THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF VETERINARY AND BIOMEDICAL SCIENCES

AN INVESTIGATION OF THE ANTI-INFLAMMATORY PROPERTIES OF METABOLITES OF EICOSAPENTAENOIC ACID

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ABSTRACT

There has been a recent upsurge of interest in the health benefits of fish oils, which are rich in omega-3 fatty acids such as eicosapentaenoic acid (EPA). These purported benefits, ranging from improved heart health to relief from arthritis, are likely due to the proven anti-inflammatory effects of omega-3's. It was unclear, however, if it was EPA itself or rather one of its metabolic breakdown products that was causing the anti-inflammatory effects. Increased consumption of fish oils is known to increase incorporation of EPA into the side chains of phospholipid molecules in the cell membrane, displacing EPA's omega-6 fatty acid analog, arachidonic acid (AA). When cells are exposed to pro-inflammatory stimuli, the fatty acids are cleaved from the phospholipids and processed by cyclooxygenase (COX) enzymes and then a succession of prostaglandin (PG) synthases. The metabolic products of AA include PGs of the 2-series, which are generally shown to be pro-inflammatory, while EPA metabolizes to PGs of the 3-series, many of which have anti-inflammatory properties. Research with 2-series prostaglandins has shown that some, most notably 15d-$\Delta^{12,14}$-PGJ_2, have unexpected anti-inflammatory effects. This project is therefore centered around the hypothesis that the 3-series analogs of these PGs may have superior anti-inflammatory capabilities. High pressure liquid chromatography coupled to UV-mass spectrometry was used to isolate and characterize the metabolic products of the *in vitro* and cell culture conversion of EPA. The products 15d-$\Delta^{12,14}$-PGJ_3 and Δ^{12}-PGJ_3 showed significant anti-inflammatory properties by mitigating expression of pro-inflammatory genes in macrophages. The results suggest that the cyclopentenone metabolites of EPA, which are endogenously produced, have potent anti-inflammatory capabilities by virtue of their chemical structures.

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
EPA	Eicosapentaenoic Acid
PG	Prostaglandin
HPLC	High-Pressure Liquid Chromatography
PUFA	Polyunsaturated Fatty Acid
COX	Cyclooxygenase
LOX	Lipoxygenase
$cPLA_2\alpha$	Cytosolic phospholipase $A_2\alpha$
NO	Nitric Oxide
LPS	Lipopolysaccharide
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
DMEM	Dulbecco's Modified Eagle's Media
PBS	Phosphate Buffered Saline
P/S	Penicillin/Streptomycin
HCl	Hydrochloric Acid
ACN	Acetonitrile
LA	Linoleic Acid
PPAR	Peroxisome Proliferator-Activated Receptors
NF-κB	Nuclear transcription factor κB
iNOS	Inducible Nitric Oxide Synthase
HSA	Human Serum Albumin
DMSO	Dimethyl Sulfoxide
UV/Vis	Ultra-violet/Visible Light
PGDS	Prostaglandin D Synthase
DNA	Deoxyribonucleic Acid
FBS	Fetal Bovine Serum
NaCl	Sodium Chloride
LC	Liquid Chromatography
MS	Mass Spectrometry
PMSF	Phenylmethylsulfonic Acid
SDS	Sodium Dodecyl Sulfate
TBS	Tris Buffered Saline
TNF-α	Tumor Necrosis Factor-Alpha
EDTA	Ethylenediaminetetraacetic Acid

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Chapter 1

Literature Review

Metabolism of Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) are a class of compounds which contain long chains of carbons with multiple carbon-carbon double bonds. They include omega-3 and omega-6 fatty acids, two well-known classes of compounds. The two types vary based on chemical structure, with omega-3s having their first double bond between carbons 3 and 4 as numbered from the methyl end of the molecule, while omega-6s have their first double bond between carbons 6 and 7. Eicosapentaenoic acid (EPA) is a classic omega-3 PUFA, and arachidonic acid (AA) is a classic omega-6 PUFA (see Figure 1-1).

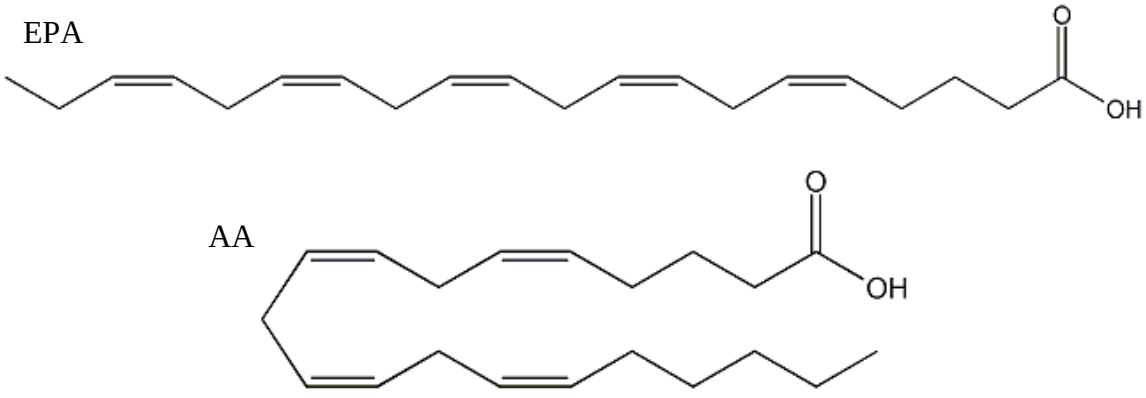


Figure 1-1. Chemical Structures of EPA (omega-3) and AA (omega-6)

The two compounds exhibit very similar metabolism both in cell cultures and *in vitro*. Both produce eicosanoids, twenty-carbon chain hormone-like lipids which play important biological roles in platelet aggregation, cell growth and differentiation, inflammatory modulation, and immune responses [3]. When cells are exposed to EPA or AA over time, the PUFAs become

incorporated into the side chains of the phospholipids making up the cellular membrane at the *sn*2-position. Since diets in the Western world are rich in omega-6 fatty acids, arachidonic acid typically occupies this position in the membrane phospholipids. However, supplementation with omega-3 fatty acids, commonly in the form of fish oils, has been shown to significantly increase the ratio of EPA to AA in the phospholipid membrane. Given pro-inflammatory stimuli, EPA or AA is cleaved off of the phospholipid side chains by cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$), which shows an equal affinity and specificity for these two compounds [7]. The freed AA or EPA is then acted upon by a class of enzyme called cyclooxygenases (COX) and converted to prostaglandins and thromboxanes of the 2- or 3-series [6]. Lipoxygenase (LOX) enzymes also act on AA and EPA to produce lipoxins, hydroxyl fatty acids, and leukotrienes of the 4- or 5-series [3].

Cyclooxygenases

Cyclooxygenase-1 was the first of the COX enzymes to be discovered, and it is constitutively expressed in the mammalian body. Though COX-2 is similar to COX-1 in structure and enzymatic activity, it is an inducible enzyme, and expression is increased by stimuli associated with inflammation. Therefore COX-1 is considered to be the normal physiological form of cyclooxygenase, and COX-2 is considered the pathological form of the enzyme [6]. Both COX-1 and COX-2 exhibit specificity for AA over EPA, but the two enzymes utilize AA equally well. In contrast, COX-2 utilizes EPA at only 30% of the rate of AA, and COX-1 does not act on EPA at all without high levels of hydroperoxide present. Instead, EPA binds to COX-1 in a conformation which does not allow for the catalytic reaction to occur, and inhibits the binding of AA [7]. Therefore, an increased EPA:AA ratio can reduce the amount of 2-series prostaglandins

produced, and increase the production of 3-series prostaglandins, especially under inflammatory conditions.

Prostaglandin Metabolism

After AA and EPA are oxidized to the hydroperoxy endoperoxide compounds PGG_2 and PGG_3 by the action of the COX enzymes, they are then further reduced to PGH_2 and PGH_3 [6]. These endoperoxide intermediates are then converted into other prostaglandins and thromboxanes by a variety of synthase enzymes (see Figure 1-3). For example, Prostaglandin D Synthase (PGDS) converts PGH_2 into PGD_2 and PGH_3 into PGD_3. Then PGD_2 and PGD_3 break down to cyclopentenone J series prostaglandins of the 2- or 3-series like PGJ, Δ^{12}-PGJ and 15-deoxy-$\Delta^{12,14}$-PGJ [5].

Experiments have shown that 15-deoxy-$\Delta^{12,14}$-PGJ_2 is endogenously produced in the body in conjunction with heightened levels of COX-2 expression, specifically in macrophage-derived foamy cells found in arteries in the early stages of atherosclerosis, considered to be a chronic inflammatory condition [5]. 15-deoxy-$\Delta^{12,14}$-PGJ_2 has also been found in both intracellular and extracellular samples from RAW 264.7 macrophages exposed to lipopolysaccharide (LPS), which provides inflammatory stimuli and results in increased expression of COX-2. Moreover, the experiments showed that 15-deoxy-$\Delta^{12,14}$-PGJ_2 could be produced *in vitro* in the absence of cells. One study showed that human serum albumin (HSA) in an aqueous buffer could catalyze the breakdown of PGD_2 into Δ^{12}-PGJ_2, 15-deoxy-$\Delta^{12,14}$-PGD_2, and 15-deoxy-$\Delta^{12,14}$-PGJ_2 [2]. However, another experiment demonstrated that when PGD_2 was placed in an aqueous buffer without any HSA, both PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 were still produced in quantity. However, the production of Δ^{12}-PGJ_2 was dependent on the presence of HSA, which also resulted in reduced production of 15-deoxy-$\Delta^{12,14}$-PGJ_2 (see Figure 1-2) [5].

