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(54) Titre : DETECTION DE L'ACTIVITE COX-2 ET DES METABOLITES D'ANANDAMIDE  
 (54) Title: DETECTION OF COX-2 ACTIVITY AND ANANDAMIDE METABOLITES

(57) **Abrégé/Abstract:**

The present invention relates generally to cyclooxygenase enzymes (COX) and more particularly the COX-2 enzyme. The present invention provides compositions, methods and articles of manufacture (kits) for determining the COX-2 activity in a sample of a subject, such as a patient or a cell culture. The present invention is useful in monitoring inflammation and cancer, or other disease processes, in patients in a clinical setting.



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(54) Title: DETECTION OF COX-2 ACTIVITY AND ANANDAMIDE METABOLITES

(57) Abstract: The present invention relates generally to cyclooxygenase enzymes (COX) and more particularly the COX-2 enzyme. The present invention provides compositions, methods and articles of manufacture (kits) for determining the COX-2 activity in a sample of a subject, such as a patient or a cell culture. The present invention is useful in monitoring inflammation and cancer, or other disease processes, in patients in a clinical setting.

WO 02/12445 A1

## DETECTION OF COX -2 ACTIVITY AND ANANDAMIDE METABOLITES

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DESCRIPTION5 COMPOSITIONS AND METHODS FOR DETECTING AND  
QUANTIFYING COX-2 ACTIVITY AND ANANDAMIDE  
METABOLITES1.0 GOVERNMENT SUPPORT CLAUSE

10 This invention was made with federal grant 5R35CA47479,  
and with partial support from the National Foundation for Cancer  
Research. The United States Government has certain rights in this  
invention.

2.0 TECHNICAL FIELD

15 The present invention relates generally to the cyclooxygenases  
and their roles in human pathology, including cancer and  
inflammation. More particularly, this invention pertains to methods  
and articles of manufacture for detecting or measuring COX-2 activity  
by detecting and measuring COX-2 specific enzymatic products  
20 including prostaglandin ethanolamides and thromboxane  
ethanolamides.

COX is a prostaglandin endoperoxide synthase enzyme  
(cyclooxygenase, COX, EC 1.14.99.1), which catalyzes the  
25 conversion of arachidonic acid to prostaglandin (PG) H<sub>2</sub>. Two  
isoforms of COX are known, COX-1 and COX-2. COX-1 is  
constitutively expressed. COX-2, however, is inducible in a variety

of cells, especially those of the central nervous and immune systems (Masferrer *et al.* 1994, *Proc. Natl. Acad. Sci. USA* 91:3228-3232; Vane *et al.* 1994, *Proc. Natl. Acad. Sci. USA* 91:2046-2050; Kennedy *et al.* 1993, *Biochem. Biophys. Res. Commun.* 197:494-500). Certain  
5 changes in COX-2 activity are associated with a variety of human inflammatory diseases. These diseases include, but are not limited to, acute appendicitis, asthma, myocardial infarction, certain immunological disease processes, infection, malignancy, endotoxemia and reperfusion injury. In addition, inappropriate  
10 COX-2 expression or over-expression is associated with certain types of cancers, including, but not limited to, carcinoma of the colon, rectum, stomach, esophagus, lung, and skin. The amount of COX-2 expression is related to the stage or progression of cancer (Fosslien, E, *et al.* 2000, *Ann. Clin. Lab. Sci.* 30:3-21). COX-2 has  
15 become a major pharmaceutical target for developing treatments for these and other diseases. Methods of detecting and measuring COX-2 activity are highly desired.

Yu *et al.* (1997) *J. Biol. Chem.* 272:21181-21186, describes the enzymatic conversion of arachidonyl ethanolamide (anandamide,  
20 AEA), to PGE<sub>2</sub>-ethanolamide in cell lines expressing COX-2 but not COX-1.

U.S. Patent 5,543,297 to Cromlish *et al.*, describes measuring total COX activity (COX-1 activity and COX-2 activity) in separate samples, with and without a COX-2 specific inhibitor, and then  
25 indirectly estimating COX-2 specific activity by subtracting the total COX activity observed with the inhibitor from the total COX activity observed without the inhibitor. One major weakness of this method is that the dynamics of enzymatic inhibition change based

upon numerous variables including time, temperature, concentration, specificity, sample preparation, etc.

U.S. patent 5,475,021 to Marnett *et al.* describes a method of measuring the activity of purified COX-2 by measuring O<sub>2</sub>-uptake during catalysis. This method requires purification of the enzyme.

U.S. Patent 6,045,773 to Isakson *et al.*, describes a method for measuring COX-2 activity in a mammal by administering a positron-emitting radioisotope-labeled COX-2 selective-binding agent to the mammal and then detecting the label by positron-emission tomography (PET). Weaknesses of this method include the invasive nature and expense of PET equipment. In addition, the method only localizes COX-2 protein but does not detect or measure activity.

What is needed, then, is a less-invasive, direct method of selectively detecting and measuring COX-2 activity in biological samples and whole animals without the need to purify the enzyme.

The present invention provides, in part, articles of manufacture and methods for selectively detecting and measuring COX-2 enzymatic activity. One object of the invention is to provide a system for distinguishing between COX-2 activity and COX-1 activity. COX-1 and COX-2 are well known to catalyze the committed step in the conversion of arachidonic acid to PGH<sub>2</sub>. Downstream events, both enzymatic and non-enzymatic, further convert the PGH<sub>2</sub> into a variety of prostaglandins and thromboxanes. The present invention provides herein a system and methods to exploit a COX-2 selective enzymatic reaction with AEA for specifically detecting and measuring COX-2 enzymatic activity.

In certain embodiments of the present invention, COX-2 enzymatic activity is detected or measured by detecting or measuring novel PGH<sub>2</sub>-EA metabolites, including, but not limited to: PGB<sub>2</sub>-EA, PGD<sub>2</sub>-EA, PGE<sub>2</sub>-EA, PGF<sub>2</sub>α-EA, TxB<sub>2</sub>-EA, 6-keto-PGF<sub>1</sub>α-EA, 15-  
5 keto-PGE<sub>2</sub>-EA, 13,14-dihydro-15-keto-PGE<sub>2</sub>-EA, PGG<sub>2</sub>-EA, PGH<sub>2</sub>-EA, PGA<sub>2</sub>-EA, PGJ<sub>2</sub>-EA, PGJ<sub>2</sub>-EA derivatives, bicyclo-PGE<sub>2</sub>-EA, TxA<sub>2</sub>-EA and PGI<sub>2</sub>-EA. In certain embodiments, the COX-2 specific enzymatic activity is compared to COX-1 activity, for example, a COX-2/COX-1 ratio is reported.

10

### 3.0 DISCLOSURE OF THE INVENTION

One aspect of the present invention is a method of detecting COX-2 activity in a biological system or subject comprising detecting a PGH<sub>2</sub>-EA metabolite in a sample of the system. In  
15 certain embodiments, detection or measurement of a PGH<sub>2</sub>-EA metabolite is performed wherein the metabolite is PGB<sub>2</sub>-EA, PGD<sub>2</sub>-EA, PGE<sub>2</sub>-EA, PGF<sub>2</sub>α-EA, TxB<sub>2</sub>-EA, 6-keto-PGF<sub>1</sub>α-EA, 15-keto-PGE<sub>2</sub>-EA, 13,14-dihydro-15-keto-PGE<sub>2</sub>-EA, PGG<sub>2</sub>-EA, PGH<sub>2</sub>-EA, PGA<sub>2</sub>-EA, PGJ<sub>2</sub>-EA, PGJ<sub>2</sub>-EA derivatives, bicyclo-PGE<sub>2</sub>-EA, TxA<sub>2</sub>-  
20 EA or PGI<sub>2</sub>-EA. The presence of the PGH<sub>2</sub>-EA metabolite in the sample is indicative of the COX-2 activity in the system or subject.

In certain embodiments of the present invention, the inventors provide standards for determining a relative or absolute measurement of COX-2 activity by comparison to a standard or a  
25 standard curve generated using the standard. For example, a standard can be used to generate a standard curve for normalization of particular test results. The present invention

provides standards including compounds related to reaction products derived from COX-2 action on AEA incorporating isotopic (e.g., radioactive), fluorescent, enzymatic labels and the like.

In certain embodiments, a determination of the amount of COX-2 activity, by the measurement of PGH<sub>2</sub>-EA metabolites, is used to detect inflammation or cancer. In certain embodiments, a relative or absolute determination of inflammation or cancer in the subject is made.

FIG. 1 is a diagram of the chemical structure for arachidonic acid (AA).

FIG. 2 is a diagram of the COX biosynthetic pathway of arachidonic acid to prostaglandins and thromboxane.

FIG. 3 is a diagram of the COX biosynthetic pathway of arachidonic acid to PGH<sub>2</sub>, showing the chemical structure of the substrate and the metabolites.

FIG. 4 is a diagram of the chemical structure for arachidonylethanolamide.

FIG. 5 is a general diagram of enzymatic oxidation of prostaglandin (or thromboxane) ethanolamide (1) and enzymatic hydrolysis of prostaglandin (or thromboxane) ethanolamide (2).

FIG. 6 is a diagram of certain metabolic pathways of PGE<sub>2</sub>-EA illustrating a selection of the type-specific enzymatic and nonenzymatic biotransformations that prostaglandin ethanolamides (PG-EAs) and thromboxane ethanolamides (Tx-EAs) undergo. This diagram of PGE<sub>2</sub>-EA biotransformations can be similarly constructed for other PG-EAs and Tx-EAs (see, FIGs. 7 and 8). Also, prostaglandin and thromboxane biotransformation pathways can be

used as a pattern by one of ordinary skill in the art for the same purpose, in light of the present invention.

FIG. 7 is a diagram of the prostaglandins enzymatically generated following COX-2 action on AEA and in the presence of  
5 enzymes downstream of COX-2 (for instance, certain *in vivo* environments).

FIG. 8 is a diagram of certain prostaglandins generated following COX-2 action on AEA *in vitro*.

FIG. 9 is a diagram of tetradeuterated prostaglandin or  
10 thromboxane ethanolamide standards.

FIG. 10 is a graph showing the recovery of PGE<sub>2</sub>-EA from human urine.

FIG. 11 is a graph showing the stability of PGE<sub>2</sub>-EA incubated for 5 hours at 37°C in bovine, canine, and human cerebrospinal fluid.

15 FIG. 12 (A) is a graph showing the stability of PGE<sub>2</sub>-EA in plasma. The PGE<sub>2</sub>-EA (4 mg/mL) remaining after incubation in rat or human plasma for 5 h at 37° C (mean ± S.E., n<sup>3</sup> = 3). (B) PGE<sub>2</sub>-EA (4 mg/mL) is incubated in rat plasma for the indicated times and subjected to LC/MS analysis with selected ion monitoring for the  
20 mass corresponding to the dehydration product of PGE<sub>2</sub>-EA (m/z 400). Chromatograms are normalized to the 2 minute sample. (C) PGE<sub>2</sub>-EA (4 mg/mL) are treated as indicated and samples are analyzed for the dehydration product by LC/MS (m/z 400). Chromatograms are normalized to the base treated sample (lowest panel). (D) Is a graph of  
25 the absorption spectra of PGE<sub>2</sub>-EA (20 mg/mL) incubated in rat plasma for the indicated times.

FIG. 13 is a graph showing the *in vivo* pharmacokinetics of PGE<sub>2</sub>-EA in a rat given 2 mg/kg PGE<sub>2</sub>-EA by IV. The calculated



plasma half-life of PGE<sub>2</sub>-EA is 380 ± 50 seconds (n = 3). A large apparent volume of distribution was observed (300-400 mL) and both PGE<sub>2</sub>-EA and 13,14-dihydro-15-keto-PGE<sub>2</sub>-EA were routinely detected at 2 hours post-dosing.

5

#### 4.0 BEST MODE FOR CARRYING OUT THE INVENTION

The present invention provides novel compositions, methods and articles of manufacture for detecting and measuring cyclooxygenase-2 (COX-2) activity in a subject or a sample thereof.

10 Certain aspects of the present invention focus on detecting and measuring metabolites of AEA, such as, but not limited to, PGE<sub>2</sub>-EA, PGD<sub>2</sub>-EA and TxA<sub>2</sub>-EA. The present invention provides novel compositions, methods and kits for detecting and measuring COX-2 activity, methods for identifying tumors in a subject, evaluating  
15 relative tumor severity, and following tumor response to therapy, and methods for detecting inflammation in a subject and evaluating relative inflammation severity. No aspect, embodiment or element, including the claims, of the present invention is bound by theory or mechanism.

20

#### 4.1 DEFINITIONS

In case of conflict, the present document, including definitions, will control. Unless otherwise indicated, materials, methods and examples described herein are illustrative only and  
25 not intended to be limiting. All references, citations, articles, publications, patents, and the like provided in this patent application are incorporated herein by reference, in their entirety.

The term *in vivo* includes the meaning of processes occurring in an animal, in tissue or cell culture, or in samples taken from an animal or culture.

The term *in vitro* includes the meaning of processes occurring  
5 in systems wholly or partially purified from the natural environment, such as with purified enzymes or defined enzyme systems.

Purified means partially or wholly isolated away from the natural milieu of factors normally associated with a particular  
10 macromolecular species. In certain embodiments, the purified factor comprises 50 percent or more (on a molar basis) of all macromolecular species present in the isolated form. In certain  
15 preferred embodiments, a purified composition will comprise more than about 80 percent of all macromolecular species present. In certain  
20 preferred embodiments, a purified composition comprises more than about 90 percent of all macromolecular species present. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by  
conventional detection methods) wherein the composition consists  
essentially of a single macromolecular species. Solvent species,  
small molecules (<500 Daltons), and elemental ion species are not  
considered macromolecular species. In non-liquid compositions,  
"purified" is based upon dry weight and the same percent purities  
stated above are embodied.

