Influence of the reducing agent triphenylphosphine on cyclooxygenase-1 metabolism of arachidonic acid

Rohini Sidhu

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Influence of the Reducing Agent
Triphenylphosphine on Cyclooxygenase-1
Metabolism of Arachidonic Acid

Rohini Sidhu
INFLUENCE OF THE REDUCING AGENT TRIPHENYLPHOSPHINE ON
CYCLOOXYGENASE-1 METABOLISM OF ARACHIDONIC ACID

by

Rohini Sidhu

Thesis

Submitted to Department of Chemistry

Eastern Michigan University

in partial fulfillment of requirements for the degree of

MASTER OF SCIENCE

in

Chemistry

Thesis Committee

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August 2010
Ypsilanti, Michigan
ACKNOWLEDGEMENTS

I take this opportunity to express my sincere gratitude and regards to my research guide Dr. Steven Pernecky for his advice, concern, guidance and constructive criticism throughout the tenure of this study.

I sincerely acknowledge the kind help rendered by my committee members, Dr. Heather Holmes and Dr. Deborah Heyl-Clegg. Under the guidance of Dr. Basu, I have learned to plan my work meticulously and take it to completion.

I wish to sincerely thank Department of Chemistry for the financial support, all the professors, especially Dr. Ruth Ann Armitage for teaching the proper use of GC-MS, and my lab mates Charles and Manal.

I also wish to thank Aditi Sengupta and Lalini Ailabonia for providing me basic data to continue on this research project.

Special thanks are due to all my friends and especially to my best friend Shridhan for believing in me and being there for me. I owe thanks to Kalyani for helping me arrange my thesis and being there for me when I needed any help for writing my thesis.

No words can describe the support rendered by a special friend Satish, himself a researcher, who not only helped me get admitted into EMU but also supported me throughout my Masters degree.

I am highly grateful to my family and especially my mom for being a constant source of encouragement and strength. I fall short of words in expressing my love and affection for them.
ABSTRACT

Cyclooxygenase (COX) metabolizes the polyunsaturated fatty acid arachidonic acid (AA) to PGG$_2$, a cyclic endoperoxide that also contains a hydroperoxide, which is subsequently reduced to an alcohol in the enzyme to yield PGH$_2$. Reactions catalyzed by COX-1 are significantly influenced by lipid hydroperoxides. The tyrosyl radical that abstracts the 13 pro(s) hydrogen from AA to initiate the COX catalytic cycle is generated by iron-oxo derivatives in the enzyme that result from lipid hydroperoxide interaction with heme iron. Commercial preparations of arachidonic acid (AA) contain lipid hydroperoxides that can be separated from AA by thin layer chromatography and readily reduced to alcohols using triphenylphosphine (TPP). The initial research objectives were to determine by GC-MS the effect of lipid hydroperoxides contained in AA preparations on COX-dependent metabolism of AA after TPP treatment and isolation of AA by TLC, and the effect of TTP treatment on the products formed during COX metabolism of AA. A novel metabolite was identified as a product of COX metabolism of AA in reactions where TPP was present; this product is a known chemo-tactic agent that influences immune function. The structural requirements of the reducing agent for production of the novel metabolite were subsequently investigated.
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Chapter 1. Introduction

1.1. Polyunsaturated Fatty Acids (PUFAs) and their Autoxidation

PUFAs have more than one double bond, hence the prefix “poly” is used before the phrase “unsaturated fatty acids”. A few of the common PUFAs are as shown in Figure 1.

Figure 1. Polyunsaturated fatty acids with their common names and structures (1).

The formula above the name represents the number of carbon atoms versus the number of double bonds present in a particular PUFA. For example, 20:4 indicates that the fatty acid has 20 carbon atoms and 4 double bonds; its common name is arachidonic acid and IUPAC name is all cis-8,11,14,17-eicosatetraenoic acid. Prefix “all cis” is used because all the double bonds in this PUFA are “cis”(2). The double bonds are separated by a methylene group, which is referred to as methylene-interrupted double bonds as shown below in Figure 2.

-CH=CH-CH2-CH=CH-CH2-CH=CH-

Figure 2. Methylene-interrupted double bonds.
These methylene groups that are separated by cis double bonds in a PUFA are readily oxidized. There is a direct relation between the number of methylene groups and the extent of the oxidation \( (1) \). Therefore arachidonic acid is more sensitive to autoxidation than linoleic acid due to the higher number of methylene groups. Double bonds also play an important role in auto oxidation; as the number of double bonds increase in a PUFA, so does the complexity of the product formed \( (1) \). Autoxidation of di-homo-\( \gamma \)-linolenic acid (3 double bonds) produces malondialdehyde and \( \beta \)-hydroxyacrolein, whereas linolenic acid (2 double bonds) does not produce similar products. Arachidonic acid that has four double bonds produces even more complex products by intramolecular cyclization via a peroxyl radical intermediate, which is an important step in fatty acid autoxidation as shown in Figure 3 \( (1) \).