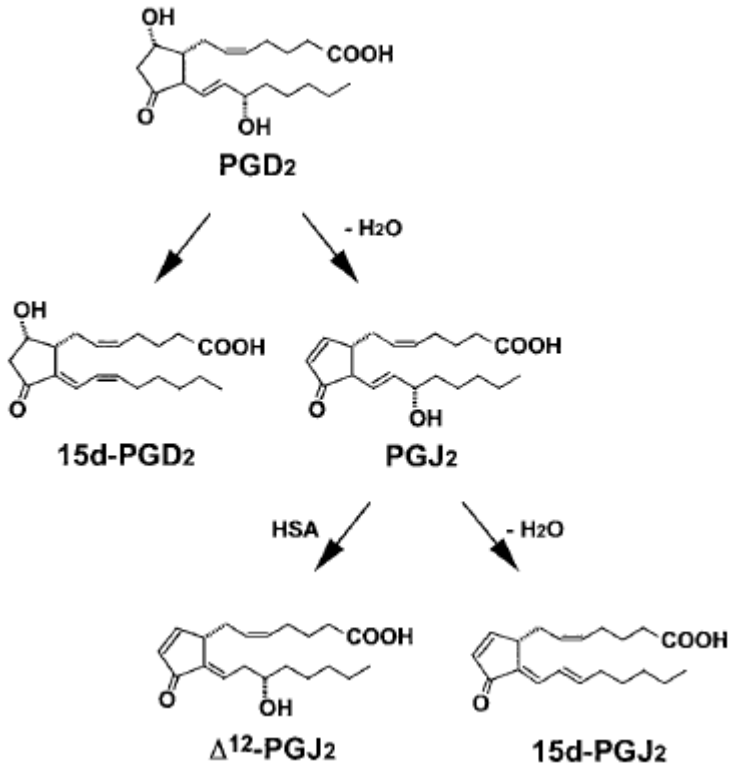


Figure 1-2. Proposed Metabolism of PGD_2 Metabolism
(Image taken from Shibata et al)

Biological activity of Eicosanoids and Prostaglandins

The various dehydration products of EPA are under scrutiny, as the metabolites of this and other omega-3 fatty acids generally have anti-inflammatory effects. This is in contrast to the metabolites of AA, an omega-6 PUFA, which are generally pro-inflammatory compounds. Due to their opposing effects, it is believed that the metabolic products of the two types of fatty PUFAs play roles in coordinating the onset and resolution of the inflammatory process, as well as the transition between acute and chronic inflammation [3].

Prostaglandins are known to regulate homeostasis and cell growth and differentiation. The production of 15-deoxy-$\Delta^{12,14}$-PGJ_2 is believed to play a role in the mitigation of the

inflammatory response once it has begun. The compound has been shown to be a high-affinity ligand of PPARγ, which may account for the reduced gene expression and production of iNOS seen in cells treated with it [5]. 15-deoxy-$\Delta^{12,14}$-PGJ_2 has also been shown to inhibit the activation of NF-κB, which can result in reduced expression of COX-2 and iNOS [1].The J-series prostaglandins also have a cyclopentenone ring system with an electrophilic carbon able to undergo a Michael addition reaction with nucleophiles in cellular proteins. This property may account for actions of these compounds which can not be attributed to the action of a specific receptor [5].

Fish Oils and Cancer

Epidemiological studies have noted lower incidence of cancers in regions of high per capita intake of fish, but results of analytical studies have not been conclusive about the relationship between intake of omega-3 fatty acids and cancer risk. Animal experiments and *in vitro* studies have shown that PUFAs such as EPA can suppress the development of mammary tumors, colon cancer, and lung cancer. Clinical studies have also shown inhibition of hyperproliferation of cells in the intestines of patients at high risk for colon cancer [3].

On the other hand, a positive association has been demonstrated between products of AA and carcinogenesis. PGE_2 promotes the survival and growth of tumors by inhibiting apoptosis, stimulating cell proliferation, and promoting angiogenesis to supply the tumor with blood. Some leukotrienes and hydroxy fatty acids produced from AA have been shown to improve tumor cell adhesion to endothelial cells, which enhances metastasis. Moreover, leukotriene B_4 increases the production of reactive oxygen species, which can result in damage to deoxyribonucleic acid (DNA). All these factors contribute to the development and progression of cancer. With this

being said, a few metabolic products of AA, such as 15d-$\Delta^{12,14}$-PGJ_2 and PGI_2 have actually been shown to have the opposite effect, inducing apoptosis and preventing proliferation of cells [3].

It is thought that omega-3 fatty acids may demonstrate anti-cancer properties because they reduce the production of omega-6 fatty acid metabolic products, which have been linked to inflammation and carcinogenesis. Omega-3 PUFAs compete with omega-6 PUFAs for elongase and desaturase enzymes which increases the level of AA via conversion of linoleic acid (LA), but will act preferentially on omega-3s instead if they are present. When supplied to cells, EPA replaces AA in the phospholipid membrane, thereby reducing the availability of AA. Omega-3s also downregulate COX-2 and compete for available cyclooxygenases. All told, these actions reduce the metabolism of omega-6 fatty acids to their eicosanoid products. When COX-2 is downregulated, its normal effects on the suppression of apoptosis are negated as well [3].

Omega-3 fatty acids and their metabolic products also have powerful effects on gene expression. Peroxisome proliferator-activated receptors (PPARs) were first noted as being involved in lipid metabolism, but are now recognized as playing a role in cell differentiation and proliferation, and inflammation. These transcription factors are activated upon the binding of a ligand, and transduce received signals into coordinated cell responses via their effects on gene expression. For PPARγ, which is expressed in epithelial tissues which are important in cancer, these ligands include AA, EPA and 15d-$\Delta^{12,14}$-PGJ_2. These PPARγ agonists have been shown to have anti-proliferative effects on cancer cells. PPARα is activated by AA and EPA as well, and both PPARγ and PPARα have anti-inflammatory properties and stem the progression of carcinogenesis. On the other hand PPARδ is activated by EPA, AA, and PGD_3, and it is thought to induce cell proliferation and promote certain types of cancer [3].

Nuclear transcription factor κB (NF-κB) also plays an important role in carcinogenesis by impacting cytokine gene expression, cellular adhesion, cell cycle control, and apoptosis. NF-κB

activation has been found to decrease significantly with omega-3 fatty acid treatment of murine macrophages [3].

Nitric oxide (NO) is produced by the enzyme inducible nitric oxide synthase (iNOS), which is expressed in quantity under inflammatory conditions. NO and its derivatives, including reactive nitrogen species, can cause DNA damage and induce mutations which can eventually result in cancer. Omega-3 fatty acids, including EPA, have been shown to suppress production of NO in a macrophage cell line [3].

The overall ability of omega-3 fatty acids to mitigate inflammation also results in reduced production of reactive oxygen species and free radicals, which can cause DNA damage and carcinogenesis. However, evidence suggests that the ratio of omega-3s to omega-6s (ideally a 1:1 or 1:2 ratio) may be the most important factor in cancer prevention, more so, in fact, than the absolute levels of omega-3s [3].

There are some concerns associated with achieving the conditions required for these preventative effects. In most of the Western world, the dietary ratio of omega-3s to omega-6s is closer to 1:10 to 1:20, so major adjustments may be required to achieve the efficacious ratio. With heavy consumption of fatty fish, however, there are worries about carcinogenic compounds such as pesticides and heavy metals which can accumulate in the fish [3]. Therefore, a balance must be found to simultaneously promote health and mitigate carcinogenesis.

