available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

# Acetylation of prostaglandin $H_2$ synthases by aspirin is inhibited by redox cycling of the peroxidase

*Manju Bala* [b], *Cindy N. Chin* [a], *Asha T. Logan* [a], *Taneem Amin* [b], *Lawrence J. Marnett* [b,c,d], *Olivier Boutaud* [b,*], *John A. Oates* [a,b]

[a] *Department of Medicine, Vanderbilt University, Nashville, TN 37232-6602, United States*
[b] *Department of Pharmacology, Vanderbilt University, 2200 Pierce Avenue, 514 RRB, Nashville, TN 37232-6602, United States*
[c] *Department of Biochemistry, Vanderbilt University, Nashville, TN 37232-6602, United States*
[d] *Department of Chemistry, Vanderbilt University, Nashville, TN 37232-6602, United States*

ARTICLE INFO

*Article history:*
Received 11 October 2007
Received in revised form
29 November 2007
Accepted 17 December 2007

*Keywords:*
Prostaglandin H synthase
Cyclooxygenase
Aspirin
Hydroperoxide
Cellular specificity

ABSTRACT

Aspirin exerts its unique pharmacological effects by irreversibly acetylating a serine residue in the cyclooxygenase site of prostaglandin-$H_2$-synthases (PGHSs). Despite the irreversibility of the inhibition, the potency of aspirin varies remarkably between cell types, suggesting that molecular determinants could contribute to cellular selectivity. Using purified enzymes, we found no evidence that aspirin is selective for either of the two PGHS isoforms, and we showed that hydroperoxide substrates of the PGHS peroxidase inhibited the rate of acetylation of PGHS-1 by 68%. Using PGHS-1 reconstituted with cobalt protoporphyrin, a heme devoid of peroxidase activity, we demonstrated that reversal by hydroperoxides of the aspirin-mediated acetylation depends upon the catalytic activity of the PGHS peroxidase. We demonstrated that inhibition of PGHS-2 by aspirin in cells in culture is reversed by 12-hydroperoxyeicosatetraenoic acid dose-dependently ($ED_{50}$ = 0.58 ± 0.15 μM) and that in cells with high levels of hydroperoxy-fatty acids (RAW264.7) the efficacy of aspirin is markedly decreased as compared to cells with low levels of hydroperoxides (A549; $IC_{50}$s = 256 ± 22 μM and 11.0 ± 0.9 μM, respectively). Together, these findings indicate that acetylation of the PGHSs by aspirin is regulated by the catalytic activity of the peroxidase, which yields a higher oxidative state of the enzyme.



## 1. Introduction

Irreversible inhibition of the prostaglandin H synthases (PGHSs) is central to the unique pharmacology of aspirin [1,2]. Aspirin covalently acetylates a serine in the catalytic pocket of the PGHSs (Ser530 in ovine PGHS-1 and Ser516 in murine PGHS-2) [3,4]. PGHSs thus acetylated are unable to synthesize $PGH_2$, the precursor for biosynthesis of all prostaglandins and thromboxanes. Because anuclear platelets are unable to synthesize new PGHS-1 in normal conditions, irreversible inhibition of the enzyme persists for the lifespan of the platelet. Thus, the cumulative inhibitory effect of daily

* *Corresponding author*. Tel.: +1 615 343 7398; fax: +1 615 322 4707.
E-mail address: olivier.boutaud@vanderbilt.edu (O. Boutaud).

*Abbreviations:* PGHS, prostaglandin $H_2$ synthase; PG, prostaglandin; 12-HPETE, 12-hydroperoxyeicosatetraenoic acid; 12-HETE, 12-hydroxyeicosatetraenoic acid; PPHP, 5-phenyl-4-pentenyl hydroperoxide; PPA, 5-phenyl-4-pentenyl alcohol; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; Tris, tris(hydroxymethyl) aminomethane; FePPIX, iron protoporphyrin IX; AA, arachidonic acid; LPS, lipopolysaccahride.

0006-2952/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2007.12.005

administration of small doses of aspirin (e.g. 81 mg) will yield marked inhibition of biosynthesis of thromboxane $A_2$ by platelets [5,6], whereas achieving this extent of inhibition by a single dose requires 160–325 mg. This selectivity for platelets resulting from accumulation of irreversible PGHS-1 inhibition during chronic administration of aspirin is amplified by exposure of platelets to the higher concentrations of aspirin in the portal circulation as compared with the systemic circulation in which concentrations of the drug are lower due to presystematic clearance [7] and dilution into its volume of distribution.

In contrast to the mechanistic insights into the relative selectivity of aspirin for the platelet, the reason why aspirin exerts little or no anti-inflammatory effect at antipyretic and analgesic doses has not been understood. The relative ineffectiveness of aspirin as an anti-inflammatory drug is mirrored at the cellular level by the demonstration that aspirin is 166 times more potent in inhibiting PGHS activity in the platelet than in the J774.2 macrophage cell line [8], leading to consideration that the action of aspirin might be selective for the PGHS-1 isoform. However, the potency of inhibition of PGHS-1 by aspirin ex vivo observed in normal platelets is lost in platelets of patients after coronary artery bypass grafting [9], and PGHS-1 from platelet homogenates is poorly acetylated by aspirin [10], suggesting differences in acetylation that cannot be attributed to isoform selectivity. Collectively, these observations reflect considerable variability in acetylation of the PGHSs by aspirin, a variability that implies a mechanistic basis for regulating the action of the drug.