![Diagram showing autoxidation of PUFAs](image)

Figure 3. Products formed by autoxidation of PUFAs.
The monocyclic and bicyclic peroxides are autoxidation products of arachidonic acid and are also known as isoprostanes, which are analogues of prostaglandins (PG’s). Isoprostanes are biomarkers for oxidative stress and lipid peroxidation and are formed in vivo primarily from arachidonic acid via free-radical-catalyzation in the absence of an enzyme (3).

1.2. **Formation of Prostaglandins from Arachidonic Acid by Enzymatic Transformation**

Arachidonic acid is present in phospholipids and is released from the membrane by the enzyme phospholipase A2. The released arachidonic acid is metabolized by the enzyme cyclooxygenase-1 (COX-1) via formation of an endoperoxide (PGG$_2$) intermediate that is reduced to hydroperoxide (PGH$_2$), which is further converted to several biological mediators (structurally similar prostaglandins) as shown in Figure 4.

![Figure 4. Cyclooxygenase in the synthesis of biological mediators (4).](image_url)
1.3. **Prostaglandins (PG’s)**

Prostaglandins are bioactive lipid mediators with specific physiological effects. Prostaglandins are oxygenated fatty acids with 20-carbon atoms that contain a 5-carbon-atom ring, and they differ from each other in the position/number of double bonds or various functional groups (keto, alcohol, etc.) present on the 5-membered ring as shown in Table 1 (2).

Table 1. Structural differences between PGE₂, PGD₂, PGF₉α, PGI₂ and 6-Keto PGF₁α and their functions.

<table>
<thead>
<tr>
<th>Prostaglandins (PGs)</th>
<th>Functions</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE₂</td>
<td>Hyperalgesia</td>
<td><img src="image1" alt="Structure of PGE2" /></td>
</tr>
<tr>
<td></td>
<td>Pyrogenic</td>
<td><img src="image1" alt="Structure of PGE2" /></td>
</tr>
<tr>
<td></td>
<td>GI tract smooth muscle contraction</td>
<td><img src="image1" alt="Structure of PGE2" /></td>
</tr>
<tr>
<td>PGD₂</td>
<td>Cytotoxic Effects</td>
<td><img src="image2" alt="Structure of PGD2" /></td>
</tr>
<tr>
<td></td>
<td>Bronchoconstriction</td>
<td><img src="image2" alt="Structure of PGD2" /></td>
</tr>
<tr>
<td>PGF₉α</td>
<td>Uterus Contraction</td>
<td><img src="image3" alt="Structure of PGF9alpha" /></td>
</tr>
<tr>
<td></td>
<td>Bronchoconstriction</td>
<td><img src="image3" alt="Structure of PGF9alpha" /></td>
</tr>
</tbody>
</table>
**Prostaglandins** have several important physiological effects like smooth muscle contraction and blood pressure regulation. They also play a role in pathological situations like initiation of pain and inflammation, broncho-constriction during an asthmatic attack, and so on. (6). The various influences on prostaglandin production and the detrimental effects of prostaglandins on a variety of cell types and function are shown in Figure 5. Cell injury results in the release of various inflammatory triggers including the release of arachidonic acid from membrane lipids, which further undergoes metabolism by enzymes (COX-1 and COX-2) to produce biologically active prostaglandins via an intermediate (PGH$_2$). Figure 5 also shows how the prostaglandins are transported or carried out from the cell with the help of prostaglandin transporter. Once they are out, with help of prostaglandin receptors (EP$_1$, EP$_2$, DP$_2$ etc.) they exert their pathological functions.
Figure 5. Detrimental effects of prostaglandins (7).

1.4. Cyclooxygenase (COX)

Cyclooxygenase (COX) is an enzyme that catalyzes the metabolism of arachidonic acid to form biologically significant mediators like prostaglandins (refer to Figures 4 & 9). COX is also referred to as prostaglandin endoperoxide H synthase (PGHS). It exists in two isoforms, COX-1, which is also known as PGHS-1, and COX-2, also known as PGHS-2. COX-1 is the constitutive form of COX due its constant presence in all mammalian tissues, whereas COX-2 is called the inducible form as it is produced in
response to external factors or injury to the cell and is undetectable in most of the mammalian tissues (8, 9).