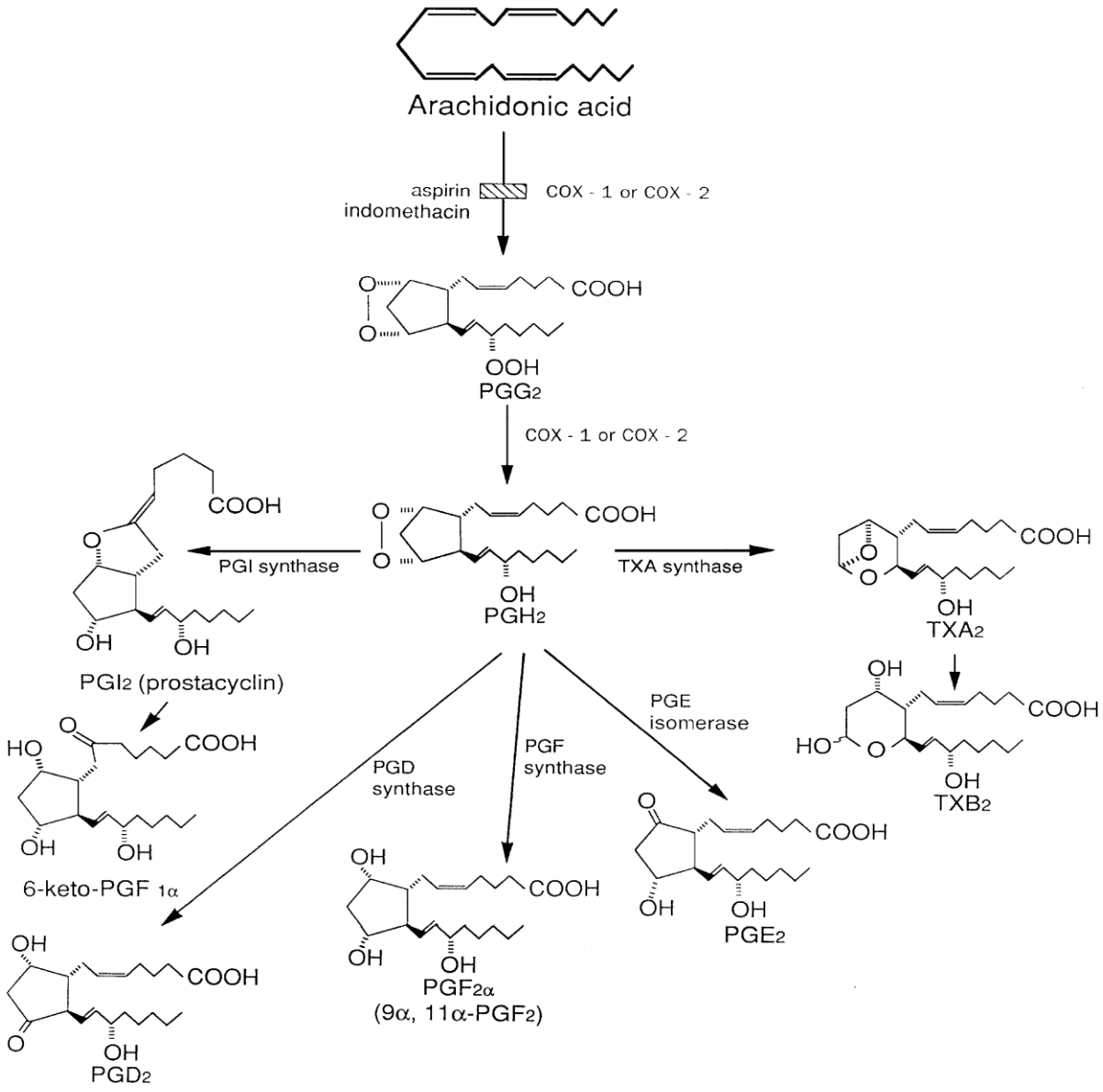


Figure 1-3. The Arachidonic Acid Cascade
(Image taken from Vane et al)

Statement of the Research Question

Since previous research had indicated that, while most 2-series prostaglandins are pro-inflammatory, some, particularly 15d-$\Delta^{12,14}$-PGJ_2, actually have anti-inflammatory properties. Structurally, 15d- $\Delta^{12,14}$-PGJ_3 closely resembles its 2-series analog, and based on the finding that 3-series prostaglandins are generally anti-inflammatory, it stands to reason that 15d- $\Delta^{12,14}$-PGJ_3 might be a particularly potent anti-inflammatory compound. The specific hypothesis being tested is this: **15d- $\Delta^{12,14}$-PGJ_3 will be produced from PGD_3 *in vitro* and in EPA-supplemented cells as a result of LPS stimulation. When this purified compound is used to treat cells, the expression of pro-inflammatory genes such as COX-2 and iNOS will be down-regulated.**

Research Goals

1. To synthesize a variety of prostaglandins from PGD_3 *in vitro* and from EPA in cell culture.
2. To isolate and collect individual prostaglandin metabolites using high-pressure liquid chromatography (HPLC), and then characterize and identify these metabolites via UV-mass spectrometry.
3. To use the isolated compounds to treat RAW 264.7 cells and assess resulting changes in the expression of inflammatory genes by NO assay and Western Blot analysis.

Chapter 2

Experimental Design

Chemicals

The following chemicals were obtained from Sigma Aldrich Chemical Company in St. Louis, MO: bacterial endotoxin lipopolysaccharide (LPS), ethyl acetate, sodium dodecyl sulfate, Trizma© tris base, sodium selenite, Griess reagent, ammonium persulfate, and polyoxyethylene sorbitan monolaurate (Tween-20). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Fitzgerald Industries, New Acton, MA. Cayman Chemicals in Ann Arbor, MI was the source for the following chemicals: PGD_3, PGJ_2, Δ^{12}-PGJ_2, EPA, 15d- $\Delta^{12,14}$-PGJ_2, COX-2 antibody, and iNOS antibody. Products purchased from Thermo Scientific in Waltham, MA included the Supersignal West Pico Chemiluminescence Kit, Restore Western blot stripping buffer, m-PER mammalian cell lysis reagent, and fetal bovine serum (FBS). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin (P/S), and phosphate buffered saline (PBS) came from Invitrogen in Carlsbad, CA, and trypsin and L-glutamine came from Mediatech, Inc. in Manassas, VA. Hydrochloric acid (HCl) and sodium chloride (NaCl) were purchased from Mallinckrodt Baker, Inc. in Phillipsburg, NJ, and glycine and acetonitrile (ACN) came from EMD Chemicals, USA. Hexane was from BDH Chemcials, Ltd, UK, and ether was purchased from Burdick and Jackson, USA.

PGD_3 Metabolism Reaction and Metabolite Bioassays

PGD_3 was added to a 0.1M, pH 7.4 phosphate buffer containing 0.9% NaCl and incubated for 24, 36, 48, or 144 hours in a 37ºC incubator on a 200 rpm shaker (procedure

modified from [2] and [5]). The mixture was acidified to ~pH 3 with HCl and extracted three times with a 1:1 mixture of hexane and ether. The organic phase extract was pooled, the solvent phase evaporated under a stream of nitrogen gas, and the residue reconstituted in a 700:300:1 ACN:H_2O:acetic acid (volume/volume) mobile phase for isolation of the products via high-pressure liquid chromatography (HPLC).

HPLC Purification

A Beckman System Gold HPLC system was operated at 29ºC with a Dynamax semi-preparative C-18 column (10.0 x 250 mm volume) and a 700:300:1 ACN:H_2O:acetic acid (volume/volume) mobile phase under isocratic elution conditions. The pumps were adjusted to a flow rate of 2 mL/minute and the system was allowed to equilibrate for around 45 minutes so that the signal from the UV detector, set to monitor a wavelength of 280 nm, could stabilize. Next, 100 µL of the product mixture resulting from each time point of the *in vitro* metabolism reaction was injected into the system. The signal from the UV detector was monitored at 280 nm and each individual peak was collected, dried down with nitrogen gas, and reconstituted in ethyl acetate for storage at -20ºC.

Mass and UV Spectrometry

Samples were dried down under nitrogen gas, and reconstituted in LC-MS grade methanol. Samples were injected into an Applied Biosystems ABI2000 triple quadruple mass spectrometer. The system was run at 200ºC with the following settings: GS1=25, GS2=25, CUR=10, IS=-4500, DP=-60. The scan was run in negative mode at high resolution. A

fragmentation profile was created for each of the collected peaks, and the pattern was used to determine the likely identity of the compounds.

Samples were also tested in a Beckman DU 7500 UV-Vis Spectrophotometer against a methanol background. Absorbance was measured from 200 to 350 nm to produce a UV profile which could be analyzed to confirm the likely identity of the compounds. The UV spectrophotometric analysis was also used to quantify the concentration of each compound, using the extinction coefficients for the corresponding 2-series isomer, for which there was data available in the literature.

Cell Culture and Treatments for Bioassay

RAW 264.7 cells, a murine macrophage-like cell line, were cultured at 37ºC in a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle's medium (DMEM), supplemented with 5% defined fetal bovine serum (FBS), 1% penicillin/streptomycin solution, and sodium selenite at a 250 nM concentration. These cells were seeded onto 12-well plates at a concentration of 1×10^6 cells per well and allowed to adhere to the plate overnight. The cells were treated for one hour with varying concentrations of EPA, PGD_3, PGJ_2. Δ^{12}-PGJ_2, and 15d-$\Delta^{12,14}$-PGJ_2 from Cayman Chemicals and Δ^{12}-PGJ_3, Δ^{13}-PGJ_3, 15d-$\Delta^{12,14}$-PGJ_3 synthesized in the previously described metabolism reaction, all prepared in dimethyl sulfoxide (DMSO). The cells were then treated with lipopolysaccharide (LPS) at a final concentration of 1 μg/mL.

After LPS stimulation, the media was harvested for use in NO assays, and the cells were washed with cold, sterile phosphate buffered saline (PBS), containing 2.67 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, and 8.06 mM $NaHPO_4$-$7H_2O$. 50 μL of M-PER, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 10 μM leupeptin, and 1 mM phenylmethylsulfonic acid

(PMSF) was added to each well of the plates. The plates were rocked for 45 minutes at 4ºC, then frozen at -80ºC.

Nitric Oxide Assay

100 µL of cell supernatant from each of the wells in the bioassay experiment was placed in a well of a 96-well plate. A standard curve was also prepared with 100, 80, 60, 40, 20, and 10 µM concentrations of NO_2 from sodium nitrate via dilution of a 100 µM stock solution. 100 µL of 1X modified Griess Reagent was added to each well and thoroughly pipet mixed. The plate was incubated for 15 minutes at room temperature and then the absorbance was read at 560 nm. The triplicate values were averaged and a two-tail t-test was used to see if the sample values varied in a statistically significant way from the values for the LPS control.

Cell Lysate Preparation and Protein Estimation

The plates were thawed on ice, and the lysed cell solution was pipetted into Eppendorf tubes. The tubes were spun down in a 4ºC centrifuge for 10 minutes at 10,000 rpm, and the supernatant was transferred into new Eppendorf tubes. 5 µL of each sample of cell supernatant was added to one well of a 96-well plate and diluted with 20 µL of Milli-Q water. 25 µL of each bovine serum albumin (BSA) protein standard (2000, 1500, 1000, 750, 500, 250, 125, and 25 µg/µL concentrations) were then added to other wells. A 50:1 (volume/volume) mixture of BCA reagent A and reagent B was prepared, and 200 µL of this mixture was added to each sample and standard and thoroughly pipet mixed. The plate was incubated at 37ºC for 30 minutes, and then read at an absorbance of 560 nm. The absorbance values were then used to calculate the concentration of protein present in each sample. Samples were frozen at -20ºC for later use.