25 A COX-2 selective substrate is a substrate that is transformed to an enzymatic reaction product by the COX-2 enzyme; but is not transformed, or is not significantly transformed, to a reaction product by the COX-1 enzyme. It is most preferred

that a COX-2 selective substrate of the present invention is not enzymatically transformed to a reaction product by COX-1. In certain embodiments, COX-1 may have some activity on the COX-2 selective substrate, but it is not significant relative to the COX-2 activity. Relatively insignificant activity can be determined, for example, by measuring the ratio of substrate oxygenation using purified COX-1 and COX-2.

In certain embodiments, the ratio of COX-1 activity *versus* COX-2 activity for a COX-2 selective substrate, expressed as a percentage, is about 50% or less; in certain embodiments, 40% or less; in certain embodiments, 30% or less; in certain embodiments, 25% or less; in certain embodiments, 20% or less; in certain embodiments, 10% or less; in certain embodiments, 5% or less; in certain embodiments, 3% or less; in certain embodiments, 2% or less; and in certain preferred embodiments 1% or less. The lower the percentage (above), the more preferred the embodiment. A highly preferred COX-2 selective substrate is metabolized by COX-2, but is not metabolized by COX-1.

The terms "COX-2 specific substrate" and "COX-2 selective substrate" are used interchangeably herein.

In general, enzyme activity refers to the rate at which substrate is consumed or product is formed in an enzymatic reaction under a given set of reaction conditions. The Standard International (SI) unit for enzyme activity is an enzyme unit (U) and is defined as the amount of enzyme needed to produce 1  $\mu$ mole product/minute. A unit may be defined differently herein (e.g., the amount of enzyme needed to produce 1 nmole product per minute or the amount of enzyme needed to consume 1  $\mu$ mole substrate per minute).

Additional determinations of enzyme activity can be compared when utilizing similar or preferably identical reaction conditions. It is understood that reaction conditions can be changed and a new enzyme activity scale determined (*e.g.*, by generating a standard  
5 curve of enzyme activity and use thereof, a process which is known to one of ordinary skill in the art). The specific activity of a particular enzyme preparation refers to the total enzyme units divided by the total amount of protein present in the preparation. A preferred unit of specific activity is U per mg of protein (U/mg).

10 As used herein, references to COX include both COX-1 and COX-2.

Arachidonyl ethanolamide (AEA) is defined herein to be a COX-2 selective substrate.

The terms "arachidonyl ethanolamide" and "anandamide" are  
15 used interchangeably herein.

As used herein, "prostaglandin ethanolamides" (PG-EAs) are included in the meaning of COX-2 selective metabolites.

Certain abbreviations include: cyclooxygenase (COX), cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), prostaglandin  
20 (PG), prostaglandins (PGs), ethanolamide (EA), prostaglandin ethanolamides (PG-EAs), arachidonyl ethanolamide (AEA), prostaglandin-ethanolamide (PG-EA), thromboxane (Tx).

As defined herein and using the abbreviations above, PG-EAs can include, but are not limited to: PGB<sub>2</sub>-EA, PGD<sub>2</sub>-EA, PGE<sub>2</sub>-EA,  
25 PGF<sub>2α</sub>-EA, TxB<sub>2</sub>-EA, 6-keto-PGF<sub>1α</sub>-EA, 15-keto-PGE<sub>2</sub>-EA, 13,14-dihydro-15-keto-PGE<sub>2</sub>-EA, PGG<sub>2</sub>-EA, PGH<sub>2</sub>-EA, PGA<sub>2</sub>-EA, PGJ<sub>2</sub>-EA, PGJ<sub>2</sub>-EA derivatives, bicyclo-PGE<sub>2</sub>-EA, TxA<sub>2</sub>-EA and PGI<sub>2</sub>-EA (also referred to herein as prostacyclin-EA).

Tumor type typically references the tissue of tumor origin, but can also refer to the current tissue in which a tumor is located (e.g., colon cancer, liver cancer, or pancreatic cancer). The stage and grade of a tumor is related to severity and medical definitions of stages and grades within a continuum are known in the art for each tumor or cancer type. Each specialty within oncology (e.g., hematology, colorectal, liver, pancreatic, lung, brain, dermatology, etc.) may have a particular standard for the stage and grade scale of the tumors used within that clinical specialty, known to one of skill in that art, which varies from the general definitions of tumor stage and grade provided below.

Tumor grade is determined by the appearance of the tumor under the microscope and how quickly the tumor is likely to grow and spread. In general, grading systems are different for each type of cancer, but are known to one of ordinary skill in the art. For example, grade I tumors are the least malignant appearing, grade II tumors are moderately differentiated with a moderately malignant appearance, grade III tumors are less differentiated and show enhanced signs of tissue invasion, and grade IV tumors display the least differentiation and are the most malignant appearing. The grade of a tumor is determined by one of ordinary skill in the art.

The stage of a tumor refers to the extent of a cancer, how advanced the tumor is in the patient (e.g., whether the disease has spread from the original site to other parts of the body). The stage of a tumor is generally determined by radiographic studies such as a computed tomography (CT) scan, magnetic resonance (MRI) imaging and/or ultrasound. Tumor staging is determined by one of ordinary skill in the art and can vary by tumor type or as a field

advances, standard staging practices may change. Certain definitions of stages for various cancers are provided in the Dictionary of Cancer Terms on CANCERNET which is a service of the National Cancer Institute available on the World Wide Web at  
5 “<http://cancernet.nci.nih.gov/dictionary/dictionaryfull.html>”, incorporated herein by reference in its entirety. A physical location for inquiry or obtaining a copy of the Dictionary of Cancer Terms is: NCI Public Inquiries Office; Building 31, Room 10A03; 31 Center Drive, MSC 2580; Bethesda, MD 20892-2580.

10 Staging refers to performing exams and tests to learn the extent of the cancer within the body, especially whether the disease has spread from the original site to other parts of the body.

#### 4.2 CYCLOOXYGENASES AND PROSTAGLANDINS

15 Cyclooxygenase (COX; prostaglandin endoperoxide synthase, EC 1.14.99.1) catalyzes the *bis*-dioxygenation of arachidonic acid (FIG. 1) generating prostaglandin (PG) H<sub>2</sub> (FIGS. 2-3). This is the committed step in prostaglandin and thromboxane biosynthesis. Two isoforms of COX have been cloned from animal cells including  
20 constitutively expressed COX-1 (DeWitt, D. L., and Smith, W. L. 1988, *Proc. Natl. Acad. Sci. USA*, 85:1412-1416; Merlie, *et al.* 1988, *J. Biol. Chem.*, 263:3550-3553; Yokoyama, *et al.* 1988, *FEBS Lett.*, 231:347-351; DeWitt, *et al.* 1990, *J. Biol. Chem.*, 265:5192-5198; and Yokoyama, C. and Tanabe, T. 1989, *Biochem. Biophys. Res.*  
25 *Commun.*, 165:888-894) and inducibly expressed COX-2 (Xie, *et al.* 1991, *Proc. Natl. Acad. Sci. USA*, 88:2692-2696; Kujubu, *et al.* 1991, *J. Biol. Chem.*, 266:12866-12872; O'Banion, *et al.* 1991, *J. Biol. Chem.*, 266:23261-23267; Hla, T. and Nielson, K. 1992, *Proc. Natl.*

*Acad. Sci. USA*, 89:7384-7388; Jones, *et al.* 1993, *J. Biol. Chem.*, 268:9049-9054; and Feng, *et al.* 1993, *Arch. Biochem. Biophys.*, 307:361-368).

Prostaglandins produced as a result of the activity of COX  
5 are known to have numerous physiological functions. These  
functions include the antithrombogenic action of prostacyclin  
released by the vascular endothelium and the cytoprotective effect  
of prostaglandins produced by the gastric mucosa (Whittle, *et al.*  
1980, *Nature*, 284:271-273). COX-2 is typically expressed following  
10 the activation of normal cells and certain atypically proliferating  
cells, by various pro-inflammatory agents including certain  
cytokines (Hla, T. and Nielson, K. 1992, *Proc. Natl. Acad. Sci. USA*,  
89:7384-7388; Feng, *et al.* 1993, *Arch. Biochem. Biophys.*, 307:361-  
368), endotoxin (Lee, *et al.* 1992, *J. Biol. Chem.*, 267:25934-25938)  
15 and certain mitogens (Kujubu, *et al.* 1991, *J. Biol. Chem.*,  
266:12866-12872; O'Banion, *et al.* 1991, *J. Biol. Chem.*, 266:23261-  
23267; and Hla, T. and Nielson, K. 1992, *Proc. Natl. Acad. Sci.*  
*USA*, 89:7384-7388).

Prostaglandins represent a class of substances produced in a  
20 wide variety of cells. In general, PGs act on the cells that produce  
them, on neighboring cells, or over short distances and can be  
classified as autocrine hormones. PGs and their relatives are  
usually thought of as potent local hormones (autocrine and  
paracrine) acting over a short lifetime. PGs, and related compounds,  
25 prostacyclin (PGI<sub>2</sub>), thromboxanes (TX), leukotrienes (LT), and  
lipoxins (LP), derive from fatty acids stored in cellular membranes  
as phospholipids or triglycerides, especially arachidonic acid, with  
an open chain, 20-carbon structure (FIGS. 1-3). Prostaglandins

generally resemble hairpins structurally with a five-membered ring and two chains extending from the ring. In general, substituents on the five-membered ring determine the subclass and activity of the prostaglandins. A series of synthetic reactions catalyzed by enzymes  
5 in the membranes, and certain non-enzymatic transformations, culminate in the release of prostaglandin product.

#### 4.3 DETECTING COX-2 ACTIVITY BY DETECTING PROSTAGLANDIN ETHANOLAMIDES

10 AEA is an unique substrate specific for COX-2 (FIG. 4). COX-2 catalyzes the conversion of AEA to prostaglandin H<sub>2</sub> ethanolamide (PGH<sub>2</sub>-EA) (see FIG. 5-8; Yu, M., et al 1997, *J. Biol. Chem.* 272:21181-21186, incorporated herein by reference). The PGH<sub>2</sub>-EA is subsequently enzymatically and nonenzymatically metabolized to  
15 a variety of compounds, such prostaglandin E<sub>2</sub> ethanolamide (PGE<sub>2</sub>-EA) and prostaglandin D<sub>2</sub> ethanolamide (PGD<sub>2</sub>-EA). These downstream metabolites will be referred to, herein, as PGH<sub>2</sub>-EA metabolites. PGH<sub>2</sub>-EA metabolites include, but are not limited to, PGB<sub>2</sub>-EA, PGD<sub>2</sub>-EA, PGE<sub>2</sub>-EA, PGF<sub>2</sub> $\alpha$ -EA, TxB<sub>2</sub>-EA, 6-keto-PGF<sub>1</sub> $\alpha$ -  
20 EA, 15-keto-PGE<sub>2</sub>-EA, 13,14-dihydro-15-keto-PGE<sub>2</sub>-EA, PGG<sub>2</sub>-EA, PGH<sub>2</sub>-EA, PGA<sub>2</sub>-EA, PGJ<sub>2</sub>-EA, PGJ<sub>2</sub>-EA derivatives, bicyclo-PGE<sub>2</sub>-EA, HETEs, TxA<sub>2</sub>-EA and PGI<sub>2</sub>-EA., which are metabolized directly from AEA by COX-2, will also be referred to as PGH<sub>2</sub>-EA  
25 metabolites in this application. In general, all prostaglandin and thromboxane ethanolamides are susceptible to enzymatic oxidation of the ethanolamide moiety and can undergo oxidation (FIG. 5). A representative example for PGE<sub>2</sub>-EA is shown in FIG. 6. In addition, individual metabolites can enter the 15-



hydroxyprostaglandin dehydrogenase/ $\Delta^{13}$ -15-keto-prostaglandin reductase pathway to generate 13,14-dihydro-15-keto metabolites, for example 15-keto  $\text{PGA}_2\text{-EA}$  and 13,14-dihydro- $\text{PGA}_2\text{-EA}$ . In general,  $\text{PGE}_2\text{-EA}$  metabolites can undergo dehydration to enter the  
5  $\text{PGA}_2\text{-EA/PGB}_2\text{-EA}$  pathway. For example, 13,14-dihydro-15-keto- $\text{PGE}_2\text{-EA}$  can dehydrate to yield 13,14-dihydro-15-keto- $\text{PGA}_2\text{-EA}$  and 13,14-dihydro-15-keto- $\text{PGB}_2\text{-EA}$ . Finally, oxidative transformation by P450-mediated  $\omega$ -oxidation (e.g., C-19, C-20) can occur.

10 It has been shown that AEA is found endogenously (Devane *et al.* 1992, *Science* 258:1946-1949, hereby incorporated by reference). The amount of  $\text{PGH}_2\text{-EA}$  metabolites in a biological sample correlates with the COX-2 specific activity in the subject from which the sample was collected. The amount of COX-2 activity  
15 is a marker of or a measurement of inflammatory or cancerous disease processes. Certain embodiments of the present invention include methods and materials for making sensitive measurements of picogram quantities of  $\text{PGH}_2\text{-EA}$  metabolites. Further embodiments of the present invention include using these  
20 measurements of COX-2 activity to clinically grade or stage a disease process and to assess therapeutic outcomes.

#### 4.4 MEASURING PROSTAGLANDIN ETHANOLAMIDES

For the purposes of this application, detecting includes  
25 determining if a substance or compound is present in a sample. In the present invention, the substance or compound being detected is a  $\text{PGH}_2\text{-EA}$  metabolite. Detecting can include measuring. In general, measuring means determining the relative or absolute amount of a

substance or compound detected. Measurement is generally, but not always, performed relative to a standard. For example, the amount of the standard may be correlated with an amount of COX-2 activity or expression. Therefore, comparing the amount of PGH<sub>2</sub>-EA metabolites measured in a sample from a subject indicates an amount of COX-2 activity or expression in the subject.