1.5. **Non-Steroidal Anti Inflammatory Drugs (NSAIDs)**

As the name suggests, non-steroidal anti-inflammatory drugs have anti-inflammatory properties, and the term non-steroidal is used to differentiate them from the steroidal drugs. The most common NSAIDs are aspirin, naproxen, and ibuprofen, which are available over the counter. The reason for pain and inflammation in most of the situations is due to prostaglandin formation from arachidonic acid, which is catalyzed by cyclooxygenase. NSAIDs act by inhibiting the enzyme cyclooxygenase (both COX-1 and COX-2) to produce a therapeutic effect. Hence they are also called non-selective NSAIDs. Cyclooxygenase plays an important role in production of a protective mucous over the gastric mucusa (10). Hence, continuous or even intermittent use of non-selective NSAIDs leads to the destruction of this layer with severe consequences like drug-induced gastritis, gastric ulcer, GI bleeding, and perforation. The latest NSAIDs like Celecoxib selectively inhibit the enzyme COX-2, which is usually responsible for pain and inflammation, but does not affect COX-1, which is primarily responsible for the laying down of mucous in the GI tract (11).

Normally, arachidonic acid travels through the hydrophobic channel to the catalytic site on COX and activates the enzyme to form prostaglandins (Figure 6-A). Aspirin blocks the access of arachidonic acid to the catalytic site by acetylation of serine to form acetyl serine, and this process is irreversible (Figure 6-B) (12).
Figure 6. (A) Active catalytic site of COX and its interaction with arachidonic acid. (B) How aspirin binds to the active site of COX to inhibit the production of PGs. COX is represented as a dimer (13).

1.6. Role of Hydroperoxide in Activation of Cyclooxygenase

A hydroperoxide (alkyl hydroperoxide) is required to initiate the catalytic cycle for cyclooxygenase enzyme to produce biologically significant lipid mediators (PGs). The ferryl-oxo complex is formed from oxidation of the heme group by a hydroperoxide. The ferryl-oxo complex then oxidizes the Tyr-385 which is present near the active site of COX to form a tyrosyl radical. The tyrosyl radical abstracts the 13-pro(S) hydrogen from arachidonic acid to start the catalytic cycle. After the abstraction of 13-pro(S) hydrogen from arachidonic acid, it undergoes a radical cycle and intramolecular cyclization by addition of two molecular oxygen atoms to form an important intermediate (PGG$_2$) as shown in Figure 7 (14).
Figure 7. Role of Hydroperoxide in Activation of Cyclooxygenase Catalytic Cycle (14).

Figure 8 shows the role of the enzyme in hydroperoxide activation. The alkyl hydroperoxide attacks the heme group present at the peroxidase active site. Arachidonic acid is then attached to the cyclooxygenase active site to undergo radical and intramolecular cyclization with the help of Tyr-385 residue.
1.7. Role of Lipid Hydroperoxide as an Intermediate

Lipid hydroperoxide is an intermediate in the catalytic mechanism of cyclooxygenase (Figure 9). Arachidonic acid metabolism involves a two-step mechanism to form prostaglandins. The first step is catalyzed by COX-1 by addition of molecular oxygen to form a hydroperoxy (–OOH group)/endoperoxide (–O–O– group as a bicyclic ring) intermediate also known as PGG₂. In the second step, PGG₂ is reduced to hydroxy (–OH group) endoperoxide, also known as PGH₂, by peroxidase activity. PGH₂ then acts as a substrate for other metabolizing enzymes to give PGE₂, PGF₂α, PGD₂, and prostacyclin (PGL₂), and by converting the bicyclic endoperoxide to a five-membered ring that has four different substituents. Thromboxane A₂ (TXA₂) has a six-membered ring with an infused oxygen atom (oxane ring) (2, 15).
Figure 9. Represents the metabolic pathway of arachidonic acid and the role of lipid hydroperoxide as an intermediate for formation of prostaglandins.

Hydroperoxides thus play an important role in the overall production of prostaglandins by activating the cyclooxygenase enzyme (section 1.6) and by acting as an intermediate (section 1.7). Hence it is worthwhile to study the hydroperoxides in this complicated mechanism by using mild reducing agents that do not affect the unsaturation of arachidonic acid or the activity of cyclooxygenase to produce the biological mediators but do reduce the hydroperoxides to alcohols.
1.8. **Research Objectives**

1. To determine, by GC-MS, the effect of lipid hydroperoxides contained in AA preparations on COX-dependent metabolism of AA. Lipid hydroperoxides are easily formed in AA preparations and, thus, understanding their effect on the studies involving the COX enzyme may be confounded by their presence.