Electrophoresis and Immunoblotting

Based on the results of the BCA protein assay, calculations were carried out to allow for the preparation of samples containing 5 μg of total protein. Three sets of samples were prepared in 1X SDS-PAGE loading dye and electrophoresed onto a 12% polyacrylamide gel using 5% SDS-PAGE buffer, containing 15.1 g Tris base, 72 g glycine, and 5 g sodium dodecyl sulfate (SDS) per 1000 mL Milli-Q water. The protein samples were then transblotted to nitrocellulose membranes for 90 minutes using TBB buffer, containing 12.1 g Tris base, 57.64 g glycine, and 800 mL methanol made up to 4 L with Milli-Q water.

The membranes were blocked for 1 hour in a 5% non-fat milk solution made in pH 7.5 Tris buffered saline, containing 12.1 g Tris base and 8.77 g NaCl per 1 liter of water, with 0.05% Tween-20 added (TBST). After the initial blocking, the membranes were treated with primary antibodies under the specified conditions (see Table 2-1). Subsequent washing of the membranes was carried out in 0.05% TBST solution for three 10 minute intervals. The membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (see Table 2-1), and given three 10 minute washes in 0.05% TBST. The bands were visualized using an enhanced chemiluminescence assay kit according to the manufacturer's directions, and imaged with radiographic film. The images were analyzed using the Image J computer program (obtained from NIH), so that the expression of the various proteins could be roughly quantitated. The densitometric values of protein bands for the three sets of samples were averaged and compared. A two-tail t-test was carried out to see whether the values for the treated samples varied in a statistically significant way from the values for the LPS control.

In between treatments with different antibodies, membranes were stripped using enough Restore Membrane Stripping Buffer to cover the membrane. The membranes were incubated at 37ºC for one hour while being rocked, then washed three times in a 0.05% TBST solution at

room temperature for ten minutes each. If needed, the membranes were stored in a 10% ethanol, 90% water solution at 4ºC.

Table 2-1. Primary and secondary antibody concentrations and incubation conditions for Western blots

Antibody	Type	1º Ab Concentration	1º Ab Treatment Duration	2º Ab Type and Concentration	2º Ab Treatment Duration
COX-2	Rabbit Polyclonal	1:5000	O/N	Anti-Rabbit 1:2500	1 hour
iNOS	Rabbit Polyclonal	1:1000	O/N	Anti-Rabbit 1:2500	1 hour
GAPDH	Mouse Monoclonal	1:300,000,000	1 hour	Anti-Mouse 1:5000	1 hour

EPA Metabolism in Cell Culture

In order to conjugate EPA to BSA to ensure uptake by cells, the required amount of EPA for a final concentration of 50 μM was dried down and reconstituted in 5 μL of absolute ethanol in a brown glass vial. 5 mL of pre-warmed DMEM cell culture media was added to the EPA and ethanol (resulting in a final concentration of 0.1% ethanol) along with 0.2 mg of fatty-acid free BSA (for a final content of 4% BSA (weight/volume)). The resulting solution was kept at 37ºC for a minimum of 1 hour before being added to cells in a 10 cm^2 Petri dish which had been plated out at 0.5 million cells per well (procedure based on [4]).

The cells were allowed to grow in the EPA-supplemented media for 72 hours prior to treatment with 50 ng/mL of LPS. After 30 minutes, the media and LPS were removed, and the cells were gently washed in fresh media to remove any traces of LPS. DMEM was heat inactivated at 60ºC for 45 minutes to prevent reduction of the prostaglandin products, and 5 mL of this media was added back to each of the plates. After 48-72 hours, the cell media was collected, acidified to ~pH 3 with HCl and extracted with a 50:50 hexane:ether mixture. The

organic phase was pipetted off, dried down under a stream of argon gas, and reconstituted in ethyl acetate for storage at -20ºC.

HPLC Isolation of Products

Samples were dried down and reconstituted in a 700:300:1 ACN:H_20:trifluoroacetic acid mobile phase. The rest of the HPLC procedure was carried out as described above.

Mass Spectrometry

Isolated compounds were dried down and reconstituted in LC/MS grade methanol, then injected and run on the mass spectrometer as described above.

Chapter 3

Results

Prostaglandin D_3 metabolites are produced by a non-enzymatic reaction *in vitro*

Published experiments on the *in vitro* metabolism of PGD_2 indicate that several prostaglandin J metabolites are produced from it in a non-enzymatic reaction under aqueous conditions. PGD_3 was incubated in a phosphate buffer for 24, 36, 48, or 144 hours, and the reaction mixtures were acidified and extracted. HPLC analysis of the organic extracts showed the formation of four distinct metabolites (see Figure 3-1). The first peak (designated peak 0) was small, and decreased in size over time. The second peak (peak 1) was the largest peak, and increased in size over time. The third peak (peak 2) was almost as large as the second peak at 24 hours, and then disappeared by 144 hours. The fourth peak (peak 3) was of moderate size, and decreased slightly over time.

UV spectra indicate likely identities of each isolated metabolite

When the UV/Vis absorbance of a compound containing double bonds is analyzed, the resulting spectrum will have features characteristic of its chemical structure. Each isolated compound was dried down and reconstituted in methanol and tested on a UV spectrophotometer. Peak 0 was determined to be unreacted PGD_3 based on size and elution time. The spectra which resulted from the other three compounds showed that the compound in peak 1 had a λ_{max} of 242 nm, the compound in peak 2 had a λ_{max} of 293 nm, and the compound in peak 3 had λ_{max}s of both 242 nm and 304 nm (see Figure 3-2). The λ_{max} of peak 1 suggested that its likely identity was

Δ^{12}-PGJ_3, while the profile of peak 2 suggested that this compound was Δ^{13}-PGJ_3, and peak 3 was identified as 15d- $\Delta^{12,14}$-PGJ_3.

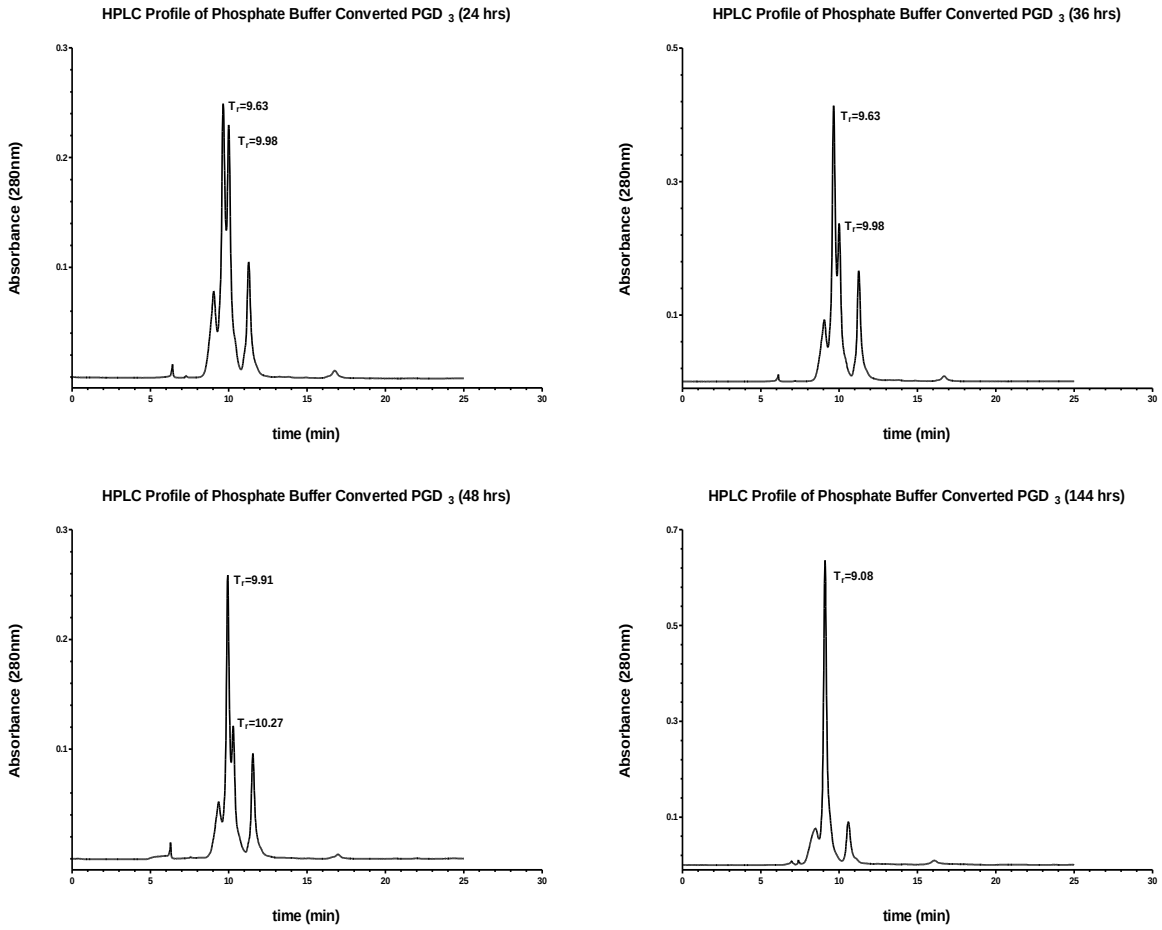


Figure 3-1. HPLC Profiles of Metabolites from PGD_3Conversion Reactions

Profiles from 24, 38, 48, and 144 hour time points vary, with peaks 0, 2, and 3 decreasing over time, and the peak 1 increasing.

