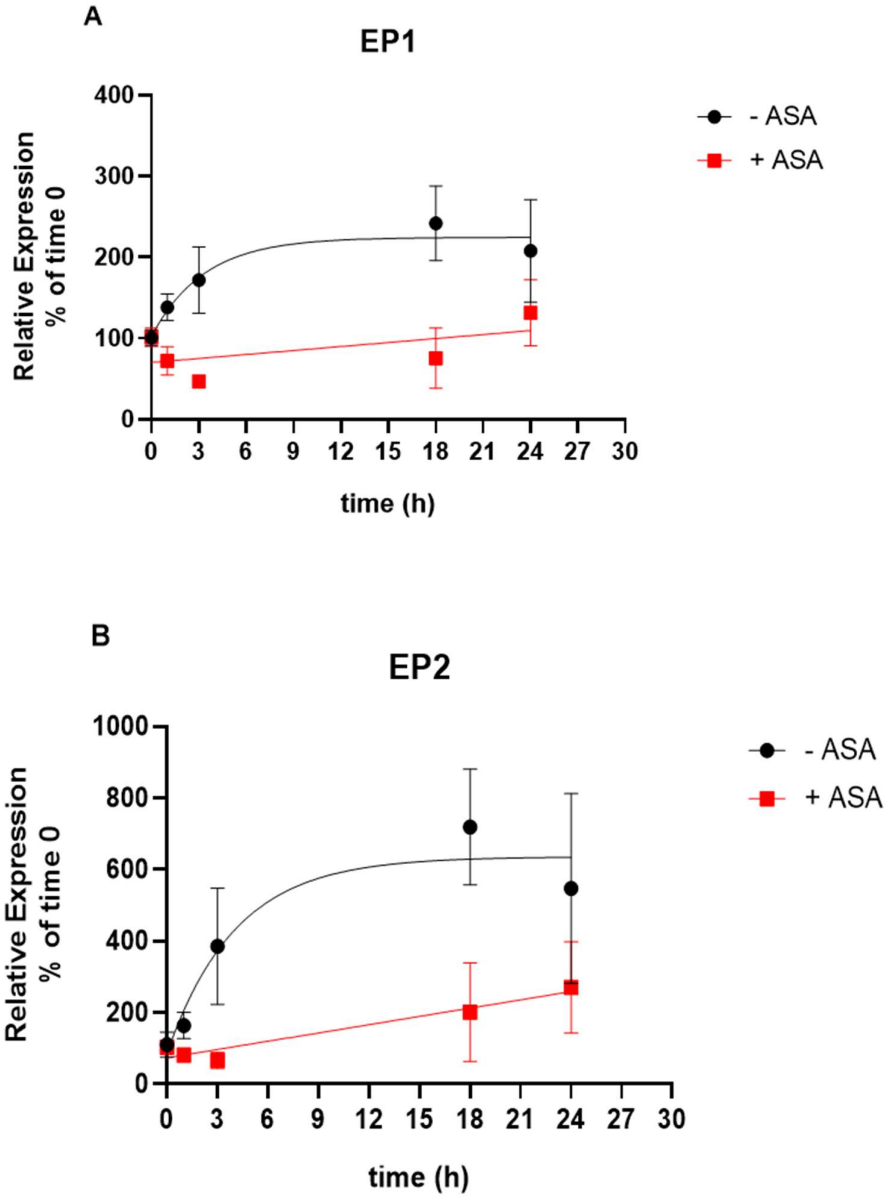
Figures 23A, 23B, 23C, and 23D show the percentage of expression for the prostanoid receptors EP1, EP2, EP3, and EP4 respectively. The expression in EP1 in the control group increased slightly with time after ACD addition until being stable around a value of 225 %, while ASA treatment prevented that increase and kept the values around 100% (figure 23A).

For EP2, the same pattern was obtained as for EP1. However, the expression in the control group increased 6 times (around 636%) compared to time 0 after the addition of ACD. The increase in the ASA-treated group was also observed, although lower 2 times (around 200%) (figure 23B). No decrease in the stability of the mRNA was observed.

Regarding EP3, the relative expression decreased in both control and ASA treatment groups. Despite having lower values of expression in the ASA-treated group

compared to the untreated cells, the half-life of the mRNA was around 6 hours in the presence of ASA compared to around 18 hours (figure 23C).

The results of EP4 show an increase in the expression over time in the control group until stabilizing around 191%, while the aspirin treatment stabilized the gene expression with no increase over time (figure 23D), suggesting that the mRNA of the gene was stable for at least 24 hours.