PGH<sub>2</sub>-EA metabolites may be detected and measured in a variety of ways (see Examples 1-6). In certain embodiments of the present invention, for example, a sample is collected from a subject, and a selective COX-2 substrate, such as AEA, is added. Then, a metabolite of the COX-2 specific PGH<sub>2</sub>-EA product is measured. In preferred embodiments, downstream metabolites are measured because the half-life of PGH<sub>2</sub>-EA in aqueous solution is approximately 20 seconds. Certain downstream metabolites are more stable and are preferred for measurement. A highly preferred downstream metabolite for measurement is PGE<sub>2</sub>-EA. In another embodiment of the present invention, for example, both a COX-1 substrate (such as arachidonic acid) and a COX-2 selective substrate (such as AEA) are added to the sample. Then the amount of downstream metabolites derived from the enzymatic actions of COX-1 and COX-2 on each substrate are measured and compared. In both examples, the samples can be incubated with the substrates over time and a series of measurements of metabolites taken and compared in relation to the amount of time that passed. In certain embodiments of the present invention, no substrate is added to the sample. Instead, the PGH<sub>2</sub>-EA metabolites derived from the endogenous AEA is detected and measured. A highly preferred downstream metabolite for measurement is 13,14-dihydro-15-keto-

PGE<sub>2</sub>-EA, a novel lipid formed by the sequential oxidation and reduction of PGE<sub>2</sub>-EA by 15-hydroxyprostaglandin dehydrogenase and  $\Delta$ 13,15-keto-prostaglandin reductase. This metabolite is formed *in vivo* following intravenous dosing of PGE<sub>2</sub>-EA to rats and is  
5 detectable in plasma by the methods described herein. In some cases, AEA is administered to the subject prior to sample collection, followed by sample collection, and detection and measurement of PGH<sub>2</sub>-EA metabolites present in the sample. In addition, in preferred embodiments, the amount of PGH<sub>2</sub>-EA metabolites  
10 measured in the sample is related to an amount of COX-2 activity in the sample or the subject.

Samples from the subject can be processed in several ways (see Examples 5-6). For example, the sample may be extracted at least one time with a solvent, to remove the PGH<sub>2</sub>-EA metabolites  
15 from the sample for analysis. Extraction can be followed by evaporation. The resulting residue may be redissolved in another solvent and analyzed. This process might involve several rounds of the extraction, evaporation and redissolving steps. Alternatively, the solution resulting from one or more extractions of the sample  
20 may be filtered and analyzed. In certain preferred embodiments, the sample is extracted, filtered and analyzed for PGH<sub>2</sub>-EA metabolite content.

One aspect of the present invention is a method of detecting COX-2 activity in a biological sample, comprising: incubation of the  
25 biological sample with AEA, extracting the sample with a solvent, evaporating the solvent to leave a residue and analyzing the residue for PGH<sub>2</sub>-EA metabolites wherein the presence of PGH<sub>2</sub>-EA

metabolites is indicative of COX-2 activity (for instance, see Examples 1, 2, and 3). In certain embodiments, the amount of PGH<sub>2</sub>-EA metabolites is measured and related to the quantity of COX-2, COX-2 expression, or COX-2 activity. In general, the analysis of PGH<sub>2</sub>-EA metabolites includes, but is not limited to, detection by liquid chromatography-mass spectrometry (Examples 1-2, 5-6).

Another embodiment of the present invention is a method of detecting COX-2 activity in a biological sample, comprising: extracting PGH<sub>2</sub>-EA metabolites present in the sample with a solvent, evaporating the solvent to leave a residue and quantifying PGH<sub>2</sub>-EA metabolites in the residue, wherein the presence of PGH<sub>2</sub>-EA metabolites is indicative of COX-2 activity. Preferably, the quantity of PGH<sub>2</sub>-EA metabolites detected are related to a quantity of COX-2 activity.

Yet another embodiment of the present invention is a method of detecting a COX-2 activity in a biological sample, comprising: extracting the sample at least one time with a first solvent, evaporating the solvent to leave a residue, dissolving the residue in a second solvent, separating at least one PGH<sub>2</sub>-EA metabolite from the dissolved residue with a separation device, lyophilizing the separated PGH<sub>2</sub>-EA metabolite, dissolving the lyophilized PGH<sub>2</sub>-EA metabolite in a third solvent and detecting the dissolved PGH<sub>2</sub>-EA metabolite with a detection device, wherein the presence of the PGH<sub>2</sub>-EA metabolite is indicative of the COX-2 activity (for instance, see Examples 1, 2, and 3). In certain embodiments, the amount of PGH<sub>2</sub>-EA metabolite is measured and related to a

quantity of COX-2 protein, COX-2 expression, or COX-2 activity. In general, the separation device for separating the PGH<sub>2</sub>-EA metabolite from a residue in the second solvent includes, but is not limited to, a liquid chromatography device.

5 Yet another aspect of the present invention is a method of detecting COX-2 activity in a biological sample, comprising: extracting at least one PGH<sub>2</sub>-EA metabolite from the sample with a solvent, evaporating the solvent to leave a residue, dissolving the residue in a second solvent, filtering the dissolved residue and  
10 detecting a PGH<sub>2</sub>-EA metabolite in the filtered solution with a detection device, wherein the presence of PGH<sub>2</sub>-EA metabolite in the sample is indicative of COX-2 activity. Preferably, the detected PGH<sub>2</sub>-EA metabolite is measured and the amount of PGH<sub>2</sub>-EA metabolite measured is related to a quantity of COX-2 activity.

15

#### 4.4.1 USE OF A STANDARD

In certain embodiments, isotopically labeled PGH<sub>2</sub>-EA metabolites, such as PGE<sub>2</sub>-EA and TxA<sub>2</sub>-EA, are used as an internal standard in quantifying PG-EAs. In certain preferred embodiments,  
20 mass spectrometric quantification of PGH<sub>2</sub>-EA metabolites is performed. One example of the synthesis of isotopically labeled ethanolamides of prostaglandins and thromboxanes is shown in FIG. 9. In general, isotopically labeled PG- or Tx-EA are prepared by coupling the target PG or Tx free acid with anhydrous,  
25 isotopically labeled ethanolamide and purified by chromatography. In another example, labeled standard ethanolamides of prostaglandins and thromboxanes can be labeled by substituting <sup>2</sup>H

or  $^3\text{H}$  for a  $^1\text{H}$  attached to at least one of the carbons 2-20 of the arachidonyl carbon chain. Alternatively, a  $\text{PGH}_2\text{-EA}$  metabolite can be labeled by substituting  $^{13}\text{C}$  or  $^{14}\text{C}$  for at least one  $^{12}\text{C}$  in the arachidonyl carbon chain. Labeled  $\text{PGH}_2\text{-EA}$  metabolites can also be  
5 made by reacting labeled AEA with COX-2, followed by purification of the reaction products. In general, labeled  $\text{PGH}_2\text{-EA}$  metabolites may also be synthesized by substituting at least one atom in the molecule with an isotope. In certain embodiments, the isotope is non-positron emitting. In certain embodiments, the isotope is  
10 positron emitting. Alternatively,  $\text{PGH}_2\text{-EA}$  metabolites can be derivatized to produce chemiluminescent or fluorescent standards, which can be used, for example, in  $\text{PGH}_2\text{-EA}$  quantitation using high performance liquid chromatographic separation coupled with fluorescence or chemiluminescence detectors.

15 Labeled PG-EA or Tx-EA standard curves, which correlate with the presence or absence of cancer in a patient, can be generated. For example, this can be accomplished by collecting samples from groups of patients with different types, stages, or grades of cancer, measuring the amount of a  $\text{PGH}_2\text{-EA}$  metabolites  
20 in the samples, and graphing the amounts of the  $\text{PGH}_2\text{-EA}$  metabolite *versus* the types of cancer. In certain embodiments, types can be varieties. In preferred embodiments, the types are a range of particular cancers measured on a clinical scale of severity or aggressiveness. Such a standard curve may indicate that the larger  
25 the amount of cancer or the more progressed the cancer in the patient, the more  $\text{PGH}_2\text{-EA}$  metabolite found in the sample from the patient. In addition, since small amounts of  $\text{PGH}_2\text{-EA}$  metabolites can be found in samples collected from people without cancer, a

threshold level of PGH<sub>2</sub>-EA metabolites will be observed at which amounts of PGH<sub>2</sub>-EA metabolites below the threshold will correlate with the lack of cancer in the patient. On the other hand, for example, amounts of PGH<sub>2</sub>-EA metabolites measured in a sample  
5 that are above the threshold level correlate with the presence of cancer in the patient.

In a similar example, a standard curve can be developed which correlates the amount of PGH<sub>2</sub>-EA metabolites observed in a sample from a patient with an amount of inflammation. Such a  
10 standard curve can be generated by collecting samples from a patient population suffering from inflammatory processes, and correlating the amounts of PGH<sub>2</sub>-EA metabolite in the samples with the severity of disease in the patient population.

In another example, no standard or standard curve is used.  
15 Instead, a series of samples may be collected from a single patient over a period of time, and the amount of PGH<sub>2</sub>-EA metabolite in each sample measured. Then the amounts of PGH<sub>2</sub>-EA metabolite measured would be compared and correlated with the amount of time which had passed between sample collections. If the amount of  
20 PGH<sub>2</sub>-EA metabolite in the samples increased over time, for example, this would indicate that the patient's disease state is worsening, or that therapeutic intervention is not effective. On the other hand, if the amount of PGH<sub>2</sub>-EA metabolite measured in the samples decreased over time, for example, this would indicate that  
25 the patient's disease state is improving or that therapeutic intervention is successful.

#### 4.4.2 DETECTION AND MEASURING DEVICE

In general, a detection device for detecting PGH<sub>2</sub>-EA metabolites includes, but is not limited to, a mass spectrometer, a chromatography-coupled mass spectrometer, an immunoassay or an enzyme-linked immunoassay, or other means for detecting PGH<sub>2</sub>-EA metabolites known in the art (see Examples 1-6). For example, in certain embodiments of the present invention, liquid chromatography/mass spectrometry (LC/MS) is conducted, preferably with a Waters 2690 Separations Module with a Zorbax RX-C18 narrow bore column (15 cm x 2.1 mm, 5 μm) interfaced to a Finnigan TSQ-7000 triple quadrupole mass spectrometer. Sodiated analytes are eluted with increasing concentrations of MeCN in 0.001% aqueous sodium acetate. Evaluation of PGH<sub>2</sub>-EA metabolites in biological samples is conducted with selected ion monitoring and quantification is accomplished using tetradeutereated standards, such as tetradeutereated PGE<sub>2</sub>-EA. Electrospray ionization (ESI) is carried out using nitrogen as sheath (76 psi) and auxiliary gas (14 psi) to assist with nebulization. A potential of 5.5 kV is applied to the ESI needle and the capillary temperature is maintained at 220 °C. Mass spectral parameters are optimized to obtain maximum sensitivity without sacrificing unit resolution.

#### 4.4.3 SEPARATION DEVICE

A variety of separation devices known in the art for separating prostaglandins from a sample may be used. In general, separation devices include, but are not limited to, extraction columns, affinity columns, filters, thin-layer chromatography plates and gels.



In certain embodiments of the present invention, a PGH<sub>2</sub>-EA metabolite in a sample may be isolated or purified (separated partially or substantially from the natural constituents of a PGH<sub>2</sub>-EA metabolite containing sample) using one or more techniques known in the art for the separation of chemical and especially prostaglandin compounds, for example, but not limited, to liquid chromatography.

#### 4.4.4 SUBJECTS

10 In certain embodiments of the present invention, the subject includes a mammal, such as a rodent, preferably a human, or a cultured cell of a mammal, including a cultured cell of a human. Other subjects include farm animals and show animals (horses, cattle, sheep, pigs and swine, goats, fowl, and the like), pets (dogs, cats, parrots, canaries and the like), animals kept in zoos and endangered species (elephants, lions, tigers, antelope, zebra, anteaters, water buffalo, pandas, cheetahs, kangaroos, ostriches, eagles, condors, finches, and the like) or a cultured cell of said animal.

20

#### 4.4.5 SAMPLES

In certain embodiments, the sample is urine or may be collected from or processed from urine. PGH<sub>2</sub>-EA metabolites can be measured in urine by isotope dilution mass spectrometry. A fixed volume of urine is treated with an appropriate internal standard (e.g., tetradeuterated PGE<sub>2</sub>-EA) and then loaded on reversed-phase extraction cartridges. The sample is then washed (e.g., 1 ml pH 4.0

25

20 mM sodium acetate) and PGH<sub>2</sub>-EA metabolites, including the added internal standard, are eluted with organic solvent (e.g., two 1 ml aliquots of MeCN). The solvent is evaporated and the residue is analyzed by LC/MS. A typical procedure wherein synthetically  
5 generated PGE<sub>2</sub>-EA and *d5*-PGD<sub>2</sub>-EA is added to fresh human urine is depicted in FIG. 10.