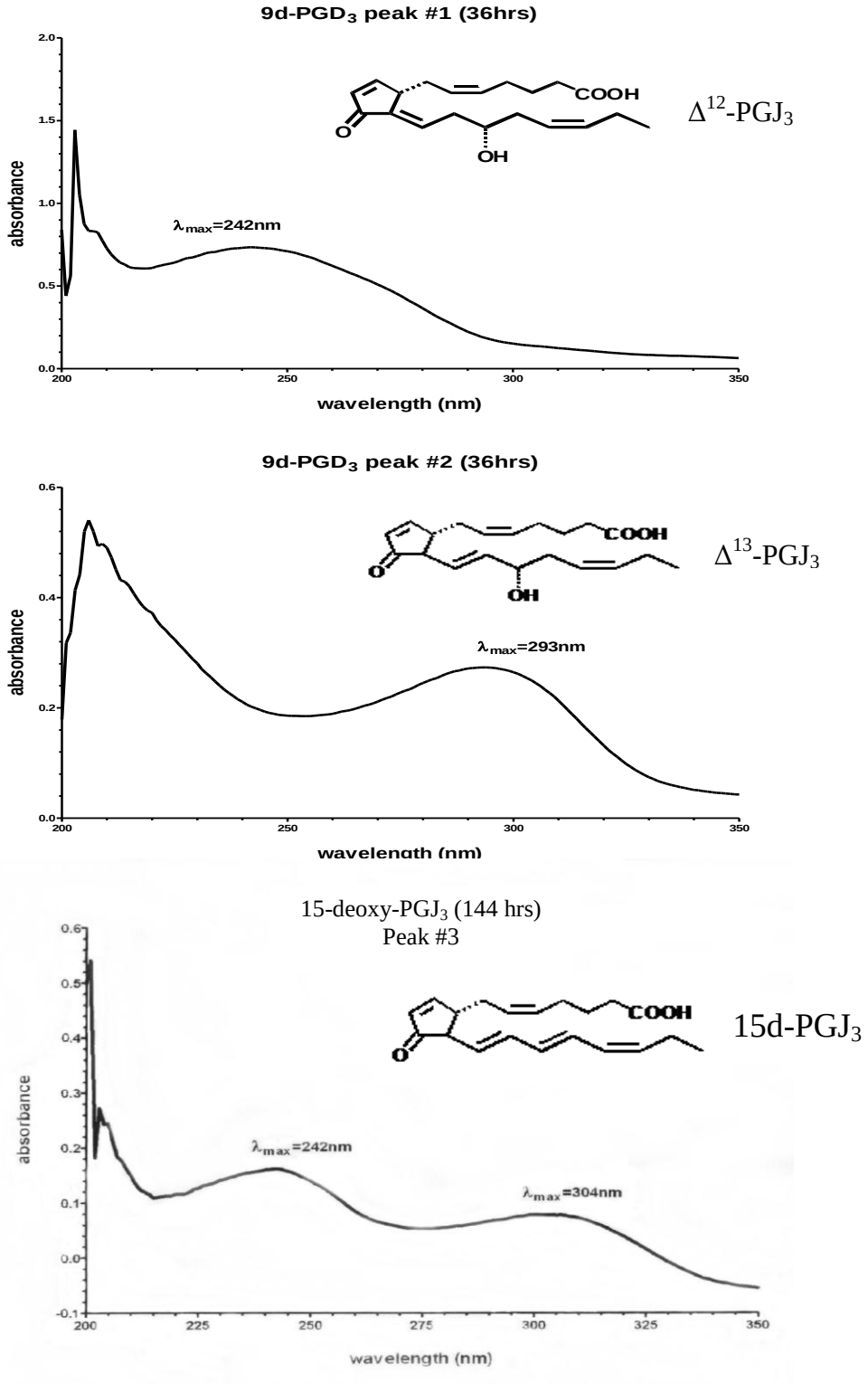


Figure 3-2. UV Spectra of each product peak from PGD_3 Conversion Reactions

Mass spectrometric profiles confirm identities of the isolated metabolites

When a compound is analyzed on a mass spectrometer, the masses of the resulting fragments, produced by ionization, indicate the likely structure of the compound. Each isolated compound (from the last three peaks) was dried down and reconstituted in methanol, then injected into the mass spectrometer. Based on these profiles, which were consistent with the suspected identities of the metabolites, it was possible to confirm peak 1 as Δ^{12}-PGJ_3, peak 2 as Δ^{13}-PGJ_3, and peak 3 as 15d- $\Delta^{12,14}$-PGJ_3. (Image not shown)

Nitric oxide assay demonstrates metabolites' anti-inflammatory capabilities

When the growth medium of cells is assayed for NO, the concentration serves as an indicator of expression of iNOS. RAW 264.7 macrophages were treated with a variety of EPA and AA metabolites and the growth medium from each well was analyzed for NO. EPA treatment alone was found to decrease NO levels by a statistically significant amount (at the $p<0.01$ level) only at 100 μM concentration, but BCA estimation showed low levels of protein consistent with cell death, so the low NO value was disregarded. 15d-$\Delta^{12,14}$-PGJ_2 showed statistically significant reductions (at the $p<0.01$ level) in NO concentration at 0.5, 1.0 and 5.0 μM concentrations. 15d-$\Delta^{12,14}$-PGJ_3 showed statistically significant reductions in NO levels at the $p<0.01$ level at the 24 and 48 hour time points, and at the $p<0.10$ level for the 36-hour time point. PGJ_2 showed statistically significant reductions in NO levels at the $p<0.01$ level at both 1.0 and 5.0 μM concentrations. "Peak 2," which was later determined to be PGJ_3, did not show a corresponding reduction in NO values. Δ^{12}-PGJ_2 did not show a statistically significant decrease in NO concentrations at the 1.0 μM concentration, but it did show a reduction at the 5.0 μM concentration which was statistically significant at the $p<0.01$ level. Δ^{12}-PGJ_3 showed

statistically significant reductions (at the $p<0.10$, $p<0.05$, and $p<0.01$ levels) in NO levels at the 1.0 μM level for the 24 hour, 144 hour, and 36 and 48 hour time points, respectively. At the 5.0 μM concentration, Δ^{12}-PGJ_3 showed statistically significant reductions in $p<0.01$ level for all time points (see Figure 3-3).

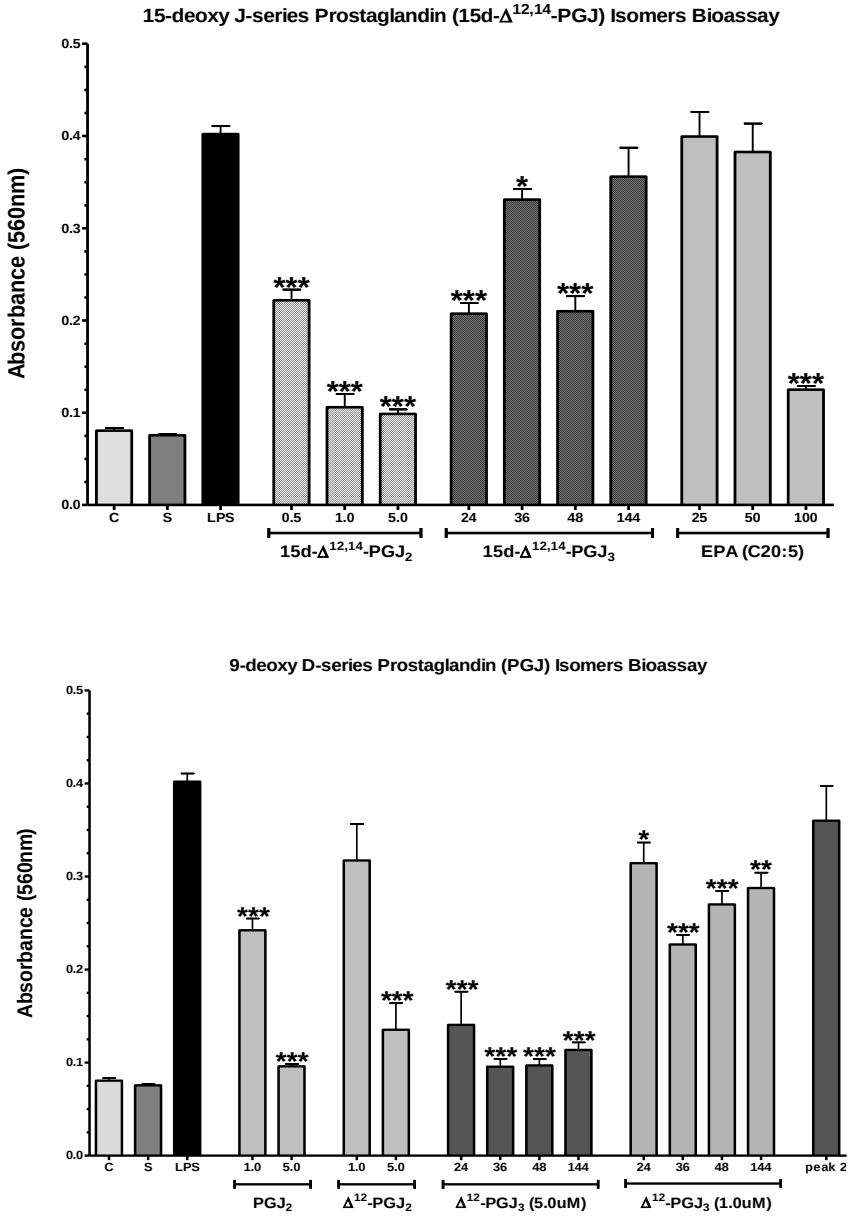


Figure 3-3. NO Assay Results

Testing cell culture media from RAW 264.7 macrophages treated with various prostaglandins and EPA.

Western blotting shows reductions in iNOS and COX-2 expression

Western blotting was employed to evaluate levels of two enzymes shown to be upregulated in the presence of inflammatory conditions. Selected samples were electrophoresed on a gel, then transferred to a membrane and tested with antibodies to iNOS and COX-2. The images (see Figure 3-4) were analyzed using Image J software and densitometric values were averaged and analyzed (see Figure 3-5). Overall, those samples which showed statistically significantly reduced levels of iNOS (at the $p<0.0.5$ level) when standardized with GAPDH were as follows: 1.0 μM 15d-$\Delta^{12,14}$-PGJ_2, 1.0 μM Δ^{12}-PGJ_3 and 5.0 μM Δ^{12}-PGJ_3 from both 24 and 144 hour time points, and 5.0 μM 15d-$\Delta^{12,14}$-PGJ_3 from the 24 hour time point. The samples shown to statistically significantly reduce levels of COX-2 (at the $p<0.05$ level), were as follows: 1.0 μM Δ^{12}-PGJ_3 and 5.0 μM Δ^{12}-PGJ_3, both from the 144 hour time point.