2. To study the effects of various reducing agents and other similar compounds on the products formed during the metabolism of arachidonic acid by the enzyme cyclooxygenase.

1.9. **Review of Literature**

The production of prostaglandins depends upon the amount of hydroperoxide, which stimulates the activity of COX-1 in arachidonic acid metabolism. In some particular experiments, when the availability of hydroperoxide is limited or decreased by certain reducing agents, the amount of prostaglandins generated is directly altered.

Various reducing agents have been used in the past, like glutathione peroxidase (17), t-butyl hydroperoxide, and 4-hydroxy-2,2,6,6-tetramethylpiperidinoxy radical (18), to modify the amount of hydroperoxides in the synthesis of prostaglandins from arachidonic acid. In another work (19), triphenylphosphine was used as a reducing agent to limit the amount of hydroperoxides during lipid peroxidation.

In our experiments, we used a series of structurally related reducing agents such as triphenylphosphine, and trimethylphosphine, and the oxidized product triphenylphosphine oxide to study the influence of reducing agents on cyclooxygenase-1 metabolism of arachidonic acid.
Chapter 2. Materials and Methodology

2.1. Arachidonic Acid (AA)

Arachidonic acid (100 mg/mL in ethanol) was purchased from Cayman Chemicals, Ann Arbor, and stored at -20°C, as per manufacturer’s instructions. A 1:100 dilution of arachidonic acid was made in ethanol to obtain a concentration of 1 mg/mL, which was prepared as a stock solution and stored at -20°C for further use.

2.2. Triphenylphosphine (TPP)

Triphenylphosphine (CAS number: 603-35-0) was purchased from Sigma Aldrich and stored at room temperature, as per manufacturer’s instructions. TPP is an organic compound with three phenyl rings attached to a phosphorus atom with a nucleophilic centre and has the ability to act as a reducing agent. A mass of 5 mg of triphenylphosphine was dissolved in 5 mL of diethyl ether to give 1 mg/mL of triphenylphosphine in ether and was prepared fresh before use.

2.3. Cyclooxygenase – 1 (COX-1)

COX-1 (Ovine) 5 Kunits was supplied in 80 mM Tris-HCl, pH 8.0 with 0.1% Tween 20 and 300 μM diethylthiocarbamate and purchased from Cayman Chemicals, Ann Arbor. COX-1 is extremely unstable to freeze-thaw cycling and hence was apportioned to 10 μl aliquotes and stored at -80°C for further use. To prevent the loss of activity of enzyme while performing the reaction at room temperature, COX-1 was kept on ice (0-4°C) throughout the experiment.
2.4. Preparation of purified arachidonic acid (P-AA)

100 μL of 1 mg/mL of arachidonic acid was incubated with 100 μL of 1 mg/mL of triphenylphosphine solution for 30 minutes at room temperature and evaporated under a stream of nitrogen followed by reconstitution in 100 μL of ether. Thin layer chromatography (TLC) was performed to obtain purified arachidonic acid.

2.5. TLC procedure

TLC (thin layer chromatography) plates with silica gel matrix supported on glass of 5 cm x 20 cm, with layer thickness of 250 μm and a particle size of 17 μm were purchased from Sigma-Aldrich. The reconstituted arachidonic acid solution was spotted as a band on the TLC plate and was developed with the solvent system of hexane, ether, and acetic acid in a ratio of 90:10:1. The purified arachidonic acid (upper band) was scraped from the TLC plate and extracted thrice from the silica with ether. Extracts were combined, dried under nitrogen, and reconstituted in 100 μL of ether. Argon was bubbled in the solution to prevent any auto-oxidation. The purified arachidonic acid (P-AA) was then stored at -80 °C for further use.

2.6. P-AA treated with TPP

100 μL of TPP solution was incubated with 100 μL of P-AA at room temperature for 30 minutes and evaporated under nitrogen to eliminate hydroperoxides still present in P-AA.

2.7. COX-1 treated with TPP

100 μL of TPP solution was first evaporated completely under nitrogen, and 1 to 2 μL of COX-1 was added followed by incubation at room temperature for 30 minutes.
2.8. Reaction of COX-1 with P-AA

Two solutions A and B were prepared and subsequently mixed to initiate the reaction. Solution A contained 100 μL of 0.5 M phosphate buffer, pH 7.8, 50 μL of 1% Tween-20: 1% octylglucoside, 50 μL of 50% glycerol, and 50 μL 2.5 mM phenol. Chemicals were mixed prior to addition of 2 μL COX-1, and the mixture was then shaken at 37°C for 2 min. Solution B contained 100 μL of P-AA evaporated under nitrogen, and suspended in 142 μL of deionized water.