PGH<sub>2</sub>-EA metabolites are stable in cell culture, bovine, canine and human CSF (FIG. 11) and human and rat plasma (FIG. 12) incubated 5 hours at 37°C. FIG. 13 shows the *in vivo* pharmacokinetics of PGE<sub>2</sub>-EA in a rat given 2 mg/kg PGE<sub>2</sub>-EA  
10 intravenously. The calculated half-life of PGE<sub>2</sub>-EA is 380 ± 50 seconds (n=3). Notably, this half-life greatly exceeds that observed for the free acid PGE<sub>2</sub> *in vivo*, highlighting one significant advantage of the methods described herein over previously  
15 described methods for COX activity assessment *in vivo*. A large apparent volume of distribution was observed (300-400 ml) and both PGE<sub>2</sub>-EA and 13,14-dihydro-15-keto- PGE<sub>2</sub>-EA were routinely detected at 2 hours post-dosing. In certain embodiments, the sample is blood, plasma, cerebrospinal fluid, saliva, sputum, bile, joint fluid,  
20 biopsy, immune cells, cancer cells, tumor cells, malignant cells, inflammatory cells, non-tumor cells, non-immune cells, cells not activated by inflammatory stimuli or may be collected from or processed from one or more of these fluids and tissues. In certain  
embodiments of the present invention, the sample may be  
25 conditioned media from a cell culture. In certain embodiments of the present invention, a plurality of samples may be collected from the subject, with a period of time being allowed to pass between consecutive collections of the samples. The amounts of PGH<sub>2</sub>-EA

metabolites present in these samples are measured, compared and related to the periods of time that had been allowed to pass between collections. In certain embodiments, the subject is a patient and the samples are taken in order to evaluate the effectiveness of anti-  
5 cancer therapy and to evaluate tumor state, severity or load in the patient.

In general, samples can be collected from or prepared from cultured cells (Example 6). A variety of cell lines known to one skilled in the art are acceptable. In addition, primary cell cultures  
10 can be used. Methods for collecting and culturing primary cell cultures are well known in the art.

#### 4.4.6 ANTIBODY SYNTHESIS

Monoclonal and polyclonal antibodies to PGH<sub>2</sub>-EA  
15 metabolites or their metabolites can be made using standard antibody generation techniques in light of the present invention (Cohen, et al., U.S. Patent Serial No. 5,589,575, herein incorporated by reference; McCafferty, *et al.* 1996, *Antibody Engineering, a Practical Approach*, IRL Press; Mernaugh & Mernaugh 1994,  
20 *Methods for the Production of Monoclonal Antibodies*, in **Molecular Methods in Plant Pathology**). For example, monoclonal antibodies against PGE<sub>2</sub> are commercially available from Cayman Chemical (118 E. Ellsworth Rd., Ann Arbor, MI 48108, 800-364-9897) and chemiluminescent ELISA kits for several PGs, HETE and  
25 TxB<sub>2</sub> are available from Assay Designs, Inc. (800 Technology Dr., Ann Arbor, MI 48108; 734-668-6113). To create antibodies that recognize PGE<sub>2</sub>-EA, for example, the cyclopentyl substituents and amide moieties of the PGE<sub>2</sub>-EA are protected using standard

techniques. Then the protected PGE<sub>2</sub>-EA is chemically linked to an appropriate conjugate (e.g., keyhole limpet hemocyanin (KLH)) preferably *via* covalent attachment at carbon 15. The hapttenized product is deprotected and the resultant immunogen is used to  
5 inoculate mice, rabbits, or goats using standard techniques. According to standard protocols, serum would be collected from the immunized animals and antibodies are purified from the serum. Alternative antibody development strategies using the same immunogen could also be employed (single-chain antibodies,  
10 hybridomas, etc.).

#### 4.5 COX-2 AND INFLAMMATORY DISEASES/DISORDERS

A wide variety of human diseases are associated with inflammation. These range from acute appendicitis to asthma,  
15 myocardial infarction, specific immunological disease processes, infection with viruses or bacteria, malignancy and metastasis, endotoxemia and reperfusion injury. Since COX-2 activity is important in the progression of these diseases, the present invention is a useful method of diagnosing or monitoring disease  
20 state. The present invention is also useful in detecting and treating non-malignant or immunological-related cell-proliferative diseases such as psoriasis, pemphigus vulgaris, Behcet's syndrome, acute respiratory distress syndrome (ARDS), ischemic heart disease, post-dialysis syndrome, leukemia, acquired immune deficiency  
25 syndrome, septic shock and other types of acute inflammation, and lipid histiocytosis. Essentially, the present invention can be used to facilitate the detection, measurement and treatment of any disorder

which is etiologically linked to the inflammatory process (for instance, see Example 4).

For example, in certain aspects of the present invention, a sample is collected from a patient suspected of having an inflammatory disease or diagnosed with an inflammatory disease and PGE<sub>2</sub>-EA in the sample is detected and measured. The amount of PGE<sub>2</sub>-EA present in the sample correlates with COX-2 activity in the patient and is a marker of the progress or severity of that disease or disease process. In another example, a series of samples are collected from the patient over a period of time. During that period of time, the patient undergoes treatment for the inflammatory disease. Changes in the amount of PGE<sub>2</sub>-EA measured over time are indicative of changes in COX-2 activity and changes in the patients disease state. This information would be used by the physician to evaluate the patient's condition as well as the effectiveness of therapeutic intervention, wherein a decrease in the PGH<sub>2</sub>-EA metabolites or COX-2 activity is indicative of an improvement in the patient's condition or effective therapy.

#### 20 4.6 COX-2 AND CANCER

Studies in human colon cancer have shown that COX-2 expression is increased in colon cancer cells compared to the adjacent colonic mucosa; similar observations have been made in experimental models of colon cancer (Eberhart, CE, *et al.* 1994, *Gastroenterology* 107:1183; Sheng, H, *et al.* 1997, *J. Clin. Invest* 99:2254; DuBois, RN, *et al.* 1996, *Gastroenterology* 110:1259). COX-2 expression is a marker for the metastatic potential of colon cancer

cells and is related to patient survival (Tsuji, M, *et al.* 1997, *Proc. Natl. Acad. Sci. USA* 94:3336; Sheehan, KM, *et al.* 1999, *JAMA* 282:1254). In one study, for example, COX-2 expression was determined in 76 patients with a variety of stages of colorectal  
5 cancer (Sheehan, KM, *et al.*, 1999, *JAMA* 282:1254). Such studies can be used to generate a standard curve for COX-2 expression in cancer and colon cancer in particular (see *supra*). Ten-year survival was significantly higher in patients with the lowest levels of COX-2  
10 expression (68 versus 35 percent). These findings suggest that COX-2 activation promotes tumor growth. Consistent with this hypothesis is a study in which human colon cancer cells that expressed high levels of COX-2 were implanted into nude mice. Treatment with a selective COX-2 inhibitor reduced tumor formation by 85 to 90 percent and inhibited colony formation of  
15 cultured cells (Sheng, H, *et al.* 1997, *J. Clin. Invest* 99:2254). This benefit was not seen with tumor cells that lacked COX-2.

Certain aspects of the present invention include methods of detecting a tumor in a patient in need thereof, comprising: obtaining a sample from the patient and detecting at least one  
20 PGH<sub>2</sub>-EA metabolite in the sample (for instance, see Example 5). The presence of the PGH<sub>2</sub>-EA metabolite in the sample is a marker for the presence of the tumor in the patient. More preferably, an amount of PGH<sub>2</sub>-EA metabolite detected in the sample of the patient will be measured, wherein the amount of PGH<sub>2</sub>-EA  
25 metabolite measured is indicative of the amount or severity of tumor present in the patient.

A further aspect of the present invention is a method of measuring and monitoring the size, grade, and/or stage of a tumor, comprising: collecting a first sample of a subject and measuring the amount of  $\text{PGH}_2\text{-EA}$  metabolites in the first sample. Then a period  
5 of time is allowed to pass, during which the subject may, or may not, undergo anti-cancer therapy. After the period of time has passed, a second sample is collected from the subject and the amount of  $\text{PGH}_2\text{-EA}$  metabolites in the second sample is measured. The amounts of the  $\text{PGH}_2\text{-EA}$  metabolites in the first and second  
10 samples are compared, wherein the difference between the amounts of  $\text{PGH}_2\text{-EA}$  metabolites in the two samples is indicative of changes in the metabolism of the cancer cell.

In certain embodiments of the present invention, a plurality of samples may be collected from the subject, with a period of time  
15 being allowed to pass between consecutive collections of the samples. The amounts of  $\text{PGH}_2\text{-EA}$  metabolites present in these samples are measured, compared and related to the periods of time that had been allowed to pass between collections. In certain  
20 embodiments, the subject is a patient and the samples are collected in order to evaluate the effectiveness of anti-cancer therapy and to evaluate tumor severity or tumor load in the patient.

In general, the effectiveness of the anti-cancer therapy is evaluated based on the changes in the amount of sample  $\text{PGH}_2\text{-EA}$  metabolite observed over time. For example, increases in the  
25 amount of  $\text{PGH}_2\text{-EA}$  metabolites over time indicate continued tumor growth and failure of therapeutic intervention; whereas decreases in the amount of  $\text{PGH}_2\text{-EA}$  metabolites over time are indicative of

therapeutic success and tumor regression. In certain embodiments of the present invention, the sample is a culture of cancer cells used as an experimental model or a culture of cancer cells taken from a patient. The cultured cancer cells may be treated with an anti-  
5 cancer therapy *in vitro* in order to evaluate the effectiveness of that therapy in relation to alternative cancer therapies. In certain embodiments, this procedure is done in order to determine an optimal anti-cancer therapy for that individual patient.

In certain embodiments, the attending health professional  
10 may characterize both an inflammatory process and a malignancy in the subject by detecting or measuring an amount of a prostaglandin ethanolamide in a sample of a subject specifically produced by the offending malignancy and inflammatory lesion.

#### 15 4.7 COX-2 AND RESEARCH

In the prior art, investigations which attempt to identify links between COX-2 expression/activity and disease processes are time- and labor-intensive and often require examination of tissue samples post-mortem. For example, attempts to study the role of  
20 COX-2 in Alzheimer's disease (AD) require post-mortem collection of brain tissue from both deceased AD and control subjects and quantitative assessment of COX-2 expression in this tissue using standard biochemical techniques (e.g., Western blotting). Such studies are also hampered by the inability to assess enzyme  
25 activity, which may or may not correlate with enzyme expression. The use of PGH<sub>2</sub>-EA metabolite quantification in this setting allows for a relatively non-invasive quantification of COX-2 activity *in*



*vivo*. This technique provides at least two fundamental benefits. First, the noninvasive nature allows for much broader testing increasing the sample size in these studies and permitting rapid and statistically significant association (or lack thereof) between  
5 COX-2 activity and the pathology under study. Second, given the possibility of testing patients before disease signs are evident will allow for assessing the role of COX-2 in disease development and progression in contrast to post-mortem studies which evaluate the role of COX-2 long after the disease process began. Quantification of  
10 PGH<sub>2</sub>-EA metabolites *in vivo* provides a simple assay for assessing the *in vivo* efficacy of newly developed COX-2 inhibitors.

In addition, there is a great need to discover and develop new COX-2 specific regulators. However, current methods are time and labor intensive. The present invention provides compositions,  
15 methods and kits for screening candidate molecules for their ability to regulate COX-2 using cultured cells, tissues, or whole animals (Examples 9-10).

#### 4.8 KITS

20 Certain embodiments of the present invention provide an article of manufacture for the detection and/or measurement of COX-2 activity by the detection and/or measurement of PGH<sub>2</sub>-EA metabolites by radioassay or immunoassay, comprising an antibody and a set of instructions delineating a process for relating a  
25 detection and/or measurement of PGH<sub>2</sub>-EA metabolites in a sample to a detection and/or measurement of COX-2 in a subject or a sample thereof. Preferably, the article of manufacture further comprises an antibody against PGH<sub>2</sub>-EA metabolites. More

preferably, the article of manufacture further comprises the standard reagents required to perform an immunoassay, such as buffers, multi-well plates, additional antibodies and the like. Still more preferably, the article of manufacture further comprises one or  
5 more solid phase extraction columns for the isolation/purification of PGH<sub>2</sub>-EA metabolites. Preferably, the article of manufacture further comprises a set of standards. More preferably, the article of manufacture further comprises an unlabeled PGH<sub>2</sub>-EA metabolite internal standard for standard curve development.

10 In further embodiments the present invention provides an article of manufacture for the detection and/or measurement of COX-2 activity by mass spectrometry, which comprises: a set of instructions delineating a process for relating a detection and/or measurement of PGH<sub>2</sub>-EA metabolites in a sample to a detection  
15 and/or measurement of COX-2 in a subject or a sample thereof and a C18 solid phase extraction column. Preferably, the article of manufacture further comprises a set of standards. More preferably, the article of manufacture further comprises an unlabeled PGH<sub>2</sub>-EA metabolite positive control, and a tetradeuterated PGH<sub>2</sub>-EA  
20 metabolite internal standard.

## 5.0 EXAMPLES

### 5.1 Example 1

25 In general, COX-2 enzymatic products can be detected or measured by a variety of methods. For example, reversed phase liquid chromatography-mass spectrometry is used to detect or

measure COX-2 enzymatic products. Selected-ion mass chromatograms of sodium adducts of oxygenated AEA metabolites can be generated by monitoring  $m/z = 386$  (HETE-EAs), 418 (PGE-EAs), and 436 (Tx-EA and 6-keto-PGF $1\alpha$ -EA). For example, 5 products are eluted with a 15-minute gradient of 20% to 100% acetonitrile and H $_2$ O (0.001% sodium acetate). LC/MS reveals the presence of five primary products in AEA/COX-2 reaction mixtures. Two closely eluting polar products each display  $m/z$  418 consistent with the non-enzymatic PGH $_2$ -EA isomerization products PGE $_2$ -EA and PGD $_2$ -EA. Tetra-deuterated standards of PGE $_2$ -EA and PGD $_2$ -EA are synthesized and coeluted with these two polar products under multiple chromatographic conditions. An intermediate polarity product with  $m/z$  346 is observed that is consistent with the PGH $_2$ -EA degradation product HHT-EA. Finally, two closely 15 eluting non-polar AEA metabolites are detected with an  $m/z$  of 386, consistent with the ethanolamides of HETEs (hydroxy-eicosatetraenoic acids that are minor products of arachidonic acid oxygenation by COX-2).