Prostaglandin D_3 metabolites are produced by EPA-treated RAW 264.7 cells

When RAW 264.7 cells are treated with EPA, it becomes incorporated into the cellular membrane and is released and metabolized upon administration of pro-inflammatory stimuli. After treatments, the cell growth media was acidified and extracted. HPLC analysis of the organic extracts showed the formation of several different metabolites (see Figure 3-6), two of which were confirmed to be Δ^{12}-PGJ_3 and 15d-$\Delta^{12,14}$-PGJ_3 via mass spectrometric analysis of the isolated peaks (see Figure 3-7).

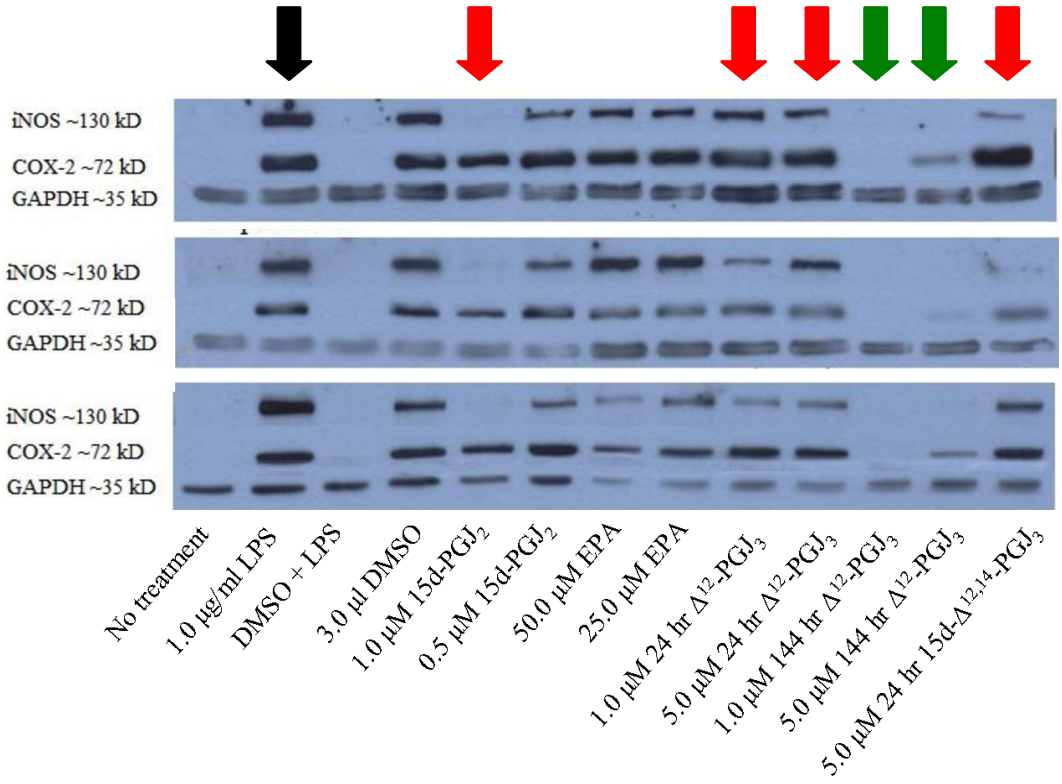
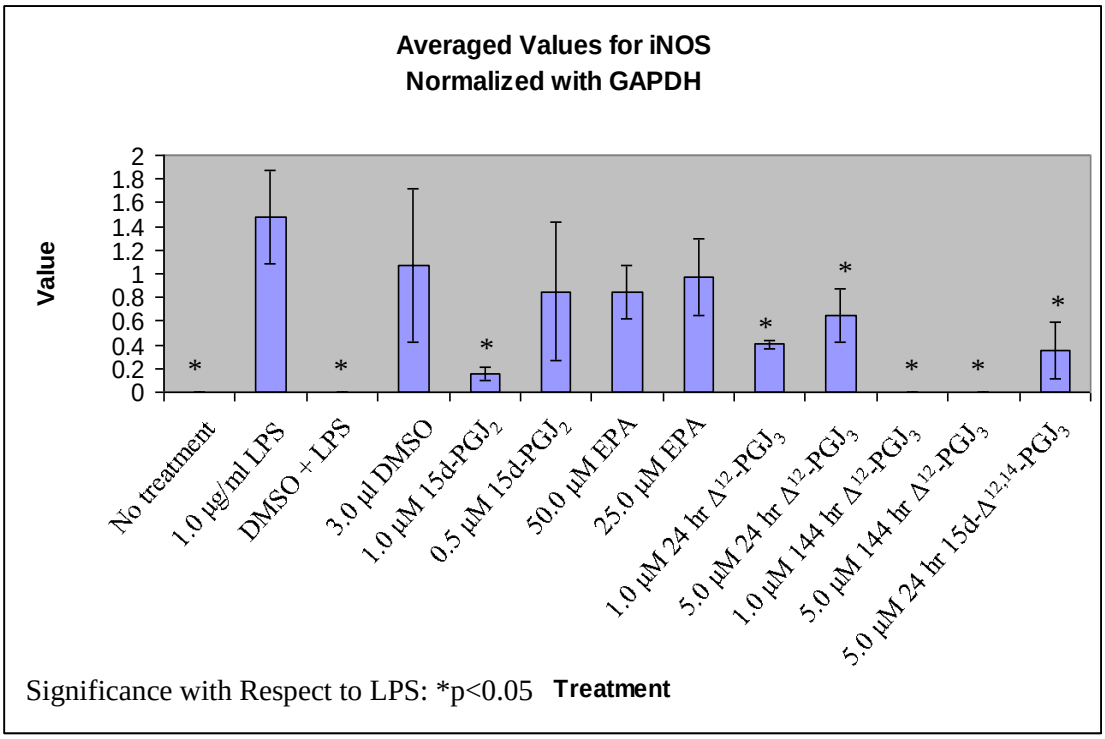


Figure 3-4. Western Blot Images

Run with selected samples which showed low levels of NO and reasonably high levels of protein as determined by BCA protein assay. Samples which showed reduced levels of iNOS only are indicated by red arrows, and samples which showed reduced levels of iNOS and COX-2 are marked by green arrows. The basis for comparison is the LPS control sample, indicated with a black arrow.



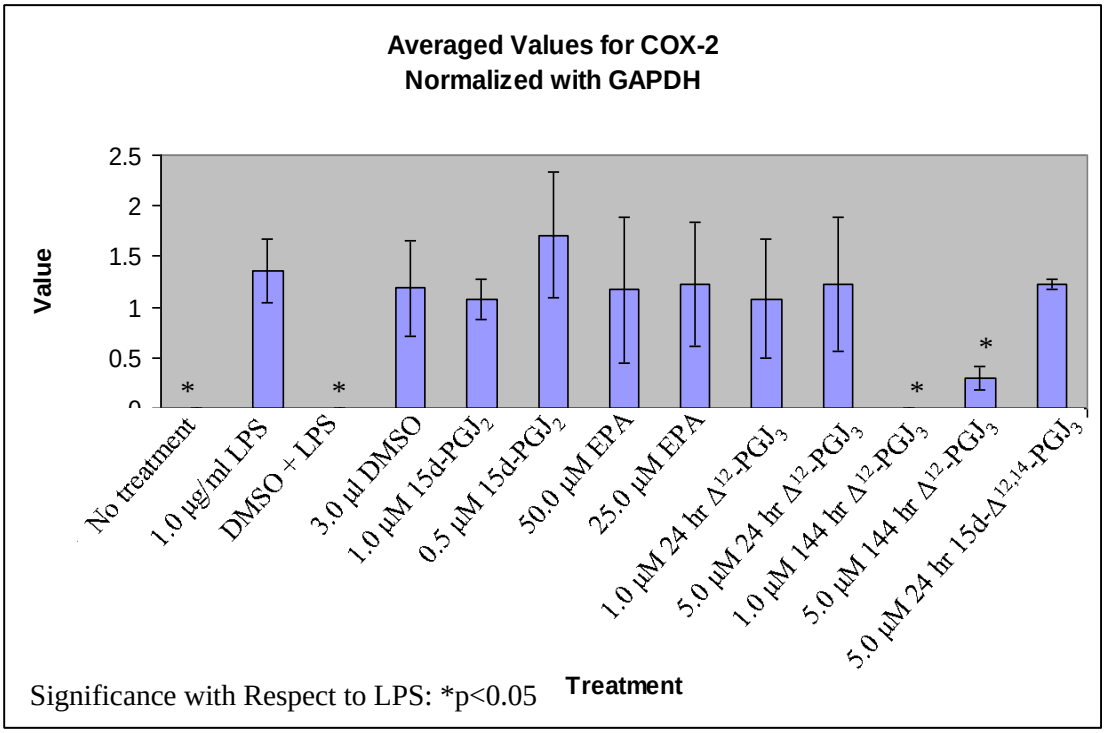


Figure 3-5. Western Blot Graphs

Averaged densitometric data from three sets of samples.