A
EP1
Relative Expression
% of time 0
400
300
200
100
0
0 3 6 9 12 15 18 21 24 27 30
time (h)
- ASA
+ ASA
B
EP2
Relative Expression
% of time 0
1000
800
600
400
200
0
0 3 6 9 12 15 18 21 24 27 30
time (h)
- ASA
+ ASA

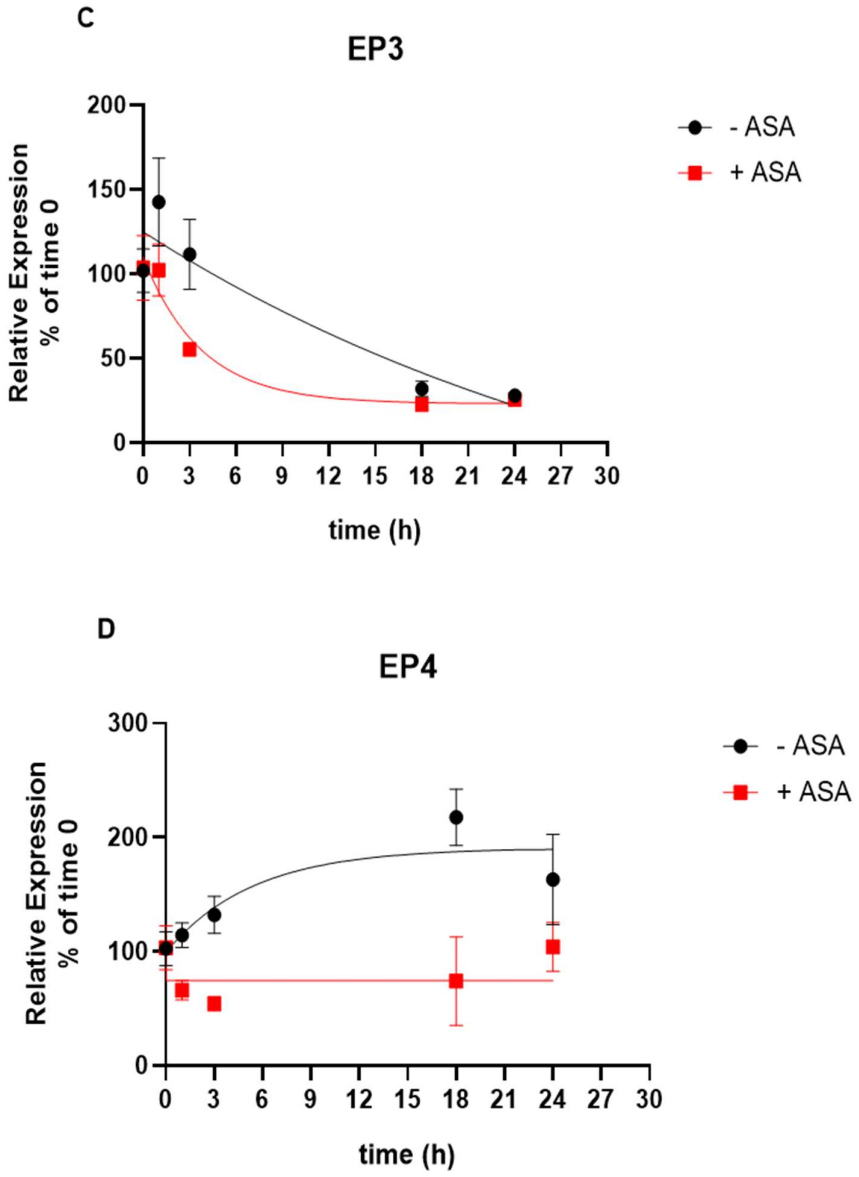


Figure 23. mRNA Decay of EP Receptors. MEG01 cells were treated with or without ASA and the half-life of mRNA was determined and data expressed as described in the legend for figure 21.

Table 3. Summary of the Modification of Gene Expression

Gene	ASA effect		ASA ½ life (24 h)	Gene expression +ACD / time	
	24	72		-ASA	+ASA
PTGS1	No change	No change	>24 h Not determined	No modification	**Slight decrease**
PTGS2	**Significant decrease**	Not significant increase	>24 h Not determined	No modification	Not beyond 50% decrease
PGES1	No change	No change	>24 h Not determined	No modification	**Slight decrease**
TXAS	No change	**Significant increase**	**3 hours**	Slow linear decrease	Beyond 50% decrease
TXAR	No change	No change	>24 h Not determined	No modification	No modification
EP1	No change	No change	>24 h Not determined	**2 times increase**	No modification
EP2	No change	No change	>24 h Not determined	**6 times increase**	2 times increase
EP3	No change	No change	**6 hours**	Slow linear decrease	**Beyond 50% decrease**
EP4	No change	No change	>24 h Not determined	**2 times increase**	No modification

CHAPTER 5

LIMITATIONS

Only one experiment done in triplicate was performed for the effect of the ASA on the stability of the mRNA messages. Additional 2 experiments are required to confirm the results. Another important point is the treatment time with ASA. Additional experiments investigating the effect of ASA at 48 and 72 hours need to be performed. Data from the recovery of the cyclooxygenase activity indicated that the MEG01 recovered their capacity to produce TXA_2 after 36 hours. Decreasing the half-life of mRNA for the enzymes responsible for the synthesis of PGE_2 or TXA_2 may delay the recovery.