## 20 5.2 Example 2

Cellular COX-2 enzymatic activity can be determined by exposing cells to exogenous AEA and measuring the COX-2 specific products. For example, unactivated RAW264.7 cells (a murine macrophage cell line) express no detectable COX-2 and low levels of 25 COX-1, but IFN- $\gamma$  and LPS induce COX-2 expression (Wadleigh, D. J. et al, 2000, *J. Biol. Chem.* 275:6259-6266). The major arachidonic acid metabolite in these cells is PGD $_2$ , so RAW264.7 cells permit the simultaneous evaluation of AEA oxygenation to PGH $_2$ -EA as well as

endoperoxide metabolism by PGD synthase (Landino, L. M., Crews, B. C., Timmons, M. D. *et al.*, 1996, *Proc. Natl. Acad. Sci. USA* 93:15069-15074). PGH<sub>2</sub>-EA metabolites are not detectable in the medium from unactivated RAW264.7 cells following addition of AEA (20 μM) (Kozak *et al.* 2000, *supra*). However, treating cells with LPS (1 μg/mL) and IFN-γ (10 units/mL) results in the synthesis and release of copious amounts of PGD<sub>2</sub>-EA, following the addition of AEA (20 μM). A LC-mass spectrogram showing the PGD<sub>2</sub>-EA production and extracellular release by activated RAW264.7 macrophages can be generated. Products are eluted with a 15-minute gradient of 20% to 100% acetonitrile in H<sub>2</sub>O (0.001% sodium acetate). Chromatograms are normalized to total ion current of AEA-treated activated macrophages. PGD<sub>2</sub>-EA biosynthesis is inhibited both by indomethacin (3 μM) and the highly selective COX-2 inhibitor, 2-[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indol-3-yl]-N-phenethylacetamide (indomethacin phenethylamide, 3 μM). No significant endogenous PGD<sub>2</sub>-EA biosynthesis is detected in the absence of exogenous substrate under these conditions.

20

### 5.3 Example 3

A sample may be urine or may be collected from or processed from urine. PGH<sub>2</sub>-EA metabolites can be measured in urine by isotope dilution mass spectrometry. A fixed volume of urine is treated with an appropriate internal standard (*e.g.*, tetradeuterated PGE<sub>2</sub>-EA) and then loaded on reversed-phase extraction cartridges. The sample is then washed (*e.g.*, 1 ml pH 4.0 20 mM sodium acetate) and PGH<sub>2</sub>-EA metabolites including the

added internal standard are eluted with organic solvent (e.g., two 1 ml aliquots of MeCN). The solvent is evaporated and the residue is analyzed by LC/MS. A typical procedure wherein synthetically generated PGE<sub>2</sub>-EA and *d4*-PGD<sub>2</sub>-EA is added to fresh human urine is depicted in FIG. 10 and demonstrates that this technique provides precise and linear quantitation over the tested range (0-40 ng PGE<sub>2</sub>-EA per ml urine).

#### 5.4 Example 4

COX-2 expression and activity are generally linked with the inflammatory process, which accompanies a plethora of pathologies including, but not limited to, arthritis/arthropathy, infectious disease, neurodegenerative disease, neoplasia and autoimmune disease. The quantification of prostaglandin PGH<sub>2</sub>-EA metabolites from biological fluids obtained non-invasively (e.g., blood, urine) will allow for the assessment of COX-2 activity *in vivo*, reflecting both inflammation and disease severity. In addition, serial testing will allow for the tracking of the natural course of the disease as well as the efficacy of anti-inflammatory therapy. A model for this application would be the ubiquitous use of C-reactive protein (CRP) in the diagnosis and assessment of diseases associated with inflammation. The benefits of using PGH<sub>2</sub>-EA metabolites in this context instead of more traditional diagnostic markers, such as CRP, involve the highly specific nature of PGH<sub>2</sub>-EA metabolite production. PGH<sub>2</sub>-EA metabolites are elevated only when COX-2 activity is elevated whereas CRP elevations, for example, are very non-specific.

**Scenario:** Elderly woman seeks medical attention for recent onset of joint pain in hands. Urine is collected and PGH<sub>2</sub>-EA metabolite quantification is conducted. PGH<sub>2</sub>-EA metabolites are elevated supporting a diagnosis of rheumatoid arthritis. COX-2 inhibitor therapy is initiated (e.g., celecoxib). After one week, symptoms are only mildly relieved and another urine sample reveals PGH<sub>2</sub>-EA metabolite levels are still elevated, indicating that the inflammatory process is still active. Following dosage increase, symptoms are relieved and urinary PGH<sub>2</sub>-EA metabolites are normalized.

#### 5.5 Example 5

COX-2 expression and activity are linked to several solid tumors, most notably colorectal adenocarcinoma. The quantification of PGH<sub>2</sub>-EA metabolites from biological fluids, described herein, provides a noninvasive “early-warning” for clinically undetectable neoplasia. In addition, serial testing following diagnosis will allow for the tracking of the natural course of the cancer as well as the efficacy of antineoplastic therapy. A model for this application would be the use of prostate specific antigen (PSA) in the diagnosis and assessment of prostate adenocarcinomas. The benefits of PGH<sub>2</sub>-EA metabolite quantification in this context include (a) relative noninvasiveness, (b) sensitivity (most cancers are advanced once symptomatic) and (c) cost (simple lab diagnostic technique versus colonoscopy for example).

**Scenario:** Elderly asymptomatic man receives annual physical examination. Urine and blood are collected and PGH<sub>2</sub>-EA metabolite quantification is conducted. PGH<sub>2</sub>-EA metabolites are

elevated in both the urine and plasma, prompting a more detailed search for possible neoplasia. Colonoscopy reveals a single tumor in the descending colon which, following biopsy, proves malignant. Standard chemotherapy is initiated. Following treatment, urinary and plasma PGH<sub>2</sub>-EA metabolite levels have normalized. Annual colonoscopies for 3 years reveal no recurrence. After 3 1/2 years, patient visits physician for an unrelated reason and urinary PGH<sub>2</sub>-EA metabolites are quantified. PGH<sub>2</sub>-EA metabolite levels are markedly elevated, indicating the recurrence of carcinoma. The physician recommends colonoscopy, which reveals the presence of carcinoma. Aggressive chemotherapy is initiated and urinary PGH<sub>2</sub>-EA metabolite levels are monitored.

#### 5.6 Example 6

The measurement of PGH<sub>2</sub>-EA metabolites from *in vitro* samples (e.g., cell culture, biopsy samples) allows for the direct quantification of COX-2 activity. Current methods which quantify cyclooxygenase activity do not directly distinguish between COX-1 and COX-2. Methods which quantify COX-2 expression (e.g., Western blotting) do not assess activity which may or may not correlate with expression levels.

**Scenario:** Researchers investigating new NSAIDS expose cultured cells expressing COX-2 to various concentrations of test compounds for predetermined periods of time. At the conclusion of the exposures, conditioned medium is collected from each culture. The samples of conditioned medium are assayed for the presence of PGH<sub>2</sub>-EA metabolite. The researchers find that most of the test compounds have no significant affect on the production of PGH<sub>2</sub>-EA

metabolite by the cultured cells. However, one compound (“compound X”) dramatically reduces the amount PGH<sub>2</sub>-EA metabolite produced by the cultured cells. Therefore, “compound X” inhibits COX-2. The researchers focus their efforts on “compound X,” which may become a new COX-2 specific treatment of inflammatory diseases or cancer. In further experiments, the researchers determine that compound X does not inhibit COX-1. Compound X is, therefore, identified as a COX-2 specific inhibitor.

#### 10 5.7 Example 7

In certain embodiments, the present invention can be employed to identify a previously undescribed small molecule modulator of COX-2 activity. This can be done with the following method. Briefly, RAW264.7 cells at 30-40% confluence are activated with lipopolysaccharide (LPS, 20 ng/mL) and treated with a series of concentrations of a test compound. Cells are incubated for 12 h at 37°C and then medium is removed and replaced with buffered saline. Cells are then treated with 50 μM AEA and incubated an additional 30 min at 37°C. Following incubation, buffered saline is collected and treated with tetradeutereated PGH<sub>2</sub>-EA metabolite standard. Buffered saline is extracted twice with equal volumes of 2:1 CHCl<sub>3</sub>:MeOH. The combined organic extract is evaporated under a stream of argon. The resultant residue is redissolved in 1:1 H<sub>2</sub>O:MeCN and analyzed by liquid chromatography-mass spectrometry (LC-MS) with selected ion monitoring of PGH<sub>2</sub>-EA metabolite peaks at  $m/z = 418$ . Quantitation of COX-2 activity is accomplished by comparing the area of the PGH<sub>2</sub>-EA metabolite peak to that of the internal standard.



REFERENCES

All references, U.S. Patents, non U.S. Patents, journal articles, and newspaper articles referred to herein are hereby made  
5 part of the specification of the present patent and incorporated herein in their entirety by reference. This includes, but is not limited to the following references:

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This invention thus being described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present  
25 invention, and all such modifications as would be obvious to one of ordinary skill in the art are intended to be included within the scope

of the claims following the detailed description of the invention. The present invention is not bound by theory or mechanism. Thus, although there have been described particular embodiments of the present invention of a new and useful “Method for *in vitro* and *in vivo* 5 determination of COX-2 activity”, it is not intended that such references be construed as limitations upon the scope of this invention except as set forth in the following claims.

CLAIMS

What is claimed is:

1. A method of selectively detecting COX-2 activity in a sample, comprising:
  - 5 a. adding a COX-2 selective substrate to the sample; and
  - b. detecting a metabolite of the COX-2 selective substrate, thereby indicating the COX-2 activity.
2. A method of measuring COX-1 activity in a sample, comprising:
  - 10 a. adding a nonselective COX substrate and a COX-2 selective substrate to the sample;
  - b. allowing a period of time to pass;
  - c. measuring a first amount of a metabolite of the COX-1 substrate in the sample and a second amount of a metabolite of the COX-2 selective substrate metabolite;
  - 15 d. comparing the first amount and the second amount.
3. A method of detecting an activity of a COX-2 enzyme in a sample, comprising: detecting a PGH<sub>2</sub>-EA metabolite in the sample, wherein the presence of the PGH<sub>2</sub>-EA metabolite in the sample indicates the activity of the COX-2 enzyme.
  - 20
4. A method of measuring an activity of a COX-2 enzyme in a sample, comprising:
  - a. quantifying an amount of a PGH<sub>2</sub>-EA metabolite in the sample; and
  - 25 b. relating the amount of the PGH<sub>2</sub>-EA metabolite to the activity of the COX-2 enzyme.
5. A method of distinguishing a COX-2 activity from a COX-1 activity in a subject, comprising:

- a. administering a COX-1 substrate and COX-2 selective substrate to the subject;
  - b. allowing a period of time to pass;
  - c. obtaining a sample from the subject;
  - 5 d. determining a first amount of a metabolite of the COX-1 substrate and a second amount of a metabolite of the COX-2 selective substrate; and
  - e. comparing the first amount to the second amount.
6. A method of detecting an activity of a COX-2 enzyme in a  
10 subject, comprising:
    - a. obtaining a sample of the subject; and
    - b. detecting a  $\text{PGH}_2\text{-EA}$  metabolite in the sample, wherein the presence of the  $\text{PGH}_2\text{-EA}$  metabolite in the sample indicates the activity of the COX-2 enzyme in the subject.
  - 15 7. The method of Claim 6, wherein the  $\text{PGH}_2\text{-EA}$  metabolite is selected from the group consisting of  $\text{PGB}_2\text{-EA}$ ,  $\text{PGD}_2\text{-EA}$ ,  $\text{PGE}_2\text{-EA}$ ,  $\text{PGF}_{2\alpha}\text{-EA}$ ,  $\text{TxB}_2\text{-EA}$ , 6-keto- $\text{PGF}_{1\alpha}\text{-EA}$ , 15-keto- $\text{PGE}_2\text{-EA}$ , 13,14-dihydro-15-keto- $\text{PGE}_2\text{-EA}$ ,  $\text{PGG}_2\text{-EA}$ ,  $\text{PGH}_2\text{-EA}$ ,  $\text{PGA}_2\text{-EA}$ ,  $\text{PGJ}_2\text{-EA}$ ,  $\text{PGJ}_2\text{-EA}$  derivatives, bicyclo- $\text{PGE}_2\text{-EA}$ , 6-keto- $\text{PGF}_{1\alpha}\text{-EA}$ ,  $\text{TxA}_2\text{-EA}$  and  $\text{PGI}_2\text{-EA}$ .  
20
  8. The method of Claim 6, wherein the subject is a mammal.
  9. The method of Claim 6, wherein the sample is urine.
  10. The method of Claim 6, wherein the sample is selected from a group consisting of: blood, plasma, cerebrospinal fluid, saliva,  
25 sputum, bile, joint fluid, biopsy, and conditioned media from a cell culture.

11. The method of Claim 6, wherein the detecting step further comprises generating a mass chromatogram of the PGH<sub>2</sub>-EA metabolites.
12. The method of Claim 6, wherein the detecting step includes an  
5 immunoassay step.
13. A method of measuring an activity of a COX-2 enzyme in a subject, comprising:
  - a. obtaining a sample of the subject;
  - b. measuring an amount of a PGH<sub>2</sub>-EA metabolite in the  
10 sample; and
  - c. relating the amount measured to the activity of the COX-2 enzyme.
14. The method of Claim 13, wherein the relating step further  
15 comprises comparing the amount measured to a standard value.
15. The method of Claim 13, wherein the relating step further comprises generating a standard curve.
16. The method of Claim 13, wherein the PGH<sub>2</sub>-EA metabolite is  
20 selected from the group consisting of PGB<sub>2</sub>-EA, PGD<sub>2</sub>-EA, PGE<sub>2</sub>-EA, PGF<sub>2α</sub>-EA, TxB<sub>2</sub>-EA, 6-keto-PGF<sub>1α</sub>-EA, 15-keto-PGE<sub>2</sub>-EA, 13,14-dihydro-15-keto-PGE<sub>2</sub>-EA, PGG<sub>2</sub>-EA, PGH<sub>2</sub>-EA, PGA<sub>2</sub>-EA, PGJ<sub>2</sub>-EA, PGJ<sub>2</sub>-EA derivatives, bicyclo-PGE<sub>2</sub>-EA, 6-keto-PGF<sub>1α</sub>-EA, TxA<sub>2</sub>-EA and PGI<sub>2</sub>-EA.
17. The method of claim 13, wherein the subject is a mammal.
- 25 18. The method of claim 13, wherein the sample is urine.
19. The method of claim 13, wherein the sample is selected from a group consisting of: blood, plasma, cerebrospinal fluid, saliva,

sputum, bile, joint fluid, biopsy, and conditioned media from a cell culture.