Solution B was added to Solution A and the volume was made to 500 μL with deionized water. The reaction was carried out on a shaker for 15 minutes at 37°C. The reaction was stopped by plunging tubes in ice and by adding 200 μL of methanol and 100 μL of 1 M citric acid. The samples were then extracted thrice with 1 mL of ether for each extraction. Approximately 50 grams of anhydrous sodium sulfate were added to the samples to remove any traces of aqueous component. The organic layer (ether) was separated from the drying agent followed by complete evaporation under nitrogen. The products formed were further analyzed using GC/MS.

2.9. Reaction of COX-1 and P-AA both treated with TPP

A similar reaction as in section 2.8 was performed using COX-1 treated with TPP and P-AA treated with TPP.

2.10. Reaction of COX-1 and P-AA both treated with MDPP/DMPP/ TBP & TBPO

A similar reaction as in section 2.8 was performed using COX-1 and P-AA treated with different reducing agents like methyldiphenylphosphine (MDPP)/
dimethylphenylphosphine (DMPP)/ tributylphosphine (TBP) and triphenylphosphine oxide (TPPO).

2.11. Reaction of denatured COX-1 with P-AA

The enzyme COX-1 was denatured by incubating at 60°C for 10 minutes. The denatured COX-1 was reacted with P-AA in a similar manner as explained above.

2.12. Preparation of derivatives for GC-MS analysis

A compound is chemically changed in a derivatization reaction so that its properties become suitable for detection in a specific analytical method. In GC-MS, derivatization is particularly done to impart volatility to non-volatile compounds, making them thermally stable and reducing their polarity for better chromatographic separations.

Three different types of derivatives were prepared for analysis in the following sequence:

1. Methyl ester derivative

The function of methyl ester derivative is to esterify carboxyl group as shown in Figure 10.

![Figure 10. The conversion of carboxyl group to acetate group by TMSD.](image)
Freshly prepared solutions were used for all steps of the derivatization. Solution 1 contained 0.02 M trimethylsilyldiazomethane (TMSD) in heptane, which was prepared by taking 10 µL of 2.0 M of TMSD solution in heptane (Aldrich) and diluting with 990 µL of heptane. Solution 2 contained 20% methanol in acetone, which was prepared by taking 600 µL methanol and diluting with 2400 µL acetone. 350 µL of each solution 1 and solution 2 were added to the sample and vortexed for 1 minute followed by incubation at room temperature for 30 minutes and complete evaporation under nitrogen for the next derivative.

2. Oxime derivative

The function of an oxime derivative is to convert a carbonyl group to a methoxime group as shown in Figure 11.

![Figure 11. Carbonyl group is converted to methoxime group by methoxylamine HCl.](image)

Solution 3 contained 8 mg of methoxylamine-HCl (Aldrich), which was dissolved in 400 µL of pyridine to give 2% w/v methoxylamine-HCl in pyridine. The methyl ester derivative was dissolved in 50 µL of the solution 3, covered with parafilm, vortexed for one minute, and incubated in a water bath at 60 °C for 90 minutes followed by drying under nitrogen.
3. BSTFA derivative

Silylation of the hydroxyl group was accomplished by reaction of alcohol groups with BSTFA (N,O-Bis(trimethylsilyl) trifluoroacetamide) as shown in Figure 12.

\[
\text{R-OH} \quad \text{BSTFA} \quad \text{1% TMCS} \quad \text{CH}_3\text{Si-CH}_3
\]

Figure 12. The conversion of hydroxyl group into trimethylsilyl group.

Volumes of 25 µL of pyridine and 100 µL of BSTFA reagent in 1% trimethylchlorosilane (TMCS) was added to the sample from the oxime derivative, covered with parafilm, vortexed for one minute, and incubated in a water-bath at 60 °C for 60 minutes followed by drying under nitrogen. The dried sample was re-suspended in 500 µL of ether for purification.

2.13. Purification of samples

Samples were purified using solid phase extraction. C-18 silica columns (Waters) were washed with hexane; samples were loaded and eluted using 2 ml of diethyl ether, which was then evaporated completely under nitrogen. The samples were then re-suspended in 100 µL of ethyl acetate for GC-MS analysis.