An important limitation is the toxicity of ACD that limits the time of ACD treatment to 24 hours. For a better understanding of the effect on each gene, studying the activity could have helped better in investigating the change that could relate to the effect on expression and kinetics of each gene. Additional experiments assessing the protein expression, mainly for COX-1 and COX-2 proteins after ASA treatment would allow paralleling the COX activity.

We tried the analysis on the cPGES-3 gene, there was a problem in the aspirin treatment and the stability experiment. The primers for cPGES-3 did show good annealing. Furthermore, studying the effect of ASA on gene expression at 24 and 72 hours resulted in a significant decrease for COX-2 and an increase for TXAS. Therefore, it is better when repeating the experiment to add the time point 48 hours treatment to compare with 24 and 72-hour cases. This experiment could be carried during a recovery period which can give more results to confirm this effect.

CHAPTER 6

DISCUSSION

Studying the level of expression of important genes of the arachidonic acid pathway is essential when evaluating the mechanisms behind the recovery of prostanoid synthesis after NSAIDs treatment. The modification of some gene expressions would affect the enzymatic activity governing PGE_2 and TXA_2 synthesis. Aspirin is one of the best drugs used with its antithrombotic effect that interfere with platelet aggregation and has a vital role in therapy [1]. Despite aspirin success, there was variability in the pharmacological response to this drug, thus bringing the term aspirin resistance to existence [2]. Therefore, it is better to study the characteristics of inhibition of ASA in cell culture especially on megakaryocytic cell lines, as determining the effects of this drug on the number of cells, activity, or gene expression. This will guide to depict the mechanisms behind the ASA resistance that may occur *in vivo* in the patient.

When studying the effect of ASA on the gene expression of COX prostanoid genes, COX-2 showed a decrease in gene expression after 24 hours in the treatment group. This may imply that the COX-2 dependent activity may be slow down whereas COX-1 dependent activity remained similar after 24-hour recovery. It is essential to note that aspirin at therapeutic concentrations can decrease COX-2 mRNA, this was shown by studies on cultural cells without fetal bovine serum for 24 hours [96]. Further examinations show that mice treated with 10 to 30 mg/kg of aspirin accompanied by lipopolysaccharide challenge, showed a decrease in COX-2 mRNA in peritoneal macrophages [96].

Eventually, future studies about whether COX-2 gene expression is altered would bring better conclusions about the mode of action of aspirin on the gene expression of COX prostanoid genes.

In our study, thromboxane A_2 synthase shows an increase in expression at 72 hours in the ASA-treated group. This might be due to the autoregulation of this enzyme after 72 hours post-treatment. When low-dose aspirin is administered, COX-1 is inhibited irreversibly, followed by an effect on platelets where there is cumulative inhibition of TXA_2 production [97]. This reduction in TXA_2 synthesis is around 95% for several days [97]. The results in our laboratory showed a recovery of TX and not PGE_2 formation 72 hours post-ASA treatment and washing (Zahraa Mallah experiments- recovery data). These results suggest that the recovery of TX involves a newly synthesized COX-1 and TX synthase. The higher levels of TX synthase may contribute to the acceleration of the recovery after ASA treatment compared to PGE_2 synthases. Future studies should investigate the contribution of TX synthase.

On the other hand, focusing on the results of the RNA stability experiment, the data from PTGS1 and PGES1 (mPGES-1) might suggest that aspirin decreased the expression over time but properly stabilize it at levels above 50% (figure 21A and 21C). The half-life of PTGS1 and PGES1 could not be determined as the levels didn't decrease below 50%, therefore no exact modification on mRNA level for these genes is observed. However, COX-2 results show more decrease in the expression when aspirin was used and the level was stabilized at lower values, with the half-life indicated to be less than 1 hour, although determining the half-life of the mRNA is difficult when its expression does not progress beyond 50% (figure 21B).

As for the TX synthase, the half-life of TXAS in the control group was around 18 hours, while in the treated group was around 3 hours. This shows that, for TXAS, the stability of the mRNA induced is low, and its participation in TX recovery after ASA is to be determined. In relation to TX signaling, no modification of the levels of TX receptor expression nor its half-life of TXAR were observed.