20. The method of Claim 13, wherein the detecting step further comprises generating a mass chromatogram of the PGH<sub>2</sub>-EA metabolites.
- 5
21. The method of Claim 13, wherein the detecting step includes an immunoassay step.
22. A method of screening for a tumor in a subject in need thereof, comprising:
- 10
- a. obtaining a sample of the subject; and
  - b. detecting a PGH<sub>2</sub>-EA metabolite in the sample; wherein the presence of the PGH<sub>2</sub>-EA metabolite is indicative of the tumor in the subject.
23. A method of screening for a tumor in a subject in need thereof, comprising:
- 15
- a. obtaining a sample of the subject;
  - b. measuring an amount of a PGH<sub>2</sub>-EA metabolite in the sample; and
  - c. relating the amount measured to an existence of the
- 20
- tumor.
24. A method of monitoring an anticancer treatment, comprising:
- a. obtaining a first sample of a patient;
  - b. measuring a first amount of PGH<sub>2</sub>-EA metabolite in the first sample;
- 25
- c. obtaining a second sample from the patient after the patient undergoes anticancer therapy;
  - d. measuring a second amount of the PGH<sub>2</sub>-EA metabolite in the second sample; and

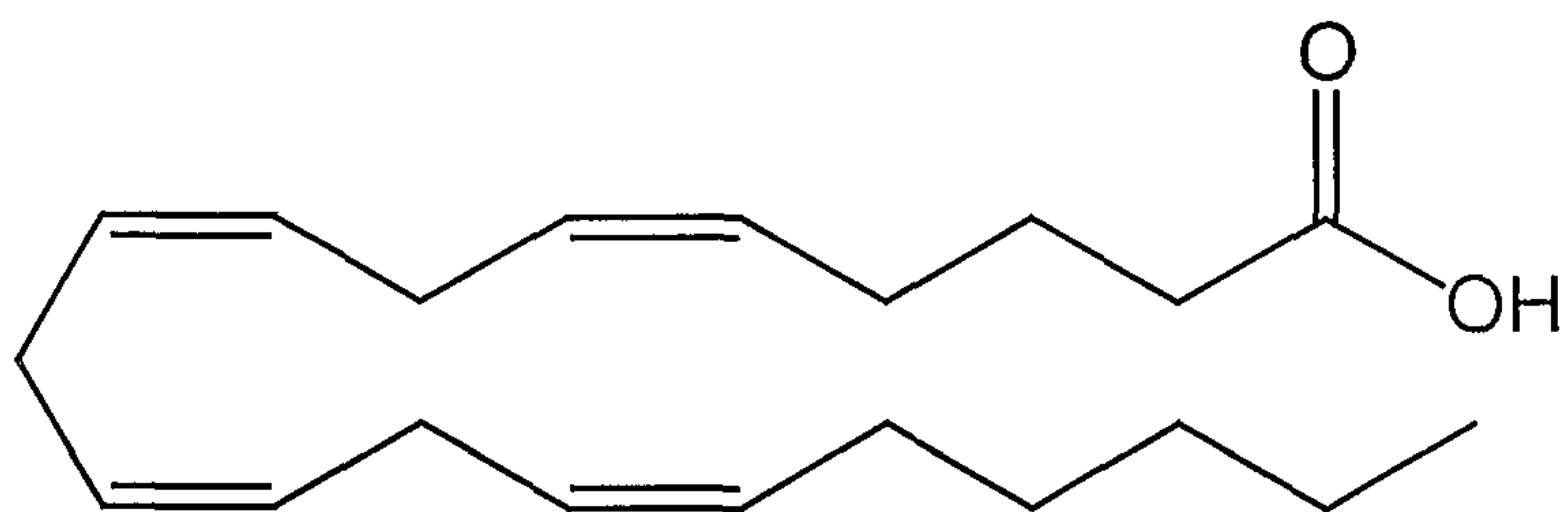


- e. determining a change in the second amount relative to the first amount, wherein the change determined is indicative of the effectiveness of the anticancer treatment.
25. A method of detecting an inflammation in a subject in need thereof, comprising:
- 5
- a. obtaining a sample of the subject; and
  - b. detecting an amount of a  $\text{PGH}_2$ -EA metabolite in the sample, wherein an inflammation is indicated when the amount detected equals or exceeds a threshold value.
- 10 26. A method of measuring an inflammation in a subject in need thereof, comprising:
- a. obtaining a sample of the subject; and
  - b. detecting an amount of a  $\text{PGH}_2$ -EA metabolite in the sample, wherein an inflammation is indicated when the amount measured equals or exceeds a threshold value.
- 15
27. A method of monitoring an anti-inflammation therapy in a subject in need thereof, comprising:
- a. obtaining a first sample from the subject;
  - b. measuring a first amount of a  $\text{PGH}_2$ -EA metabolite in the first sample;
  - c. obtaining a second sample from the patient after the anti-inflammation therapy;
  - d. measuring a second amount of the COX-2 specific metabolite in the second sample; and
  - e. determining a change in the second amount relative to the first amount, wherein the change determined is indicative of the effectiveness of the anti-inflammation therapy.
- 20
- 25

28. A composition comprising: a label for detecting a PGH<sub>2</sub>-EA metabolite.
29. The composition of Claim 28, further comprising an isolated PGH<sub>2</sub>-EA metabolite including an isotopic label.
- 5 30. The method of Claim 28, wherein the PGH<sub>2</sub>-EA metabolite is selected from the group consisting of PGB<sub>2</sub>-EA, PGD<sub>2</sub>-EA, PGE<sub>2</sub>-EA, PGF<sub>2</sub>α-EA, TxB<sub>2</sub>-EA, 6-keto-PGF<sub>1</sub>α-EA, 15-keto-PGE<sub>2</sub>-EA, 13,14-dihydro-15-keto-PGE<sub>2</sub>-EA, PGG<sub>2</sub>-EA, PGH<sub>2</sub>-EA, PGA<sub>2</sub>-EA, PGJ<sub>2</sub>-EA, PGJ<sub>2</sub>-EA derivatives, bicyclo-PGE<sub>2</sub>-EA, 6-keto-PGF<sub>1</sub>α-EA, TxA<sub>2</sub>-EA and PGI<sub>2</sub>-EA.
- 10 31. The composition of Claim 28, further comprising an isolated PGH<sub>2</sub>-EA metabolite including a non-positron emitting isotopic label.
32. The composition of Claim 28, further comprising an isolated  
15 PGH<sub>2</sub>-EA metabolite including an isotopic label selected from the group consisting of <sup>2</sup>H, <sup>3</sup>H, <sup>13</sup>C, and <sup>14</sup>C.
33. The composition of Claim 28, further comprising an PGH<sub>2</sub>-EA metabolite including a fluorescent label.
34. A process for making an isolated PGH<sub>2</sub>-EA metabolite  
20 including a label comprising: reacting a COX-2 metabolite with a labeled ethanolamide.
35. The process of Claim 34, wherein the label is isotopic.
36. The process of Claim 34, wherein the label is nonpositron emitting.
- 25 37. The process of Claim 34, wherein the label is selected from the group consisting of <sup>2</sup>H, <sup>3</sup>H, <sup>13</sup>C, and <sup>14</sup>C.
38. The process of Claim 34, wherein the label is fluorescent.

39. A process for making an isolated  $\text{PGH}_2\text{-EA}$  metabolite including a label comprising: reacting a labeled COX-2 metabolite with ethanolamide.
40. The process of Claim 39, wherein the label is isotopic.
- 5 41. The process of Claim 39, wherein the label is nonpositron emitting.
42. The process of Claim 39, wherein the label is selected from the group consisting of  $^2\text{H}$ ,  $^3\text{H}$ ,  $^{13}\text{C}$ , and  $^{14}\text{C}$ .
43. The process of Claim 39, wherein the label is fluorescent.
- 10 44. An article of manufacture comprising, packaged together:
- a. a vessel containing an isolated antibody against a  $\text{PGH}_2\text{-EA}$  metabolite; and
  - b. a set of instructions delineating a process of measuring a COX-2 specific activity.
- 15 45. An article of manufacture comprising, packaged together:
- a. a vessel containing at least one labeled  $\text{PGH}_2\text{-EA}$  metabolite; and
  - b. a set of instructions delineating a process of measuring a COX-2 specific activity.
- 20 46. An antibody that binds specifically to a COX-2 metabolite ethanolamide.
47. An antibody that binds specifically to a  $\text{PGH}_2\text{-EA}$  metabolite.
48. An antibody that binds specifically to a  $\text{PGE}_2$  ethanolamide.
49. A process of making an antibody that binds specifically to
- 25  $\text{PGH}_2\text{-EA}$  metabolites from a prostaglandin with substituted cyclopentyl and amide moieties, comprising:
- a. protecting the cyclopentyl substituents and ethanolamide moiety of the prostaglandin to produce a protected PG-EA;

- b. chemically modifying the protected PG-EA with an appropriate conjugate to produce a protected, conjugated PG-EA;
  - c. deprotecting the conjugated PG-EA to generate an immunogen; and
  - d. purifying the immunogen.
50. A method of measuring an activity of a COX-2 enzyme in a subject, comprising:
- a. administering an amount of a AEA to the subject;
  - b. obtaining a sample of the subject;
  - c. measuring an amount of a PGH<sub>2</sub>-EA metabolites in the sample; and
  - d. relating the amount of the PGH<sub>2</sub>-EA metabolites to the activity of the COX-2 enzyme.
51. The method of Claim 50, wherein the AEA includes a label.
52. The method of Claim 50, further comprising the step of comparing the amount measured to a standard.
53. A composition comprising: a prostaglandin D<sub>2</sub>-ethanolamide and pharmaceutically acceptable salts thereof.
54. A composition comprising: a 6-keto-prostaglandin F<sub>1α</sub>-ethanolamide and pharmaceutically acceptable salts thereof.



Arachidonic Acid

FIG. 1

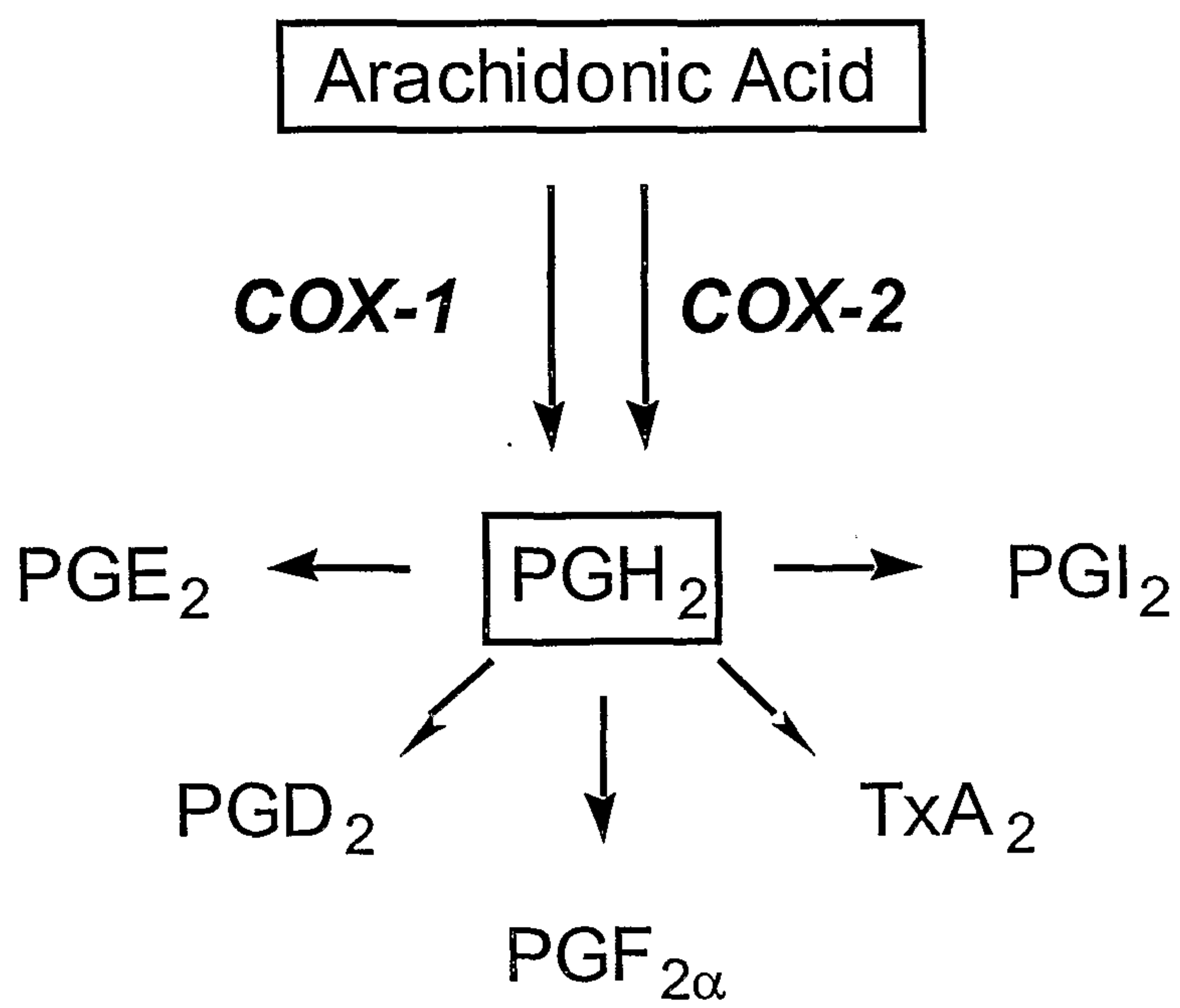
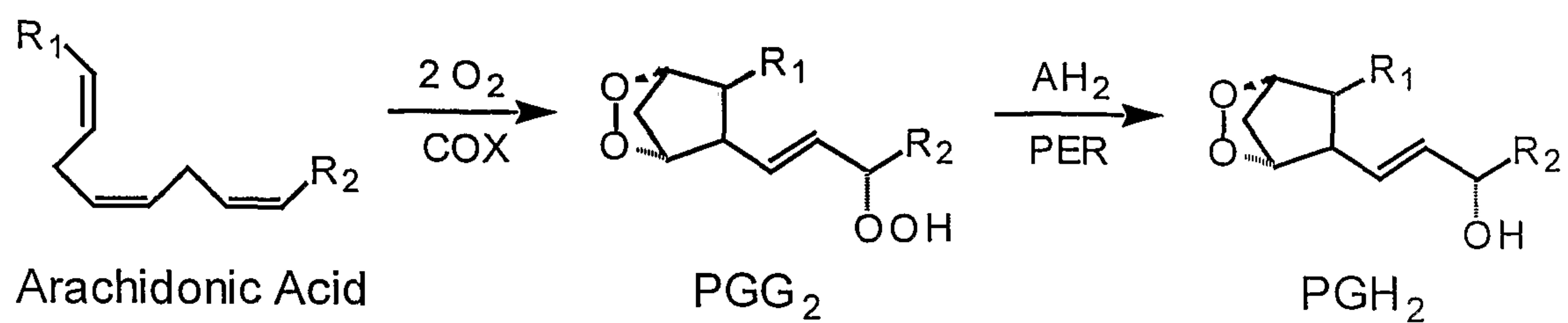