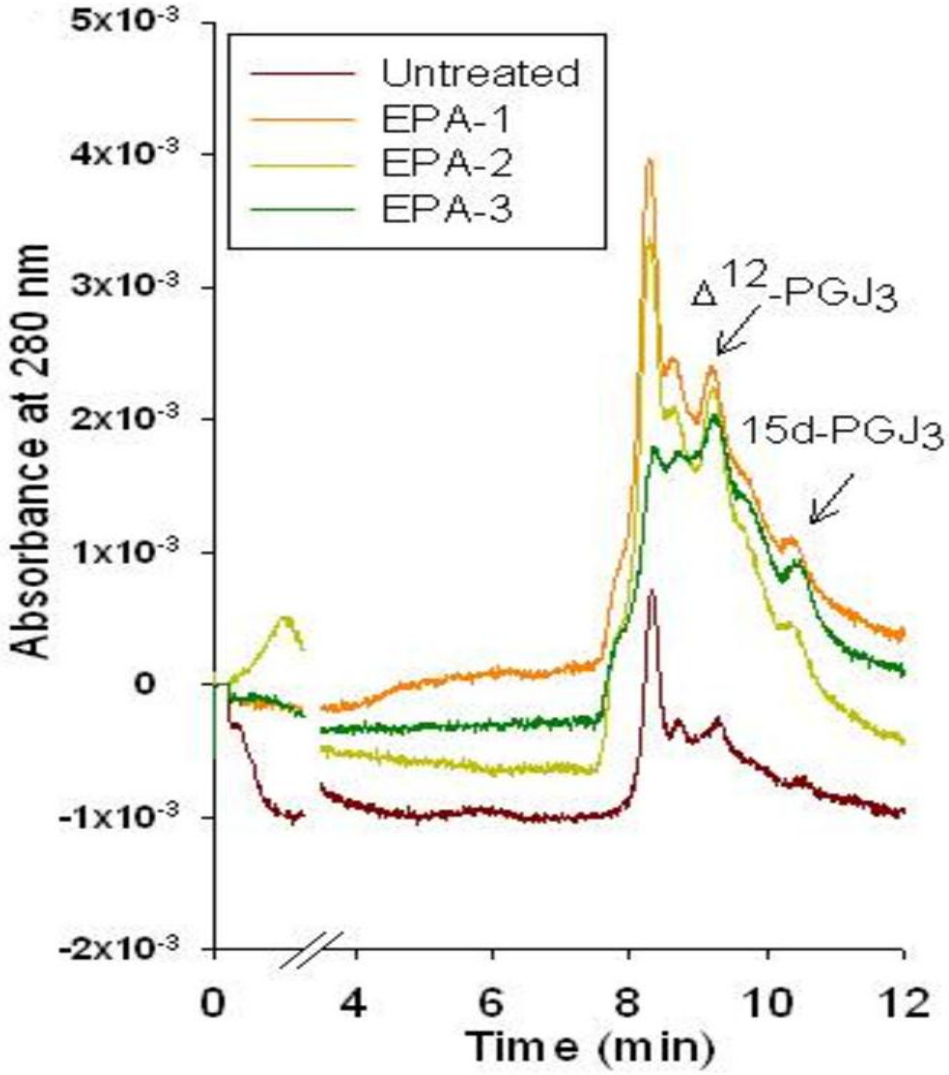


Figure 3-6. HPLC Profiles of extracts from cell culture media

The organic extracts from the media of cells cultured in EPA show the presence of both Δ^{12}-PGJ_3 and 15d-$\Delta^{12,14}$-PGJ_3.

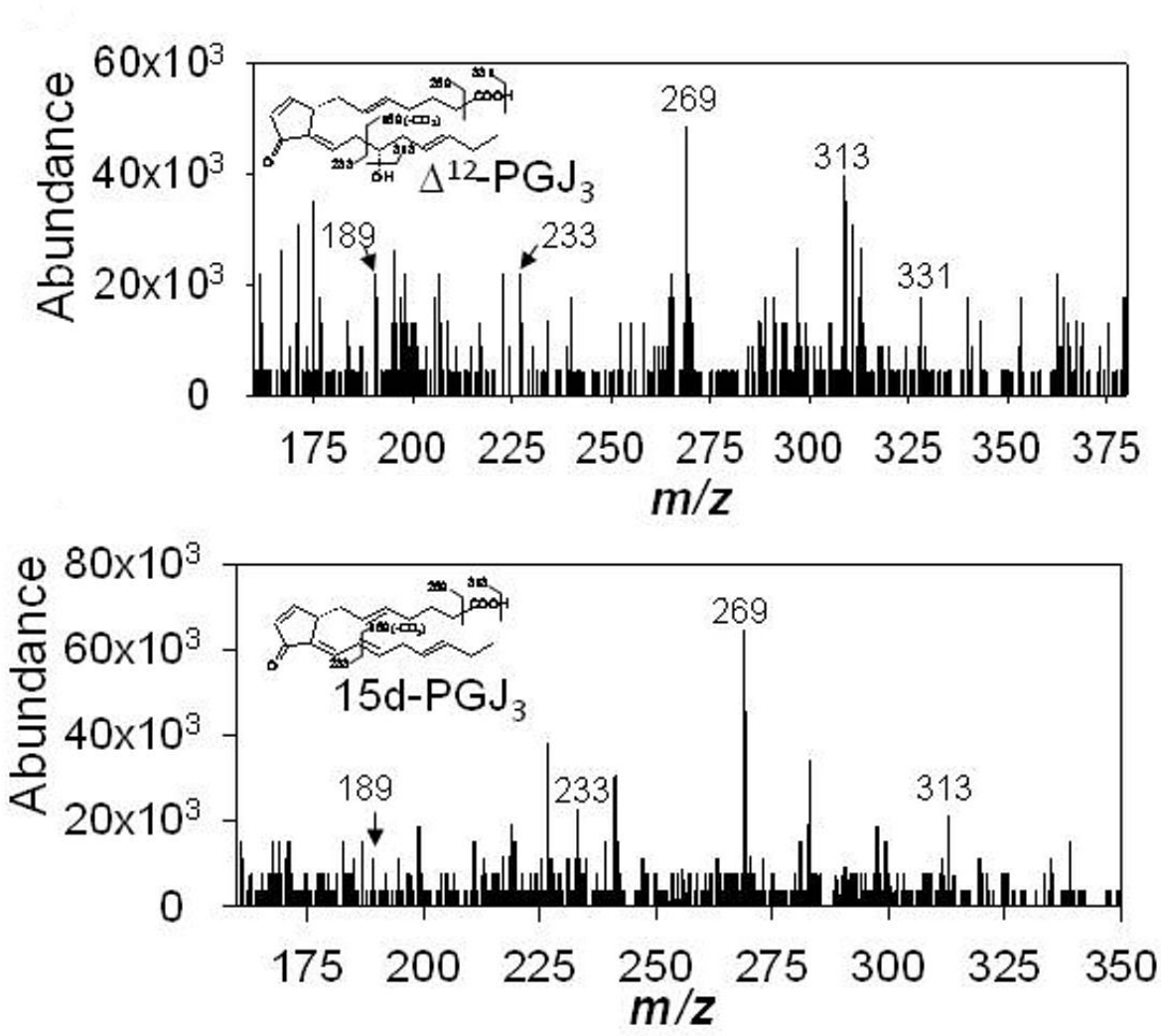


Figure 3-7. Mass Spectrometric Fractionation Patterns for Cell-Produced Compounds

The isolated compounds from the cells treated with EPA produced fractionation patterns consistent with Δ^{12}-PGJ_3 and 15d-$\Delta^{12,14}$-PGJ_3, as expected.

Chapter 4

Discussion

The process by which PGD_2 is metabolized into its end products in non-enzymatic reactions [2, 5] and how AA and EPA are metabolized in cell cultures have been described in the literature [7]. Studies also detail the anti-inflammatory properties of the cyclopentenone compound 15d-$\Delta^{12,14}$-PGJ_2, but research on the production and biological activity of the 3-series cyclopentenone prostaglandin J analogs from EPA has yet to be published. This project is focused on filling in this gap in the field of lipid research by investigating the production of metabolites from PGD_3 and EPA *in vitro* and in cell cultures, and then exploring the anti-inflammatory capabilities of these compounds.

As described in [5], PGD_2 breaks down into PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 in aqueous solution, and albumin is not required to catalyze the reaction. However, Δ^{12}-PGJ_2 was only produced in quantity in the presence of albumin. Another experiment, described in [2], utilized human serum albumin to catalyze the dehydration of PGD_2. To determine if PGD_3 was metabolized in the same way as PGD_2, a procedure was modified from [2] and [5] in which PGD_3 was added to a phosphate buffer solution without albumin. The reactions were acidified and extracted after various periods of time to produce a time course of the PGD_3 metabolism reaction. The organic extracts were separated into individual metabolites by HPLC, and peak size was used as an indicator of relative quantities of each metabolite. Metabolites were identified based on UV spectra and mass spectrometric profiles.

It was noted that peak 0 in the HPLC profile (see Figure 3-1), identified as unreacted PGD_3, was a small peak, and also showed a clear decline in quantity over the time course. The small size of this peak indicates that the metabolism reaction proceeds almost to completion by

the end of the 144 hour time course. Peak 1, identified as Δ^{12}-PGJ_3, increased in relative quantity over the course of the reaction, suggesting that this compound may be the most stable and was therefore best able to accumulate during the metabolism reaction. It is interesting to note that, even in the absence of albumin, Δ^{12}-PGJ_3 was produced in quantity, while, in published experiments [5], albumin was needed to produce large quantities of Δ^{12}-PGJ_2. Peak 2, identified as Δ^{13}-PGJ_3, decreased dramatically over the time course, and had completely disappeared by the 144 hour time point. Similarly, the final peak, identified as 15d-$\Delta^{12,14}$-PGJ_3, was a medium-sized peak and decreased over the time course. This indicates that these compounds may be produced early in the metabolism reaction (in a limited quantity, in the case of 15d-$\Delta^{12,14}$-PGJ_3), and that they subsequently break down due to instability and/or are converted to other metabolites as the reaction continues. Based on the results of the time course, and what is known about the metabolism of PGD_2 (see Figure 1-2), a theory involving progressive dehydration and rearrangement metabolism reactions is proposed (see Figure 4-1).