2.14. GC-MS methodology

A 3800 Saturn Varian Gas Chromatograph and 2200 Varian Mass Spectrometer were used to analyze the purified samples. A volume of 1 µL of sample was injected in split/splitless injection with a split ratio of 1:5 and injector temperature of 200 °C into a
fused silica column (5% phenyl and 95% dimethylpolysiloxane) having dimensions of 30 meters length, 0.25 mm diameter, and with 0.25 µm film thickness. Helium was used as the mobile phase. The column oven was set initially at 200 °C for 2 minutes followed by first ramp up to 240 °C at the rate of 40 °C/minute and a second ramp up to 275 °C at the rate of 8 °C/minute for 4.5 minutes. Electron Impact Ionization was used with an ion trap detector. The mass range of 50 to 650 was selected and the transfer line was kept at 250 °C, while the ion trap was maintained at 180 °C.
3.1. Reaction of COX-1 with P-AA

The aim of this reaction was to confirm the products formed by arachidonic acid metabolism via COX-1. Two major peaks in the chromatogram were seen at retention times of 6.10 and 7.30 minutes after injection of the sample on the GC column as shown in Figure 13.

![Reaction of COX-1 with P-AA and Derivatized Standard for PGE2](image)

Figure 13. Gas chromatogram of derivatized products formed when P-AA is incubated with COX-1 is shown above the chromatogram of derivatized standard PGE2.

The product with a retention time of 7.30 minutes was identified as PGE2 and confirmed by comparison to a derivatized PGE2 standard. The peak at 6.10 minutes was identified as 6-keto PGF1α by comparing it with a derivatized standard of 6-keto PGF1α, as shown in Figure 14.
Figure 14. (A) Gas chromatogram of derivatized standard 6-Keto PGF$_{1\alpha}$. (B) Gas chromatogram of derivatized products formed at 6.10 minutes when P-AA is incubated with COX-1.

The derivatization reaction of PGE$_2$ and 6-keto PGF$_{1\alpha}$ is as shown in Figure 15 (A) and (B).
Figure 15. Scheme for synthesis of the methyl ester-oxime-silylated derivative of (A) PGE$_2$ and (B) 6-keto PGF$_{1\alpha}$.

Figure 16. Comparison between the mass spectra of the product formed at 6.13 min with derivatized PGE$_2$ standard.
Formation of PGE$_2$ and 6-keto PGF$_{1\alpha}$ during P-AA metabolism by COX-1 was confirmed by comparing mass spectra with derivatized standards as shown above in Figure 16 and Figure 17.

The circles on the mass spectra indicate the major fragment (Base peak) and daughter fragments that are common to both mass spectra.

![Mass Spectrum of 6-Keto PGF$_{1\alpha}$ formed by reaction of COX-1 with P-AA](image1.png)

![Mass Spectrum of Derivatized Standard for 6-Keto PGF$_{1\alpha}$](image2.png)

Figure 17. Comparison between the mass spectra of the compound formed at 6.14 min with derivatized 6-keto PGF$_{1\alpha}$ standard.

### 3.2. Reaction of denatured COX-1 and P-AA

The aim of this reaction was to confirm that the production of PGE$_2$ and 6-keto PGF$_{1\alpha}$ from arachidonic acid catalyzed by COX-1 is a function of the enzyme rather than a contamination in commercially available arachidonic acid preparations (refer to Figure...
18 ([A] and [B]). Indeed, no peaks corresponding to those found to be present in the chromatogram were evident when the intact COX-1 was replaced with the heat-denatured form.

Figure 18. (A) Gas chromatogram and (B) A mass spectrum of the products of a reaction between the denatured COX-1 and P- AA, showing absence of peaks and ions respectively, concluding absence of 6-Keto PGF$_{1\alpha}$ and PGE$_2$.

3.3. **Reaction of COX-1 and P-AA both treated with TPP**

The aim of this reaction was to reduce any peroxide present in COX-1 and P-AA by treating both with TPP. The chromatogram obtained by this reaction was similar to
Figure 13. Interestingly, an additional peak at a retention time of 5.16 minutes was observed in the chromatogram, which was not present in the chromatograms for COX reactions conducted in the absence of added TPP.

Figure 19. (A) Gas chromatogram of derivatized products formed when TPP-treated COX-1 was incubated with TPP treated P-AA. (B) Gas chromatogram of derivatized standard 12-HETE.

A new peak in the chromatogram at a retention time of 5.16 minutes indicates the production of a novel metabolite that was tentatively identified as 12-hydroxyeicosatetraenoic acid (12-HETE) by comparison with 12-HETE standard as shown in Figure 19 (A) and (B).
The derivatization reaction of 12-HETE is shown in Figure 20.