FIG. 2



R<sub>1</sub> = CH<sub>2</sub>CH=CH(CH<sub>2</sub>)<sub>3</sub>CO<sub>2</sub>H; R<sub>2</sub> = C<sub>5</sub>H<sub>11</sub>; AH<sub>2</sub> = reducing substrate

FIG. 3

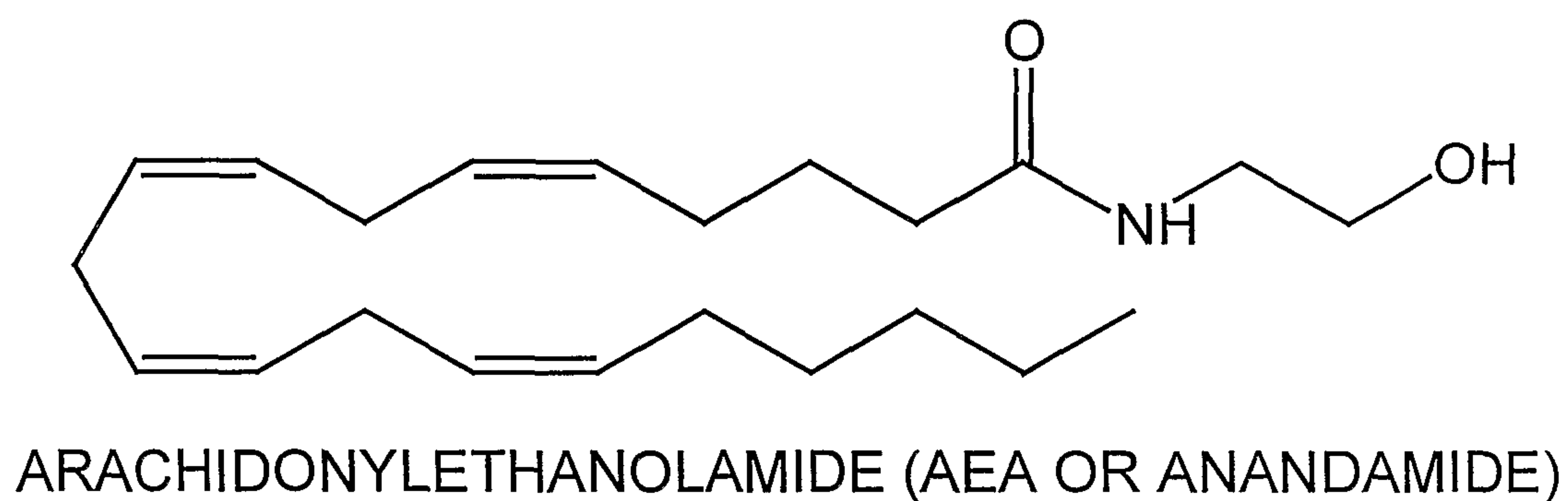


FIG. 4

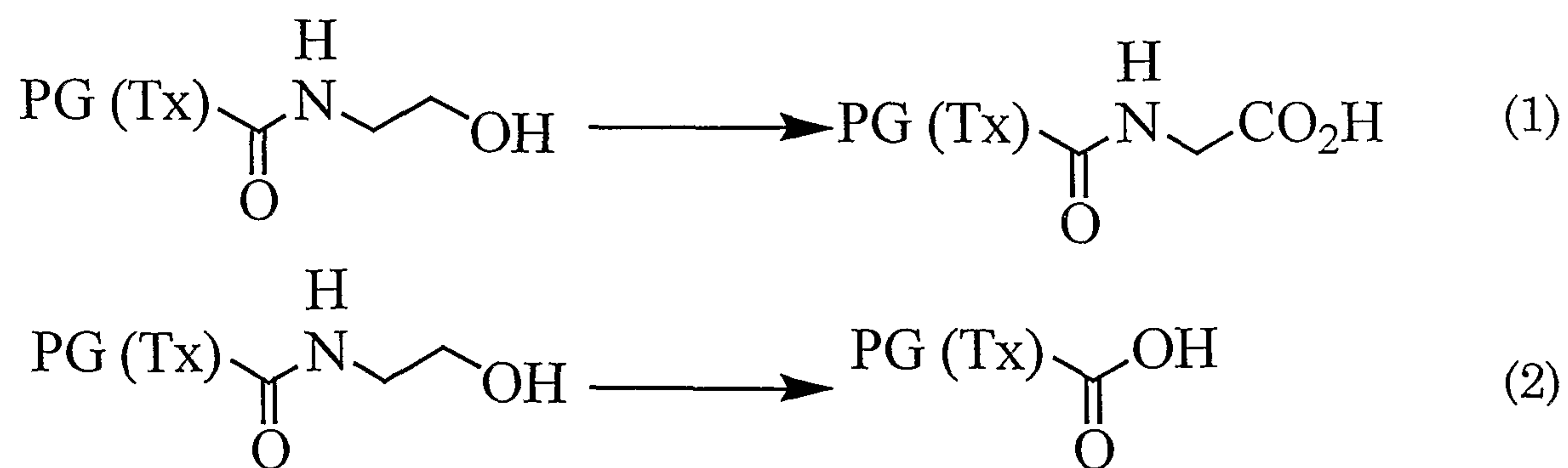


FIG. 5

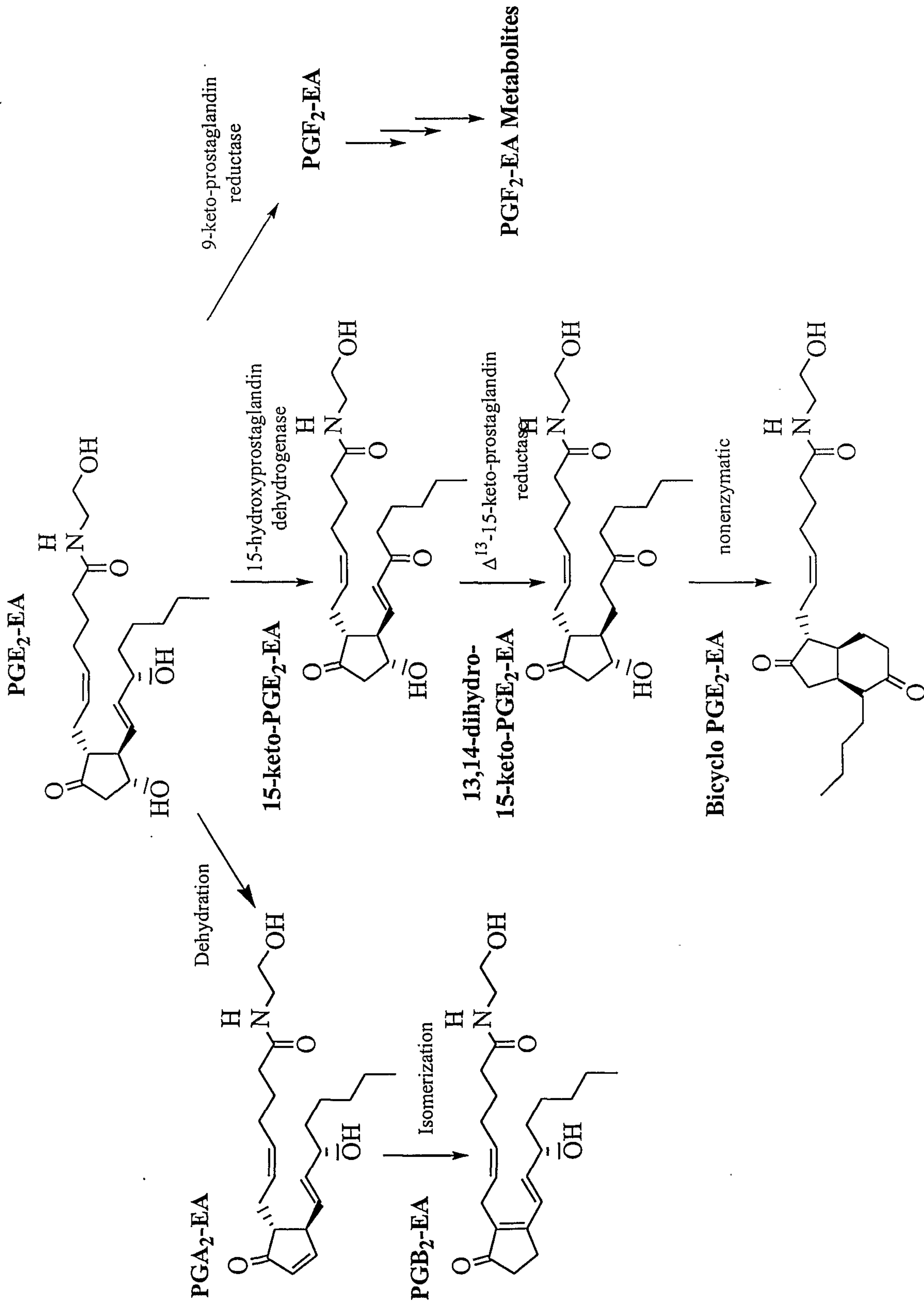