The bifunctional nature of the PGHSs provides a conceptual framework for consideration of the regulation of acetylation of the enzyme by aspirin. The PGHSs contain both a heme-containing peroxidase and a cyclooxygenase catalytic site. Reduction of a hydroperoxide by the peroxidase oxidizes the heme to a protoporphyrin radical cation. This initial event increases the overall oxidative state of the enzyme by generating radical species in protein [11,12] through intramolecular electron transfer. Generation of the Tyr385 radical in the cyclooxygenase catalytic pocket [13] leads to abstraction of hydrogen from arachidonic acid, which initiates the oxygenation that leads to formation of prostaglandin $G_2$ ($PGG_2$). We previously found that hydroperoxide substrates that drive the peroxidase activity antagonize the inhibition of PGHSs by sodium salicylate [14]. This evidence led to the hypothesis that acetylation of the PGHSs by aspirin also could be dependent on the catalytic activity of their peroxidases. The present report describes investigations that provide evidence in support of that hypothesis.

## 2. Materials and methods

### 2.1. Materials

PGHS-1 was purified from ram seminal vesicles as described previously [15]. Wild type murine PGHS-2 was expressed in SF-9 cells (Novagen) and purified as previously described [16]. Ovine COX-1 is readily available from sheep seminal vesicles and is the closest species in amino acid sequence to human COX-1. The latter is poorly expressed in insect cells and exhibits low specific activity, which makes it unsuitable for routine inhibitor assays. In contrast, mouse and human COX-2 are highly expressed in insect cells and exhibit excellent specific activity. Recombinant mouse COX-2 crystallizes readily and a number of crystal structures of mouse-COX-2-inhibitor complexes have been determined. The availability of such structural information makes mouse COX-2 a good choice for inhibitor studies. A-549 cells were a generous gift from Dr. Robert Coffey (Vanderbilt University) and HUVECs were gifted by Dr. Douglas E. Vaughan (Vanderbilt University). RAW 264.7 cells were purchased from American Type Culture Collection (Rockville, MD). Human blood was obtained following a protocol approved by the Institutional Review Board of Vanderbilt University. Washed platelets were prepared as described previously [17]. 12-Hydroperoxyeicosatetraenoic acid (12-HPETE), 12-hydroxyeicosatetraenoic acid (12-HETE), 5-phenyl-4-pentenyl hydroperoxide (PPHP) and 5-phenyl-4-pentenyl alcohol (PPA) were purchased from Cayman Chemicals (Ann Arbor, MI). Medium 199, Hanks' Balanced Salt Solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), aspirin (acetyl salicylic acid), salicylic acid, butylated hydroxyanisole, iron protoporphyrin IX (Heme), phenol and tris(hydroxymethyl) aminomethane (Tris), lipopolysaccharide (LPS), INF-γ and IL-1β were purchased from Sigma-Aldrich (St. Louis, MO). Cobalt protoporphyrin IX was obtained from Porphyrin Products (Logan, UT). Diethyl ether was purchased from Aldrich Chemical Co. (Milwaukee, WI). Silica gel 60A thin layer chromatography plates were purchased from Whatman (Clifton, NJ). [$^{14}$C]arachidonic acid was from Perkin Elmer Life Sciences (Boston, MA). [*Acetyl*-1-$^{14}$C] aspirin was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO).

### 2.2. Acetylation of PGHS-1 and PGHS-2 with [1-$^{14}$C] acetyl salicylic acid

Ovine PGHS-1 (1.5 μM; specific activity: oxygenation of 202 mol of AA/min/mol of enzyme) or wild-type murine PGHS-2 (2.7 μM; specific activity: oxygenation of 109 mol of AA/min/mol of enzyme) was reconstituted in 100 mM Tris–HCl buffer, pH 8.0 containing 500 μM phenol in presence of 2 M equivalents of hematin (Fe-PPIX) or cobalt protoporphyrin (Co-PPIX) at 4 °C for 30 min. This solution was warmed for 2 min at 37 °C. [*Acetyl*-1-$^{14}$C] aspirin was added to a final concentration of 90 μM with or without the designated concentration of 12-HPETE or 12-HETE or PPHP or PPA. At different time intervals, aliquots were taken out, mixed with SDS-PAGE sample loading buffer and the proteins were denatured by heating at 70 °C for 10 min. Samples were loaded on 10% SDS-PAGE gel for electrophoresis followed by staining with Coomassie blue. The gels were dried and the radioactivity associated with the proteins was determined by autoradiography using PhosphorScreen (Amersham Biosciences, Piscataway, NJ). The data was analyzed on Typhoon 9400 using the software Typhoon Scanner Control (Amersham Biosciences, Piscataway, NJ). The radioactivity associated with each band was quantified using the software ImageQuant 5.2 (Amersham Biosciences, Piscataway, NJ) and was expressed as a percentage of the intensity of the band at 2, 4, or 8 min in control conditions (no hydroperoxide added to the enzymatic reaction). Preincubation with