Regarding EP1, EP2, and EP4, the mRNA reaches a lower plateau in the treatment group compared to the control group and the half-life could not be detected. Also, in some cases, ACD might inhibit the synthesis of elements that are responsible for the decay of some studied genes, therefore the expression will be stabilized at higher values preventing the estimation of the half-life [98]. However, in EP3 the half-life of the mRNA was around 6 hours in the presence of ASA compared to around 18 hours without ASA.

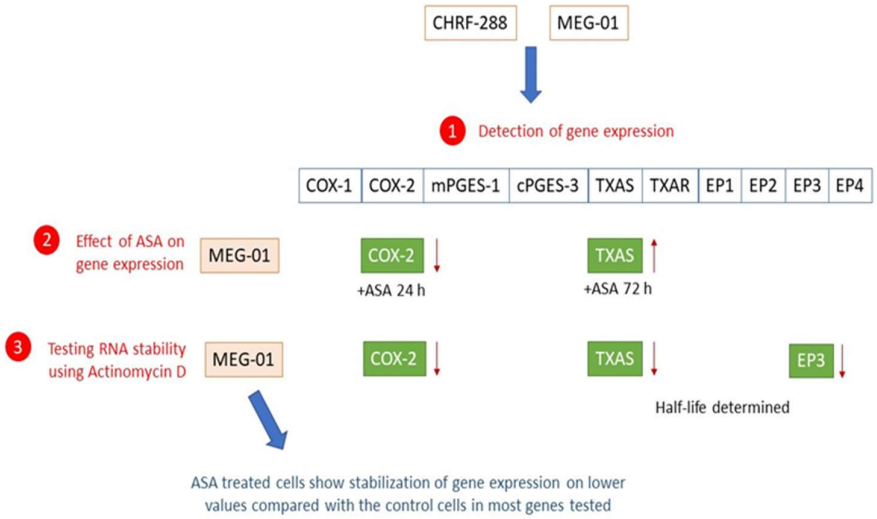


Figure 24. Summary of the Study's Major Findings

Further investigations are needed where we :

1- Repeat these experiments with the selected genes that showed promising modification in expression/ or half-lives in response to ASA.
2- Perform the analysis of the half-life at the 72 hours recovery point to check whether there is a change in other genes' half-lives.
3- Correlate the modification of decay by ASA to the prostanoid synthesis.

Indeed, the gene expression analysis by RT-PCR is not ideal to evaluate changes in half-lives and decays after ACD treatments. Our data needs to be checked after analysis of RNA by other techniques such as RNA seq.

6.1. Aspects of mRNA Half-life Experiments

These studies can be triggered using a DNA-intercalating transcription inhibitor that forms an extremely stable compound with DNA, preventing the double-helix from unwinding and so limiting the activity of DNA-dependent RNA polymerase [99]. In the study of mRNA decay, more modern techniques such as the use of inducible promoters to control transient transcription have shown to be superior to the potentially harmful effects of Actinomycin D or other transcription inhibitors [100].

Hence, whether the cells of the CHRF-288 or MEG01 cell lines were treated with ASA or not, treating them with a transcription inhibitor will provide fascinating information as to whether ASA impacts the stability of COX-1 and COX-2 mRNA genes in megakaryocytes. This will also help researchers determine whether COX-1 and COX-2 decay at different rates and how aspirin affects the half-life of cyclooxygenases in vitro.

6.2. Future Perspectives

For preferable conclusions, gene expression studies *in vitro* can be established during recovery periods, where arachidonic acid is introduced as activity and RT-PCR will be conducted on this type of experiment. Moreover, focusing on how aspirin treatment and withdrawal can influence the prostanoid synthesis in megakaryocytes, might explain the mechanism of recovery of COX activity in these cells. Gene expression analysis for aspirin treatment experiments, can include other timing points like 36 hours or beyond to compensate what is the exact change that is happening to the arachidonic acid pathway genes.

De novo synthesis of proteins could be active in pre-platelets that contain RNA and are derived from their megakaryocytic progenitors, so testing the involvement of newly synthesized COXes is an interesting project that will need the usage of cycloheximide that block the translation process, thus determining whether *de novo* synthesis plays a role in the recovery process. Therefore, COX-1 and COX-2 expression (gene and protein) can be conducted with the examination of the activity.

Subsequently, studying the contribution of COXes on the recovery of other genes such as PGE_2 and TXB_2 can be characterized using inhibitors specific to COX-1 and COX-2. The recovery period examined can be at 24, 36, 48, and 72 hours considering the features of the used cell line.

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