Figure 20. Scheme for synthesis of methyl ester-silylated derivative of 12-hydroxyeicosatetraenoic acid (12-HETE).

Formation of 12-HETE was further confirmed by comparing the mass spectrum with the derivatized standard of 12-HETE. The match between the base peaks with m/z 262.2 as well as the other ions with m/z of 51.0, 77.0, 108.1, 152.2, 183.2, 445.2, and 502.6 in both the spectra, confirms the formation of 12-HETE as shown in Figure 21.
Figure 21. Comparison between the mass spectra of derivatized 12-HETE formed by the reaction of COX-1 with P-AA both treated with TPP and the standard derivatized 12-HETE.

3.4. Derivatization of P-AA treated with TPP

P-AA, treated with TPP, was derivatized and analysed using GC-MS. No peak corresponding to 12-HETE was seen conforming that the production of 12-HETE is dependent on COX-1 and not on P-AA treated with TPP alone as shown in Figure 22.
Figure 22. Chromatogram for derivatized P-AA treated with TPP and derivatized standard 12-HETE.
Chapter 4. Interpretation and Comparison of Results

4.1. Purified Arachidonic Acid P- AA

P- AA was prepared as described in section 2.5 by using TLC. Two TLC plates were used to show the commercially purchased AA and AA that was treated with TPP, i.e. P - AA. Commercially purchased AA was spotted as a band on plate one and AA treated with TPP was spotted as a band on plate two. The plates were developed in the same chamber and phosphomolybdic acid was used to stain the plates to visualize the compounds as shown in Figure 23.

Figure 23. Thin layer chromatography plates of arachidonic acid samples before (Plate 1) and after (Plate 2) treatment with triphenylphosphine.

Plate 1 showed a single band corresponding to the commercially available AA that has hydroperoxides in it. However, Plate 2 shows two bands. The lower band
corresponds to purified AA while the upper band corresponds to the alcohol formed due to reduction of hydroperoxides present in AA by TPP.

4.2. **Comparison of products formed from reaction of COX-1 with P - AA and reaction of TPP treated COX-1 with TPP treated P - AA**

Two major observations were seen in this comparison as shown in Figure 24. First, the amount of PGE\(_2\) produced by reaction of COX-1 with P - AA treated with TPP was not appreciably altered when compared to reaction of untreated COX-1 with P - AA. Production of a novel metabolite 12- HETE was seen in the reaction of TPP-treated COX-1 with TPP-treated P - AA.

![Figure 24. Comparison of GC Chromatogram for products formed from reaction of COX-1 with P - AA and reaction of TPP-treated COX-1 with TPP-treated P - AA](image)

4.3. **Comparison of TPP with other reducing agents**

To determine the specificity of TPP in the production of 12-HETE, other reducing
agents like methyl diphenylphosphine (MDPP) and di-methyl phenylphosphine (DMPP) were used instead of TPP (refer to Figure 25).

Figure 25. Comparison of GC chromatogram for products formed from reaction of COX-1 with P - AA both treated with TPP, MDPP or DMPP.

A few major observations were made. First, the amount of PGE\(_2\) produced by using TPP, MDPP, or DMPP in the reaction of COX-1 and AA both treated with these chemicals were the same. Second, a similar peak to 12-HETE was seen with reaction of MDPP. Third, the amount of 12-HETE produced by MDPP was appreciably lower than the amount produced by TPP. Finally, virtually no 12-HETE was seen with reaction of DMPP. To confirm the production of 12-HETE by MDPP, mass spectra were compared as shown in Figure 26.
Figure 26. Comparison of mass ions for 12-HETE produced by standard 12-HETE and by the reaction COX-1 with P- AA both treated with TPP, MDPP, or DMPP.
4.4. **Comparison of TPP with other oxide reducing agents**

Reactions of COX-1 with P - AA both treated with tributylphosphine (TBP), tributylphosphine oxide (TBPO), and triphenylphosphine oxide (TPPO) were carried out to see whether 12-HETE is produced with oxide reducing agents.

No 12-HETE was formed with the reactions of TBP, TBPO or TPPO, as seen in Figure 27.

![Figure 27](image)

Figure 27. Comparison of gas chromatogram of products of reaction between COX-1 and P-AA both treated with 1. TBPO, 2. TPPO, 3. TBP, and 4. TPP.
Chapter 5. Conclusions

Thin layer chromatography clearly indicates that arachidonic acid, when treated with a reducing agent like triphenylphosphine, produces lipid alcohols; these lipid alcohols are produced due to the presence of lipid hydroperoxides in commercially available arachidonic acid preparations as shown in Figure 28.