Identification of the peaks was based on comparisons of the metabolites' UV profiles (see Figure 3-2) with the known UV profiles of the 2-series analogs of Δ^{12}-PGJ_3, Δ^{13}-PGJ_3, and 15d-$\Delta^{12,14}$-PGJ_3. Since key features of the molecules, including double bonds and groups off the main carbon chain, are similar, the UV profiles were found to be quite similar. For those compounds with double bonds that are not part of a conjugated system in the primary resonance structure (Δ^{12}-PGJ_3 and Δ^{13}-PGJ_3), only one λ_{max} was observed. In comparison, the extended system of conjugated bonds found in 15d-$\Delta^{12,14}$-PGJ_3 resulted in two λ_{max} values. The mass spectrometric profiles (see Figure 3-7) were analyzed based on theory of how the metabolites should fractionate, and served to confirm the identities of the metabolites.

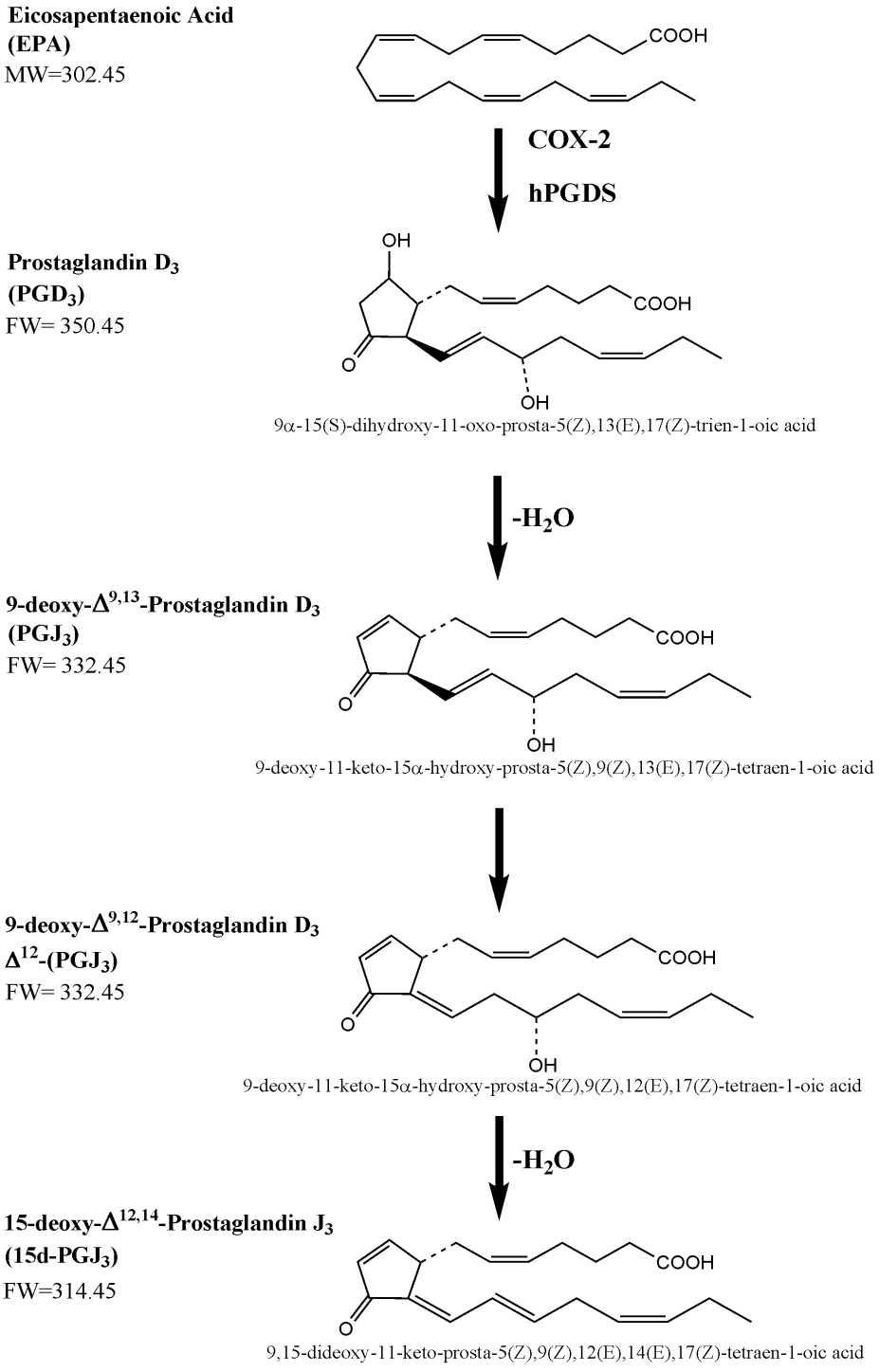


Figure 4-1. Proposed metabolism reaction of EPA and PGD_3

It is well-established that upon stimulation with pro-inflammatory compounds, such as LPS, production of COX-2 and iNOS is increased in RAW 264.7 cells. Compounds which exert an anti-inflammatory effect on the cells will be able to suppress this upregulation, resulting in lower levels of inflammatory proteins. In order to test the anti-inflammatory effects of the PGD_3 metabolites, cells were treated with the compounds before exposure to LPS. The production of NO, and therefore the activity of iNOS, was measured by an NO assay (see Figure 3-3). Treatments which showed promising reductions in NO production, but were not associated with low levels of protein (indicative of cell death and low cell numbers as the real explanations for low NO levels), were selected to be run on a gel and be analyzed via Western blot (see Figure 3-4 and Figure 3-5).

It should be noted that Δ^{12}-PGJ_3 was efficacious in significantly reducing levels of iNOS, but that this compound collected at the 144 hour time point specifically achieved a larger decrease in iNOS levels and also was the only compound to successfully reduce production of the COX-2 enzyme. It is possible that a time-dependent isomerization could have contributed to its enhanced bioactivity; however, this needs to be further confirmed using analytical techniques such as chiral-phase chromatography and perhaps nuclear magnetic resonance studies. It is also interesting to note that there was no dose-dependent effect (where higher concentrations of the compound have a greater anti-inflammatory effect) with Δ^{12}-PGJ_3, since both 1.0 and 5.0 μM concentrations were equally efficacious. This is not the case with 15d-$\Delta^{12,14}$-PGJ_2.

While 15d-$\Delta^{12,14}$-PGJ_2 and 15d-$\Delta^{12,14}$-PGJ_3 are both effective at reducing production of iNOS, they do not seem to have any effect on production of COX-2. It is also unclear why this is the case, since according to theory, production of both iNOS and COX-2 should be decreased by compounds which are anti-inflammatory. It is possible that Δ^{12}-PGJ_3 has a different mechanism of action than that of 15d-$\Delta^{12,14}$-PGJ_2 or 15d-$\Delta^{12,14}$-PGJ_3, and this is something worth investigating in the future.

This research and others have resulted in a suggested metabolism reaction for EPA (see Figure 4-1), but it was important to prove that the same metabolites which were produced in the *in vitro* experiment were also produced in cell culture. While the HPLC profiles for the extracts from the cell media had somewhat indistinct peaks (see Figure 3-6), this is to be expected. After all, the media contained a variety of compounds soluble in the organic phase used to extract the PGD_3 metabolites, which would all appear, to some degree, on the HPLC profile. However, two peaks could be discerned, and were identified to be Δ^{12}-PGJ_3 and 15d-$\Delta^{12,14}$-PGJ_3 by mass spectrometry (see Figure 3-7). The fractionation patterns were very representative of what would be expected for these compounds, based on theory about how they would break down upon ionization.

Overall, the conclusions which can be drawn from the above experiments are as follows:

1. The prostaglandin J metabolites Δ^{12}-PGJ_3, Δ^{13}-PGJ_3, and 15d-$\Delta^{12,14}$-PGJ_3 can be produced from PGD_3 via non-enzymatic reactions and from EPA in cell culture, as would be expected from the published research on their 2-series analogs.
2. These metabolites do have biological effects on RAW 264.7 cells, resulting in lower levels of NO in cell media and lower levels of iNOS and COX-2 proteins in the cell cytosol.
3. While it was originally hypothesized that 15d-$\Delta^{12,14}$-PGJ_3 would be a particularly potent anti-inflammatory agent, it was actually Δ^{12}-PGJ_3 from the end point of the reaction which had a strong suppressive effect on the production of inflammatory mediators.

Chapter 5

Future Directions

Based on the promising results of this experiment, and given the potential of these metabolites to benefit patients suffering from inflammation-related diseases of many kinds, it will be important to continue research in this area. As mentioned above, it will be important to further investigate the effect of time on the structure and biological activity of the metabolites, since the greater anti-inflammatory properties seen in Δ^{12}-PGJ_3 from the 144 hour time point can not currently be explained.

There are also plans to carry out experiments to determine the effects of these compounds on other measures of inflammation, such as levels of tumor necrosis factor-alpha (TNF-α), and on proteins of the NF-κB family, which are known to play an important role in gene expression in response to inflammation. This research will also serve the greater goal of gaining a better understanding of the mechanism of action of these metabolites.

To determine whether the research conducted so far will be generalizable to living systems, it will be important to test production of these metabolites in an EPA-supplemented mouse system, and possibly analyze the serum of human volunteers to test for the presence of these metabolites in a non-supplemented subject.

Research should certainly continue to be carried out on this important topic, both to increase knowledge about prostaglandin metabolites which have not been previously investigated and to provide insight into how these metabolites can be beneficial to the health of animals and humans.

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