Figure 28. Conversion of arachidonic acid 5-hydroperoxide to arachidonic acid 5-hydroxy by triphenylphosphine. Triphenylphosphine oxide is formed as a by-product.

The incubation of triphenylphosphine and methyl diphenylphosphine with COX-1 and purified arachidonic acid resulted in a new metabolite, 12-HETE, and did not appreciably affect the production of PGE\(_2\) and 6-Keto PGF\(_{1\alpha}\). However, when the same experiment was conducted treating both reactants with tributylphosphine,
tributylphosphine oxide or triphenylphosphine oxide, no 12-HETE was produced and COX-1 dependent production of PGE$_2$ and 6-Keto PGF$_{1\alpha}$ was still supported.

The presence of hydroperoxides in a reaction of arachidonic acid with COX ensures that the cyclooxygenase enzyme remains in a catalytically active state and the conversion of arachidonic acid to physiologically significant prostaglandins proceeds in an uninhibited fashion. Hence it is imperative to control the presence of hydroperoxides in the reaction of arachidonic acid with COX. This work shows that removal of hydroperoxides by TPP controls their presence in the reaction and, thus, controls the production of biologically significant prostaglandins.
Chapter 6. Discussion

Arachidonic acid conversion can be catalyzed by two different enzymes, cyclooxygenase and lipoxygenase, producing distinct biologically significant lipid mediators. Arachidonic acid, when metabolized by the enzyme 12-lipoxygenase, produces an intermediate called 12-HETE. The production of 12-HETE is not known to occur when arachidonic acid is metabolized by the enzyme cyclooxygenase. An attempt at verification of 12-HETE production was done by HPLC-MS in collaboration with Dr. Paul Kennedy at Cayman Chemical, Ann Arbor. Preliminary results suggest that 12-HETE is not formed during the metabolism of AA by COX-1 in the presence of TPP (Steve Pernecky, personal communication). Further experiments will have to be conducted to determine whether the preparation of derivatives for GC-MS analysis may have yielded a product with a similar chromatography and mass spectrum of 12-HETE.

The results of the current study indicated that 12-HETE is produced as a result of arachidonic acid metabolism catalyzed by COX-1 in the presence of TPP or MDPP. This is supportive of the possibility that TPP or MDPP stimulates a novel activity in the enzyme (COX-1) that results in production of 12-HETE. Since the reducing agents, TBP, TBPO, or TPPO, do not support production of 12-HETE, this suggests that the generation of the novel metabolite (12-HETE) is not attributed to the reductive capacity of the reducing agent. In addition, the control reaction with denatured COX indicates that the intact enzyme plays some role in the production of 12-HETE. 12-HETE is also a product of non-enzymatic oxidation of arachidonic acid, but in this study arachidonic acid was treated with a reducing agent (TPP) to remove hydroperoxides so that the chances of non-enzymatic oxidation of arachidonic acid was eliminated.
The hypothetical pathway for the production of 12-HETE is as shown in Figure 29.

![Pathway Diagram](image)

Figure 29. 12-HETE can be formed via formation of 12-HpETE or directly to 12-HETE. Both pathways involve reaction of P-AA and COX-1 in presence of TPP/MDPP.

The production of 12-HETE is referred to as a novel metabolite because 12-HETE has not been previously observed in the metabolic pathway of arachidonic acid catalyzed by COX-1.

The structure of the reducing agent might have an important role in supporting the production of 12-HETE (refer to Figure 30).

![Structure Comparison](image)

Figure 30. Structure comparison of TPP and MDPP.
TPP has three phenyl rings attached to a phosphorus atom, whereas MDPP has two phenyl rings, the third phenyl ring being replaced by a methyl group. This indicates that at least two phenyl rings attached to one phosphorous atom are needed to produce 12-HETE, as other reducing agents with slight dissimilarity in structure (refer to Figure 31) don’t produce 12-HETE.

![Chemical structures of DMPP, TBPO, TBP, and TPPO.](image)

Figure 31. Structure of DMPP, TBPO, TBP, and TPPO.

Some natural and dietary compounds, which have structural similarities to TPP and MDPP, can control the function of the enzyme to produce 12-HETE, which is known as a chemotactic agent, influencing the immune system (20, 21). Hence it is important to
explore the structurally similar natural/dietary compounds to see whether they influence the catalytic activity of enzyme (metabolism of arachidonic acid via COX-1) to produce 12-HETE. Our work thus explores a novel pathway in arachidonic acid metabolism towards the synthesis of HETE through COX-1.
References


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