FIG. 6

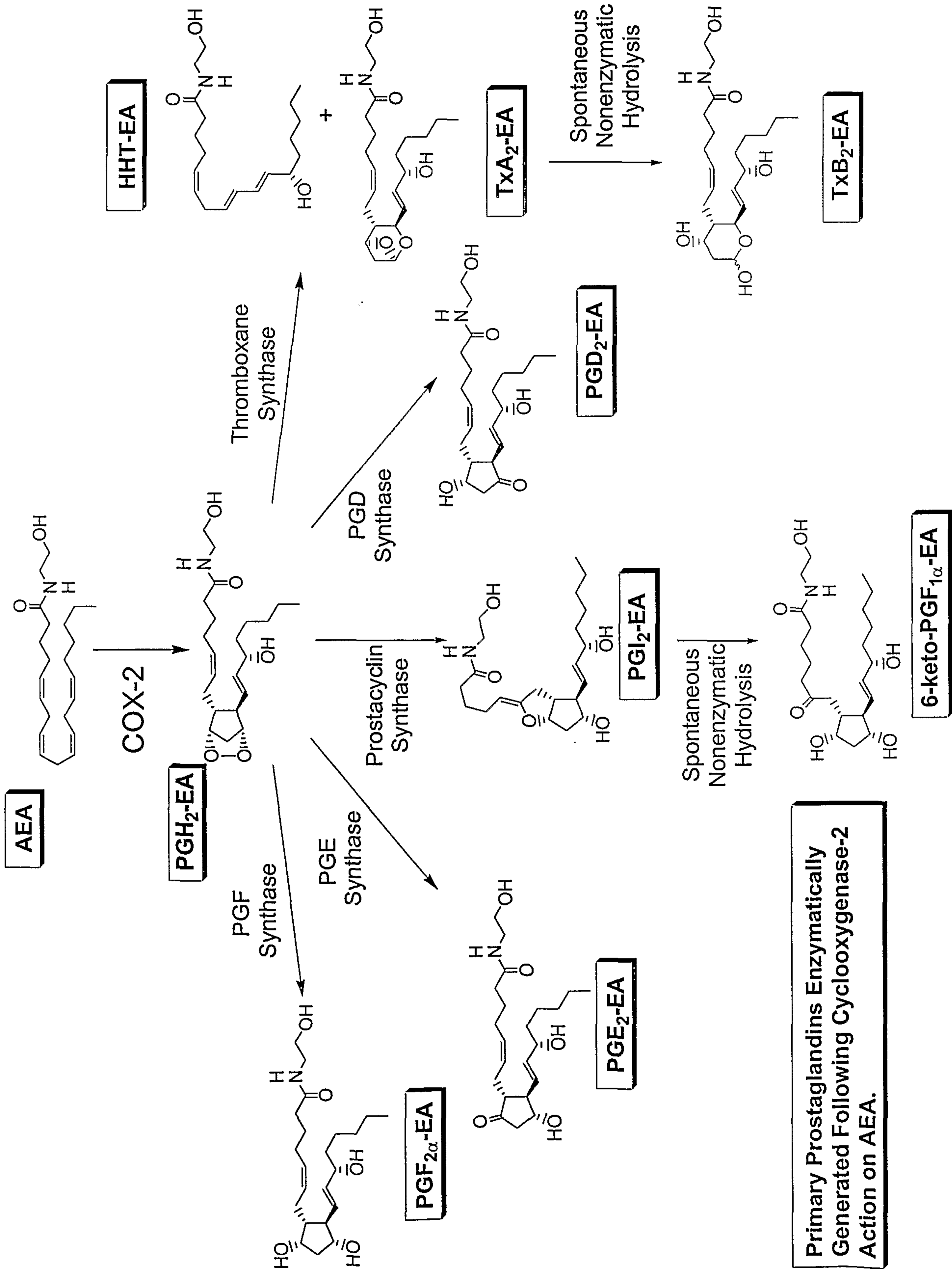


FIG. 7



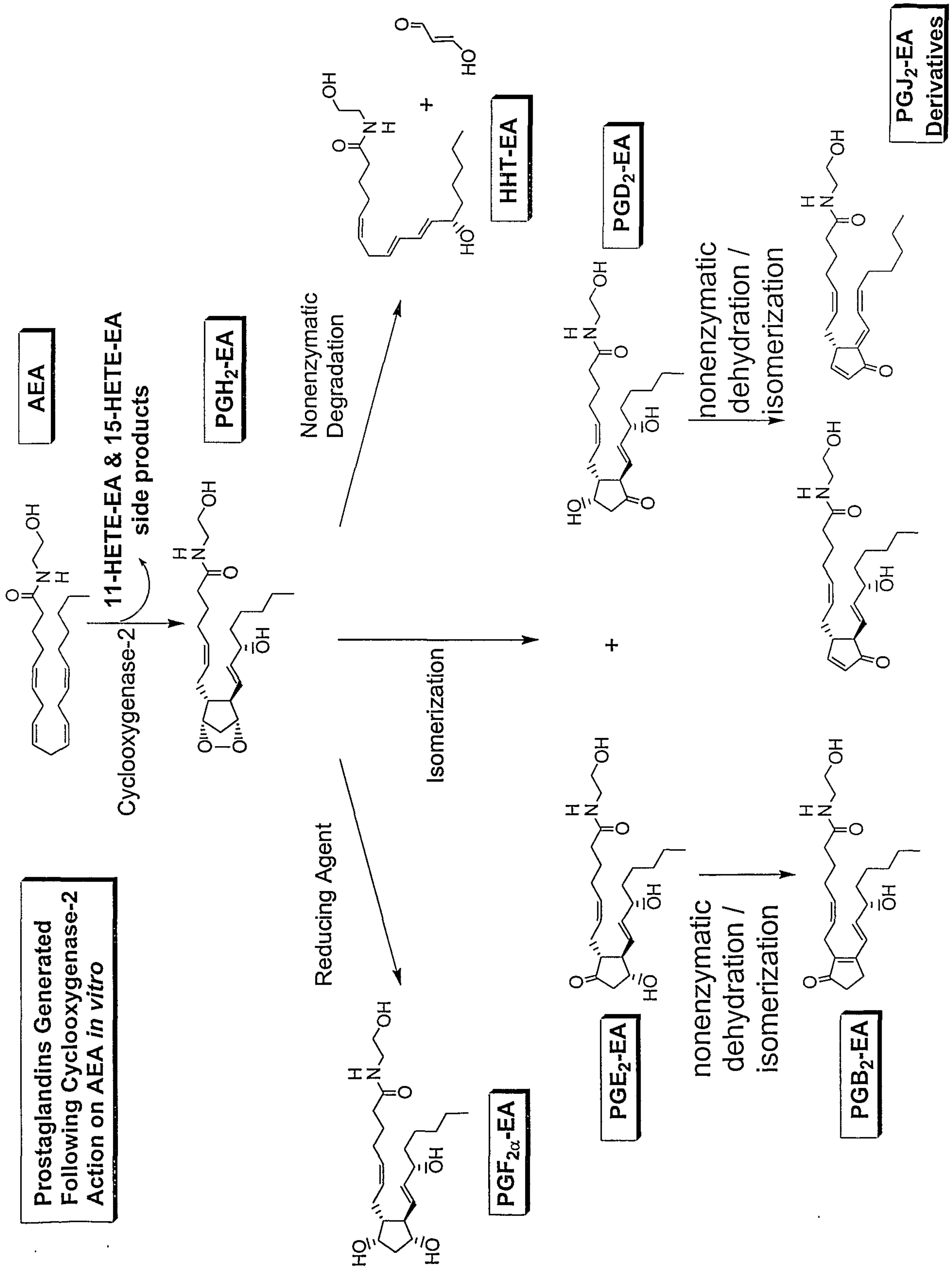


FIG. 8

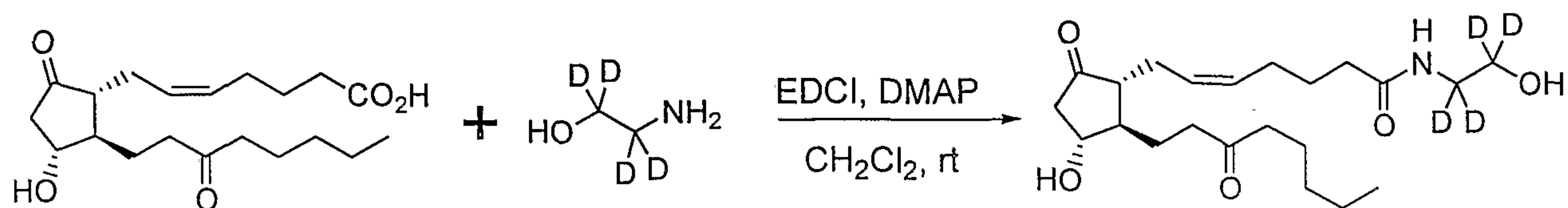


FIG. 9

### Urinary Recovery of Prostaglandin Ethanolamide

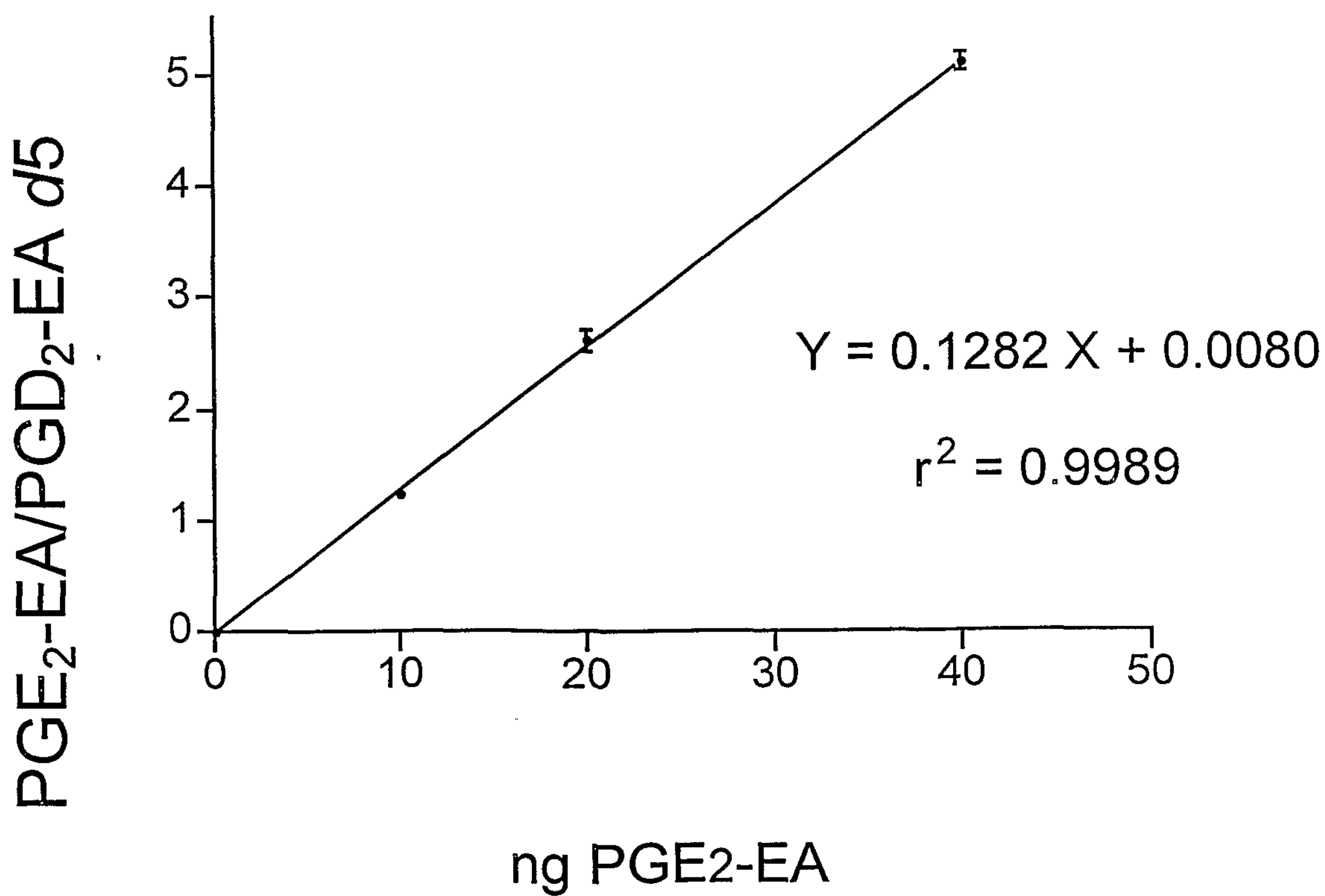


FIG. 10

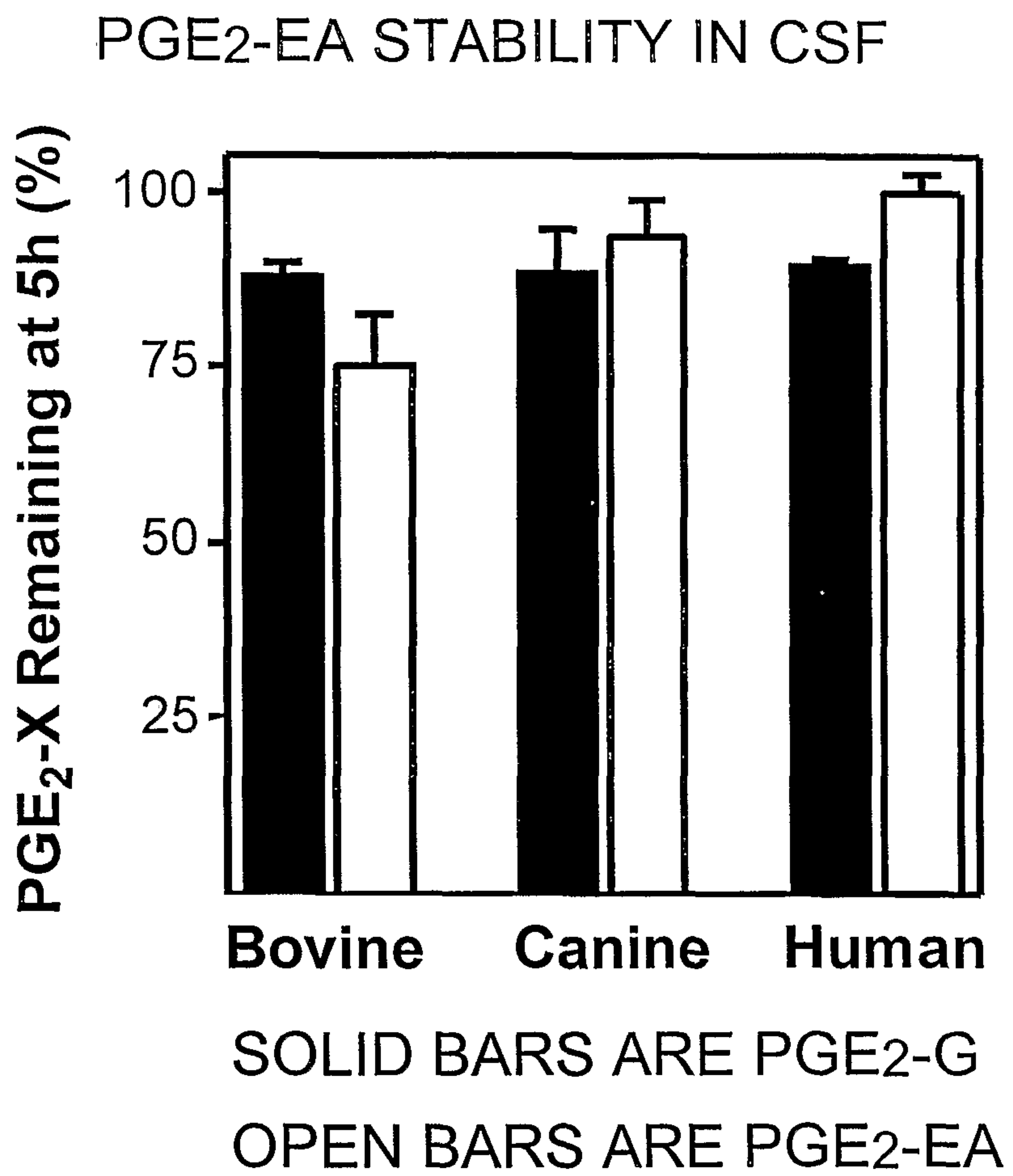


FIG. 11

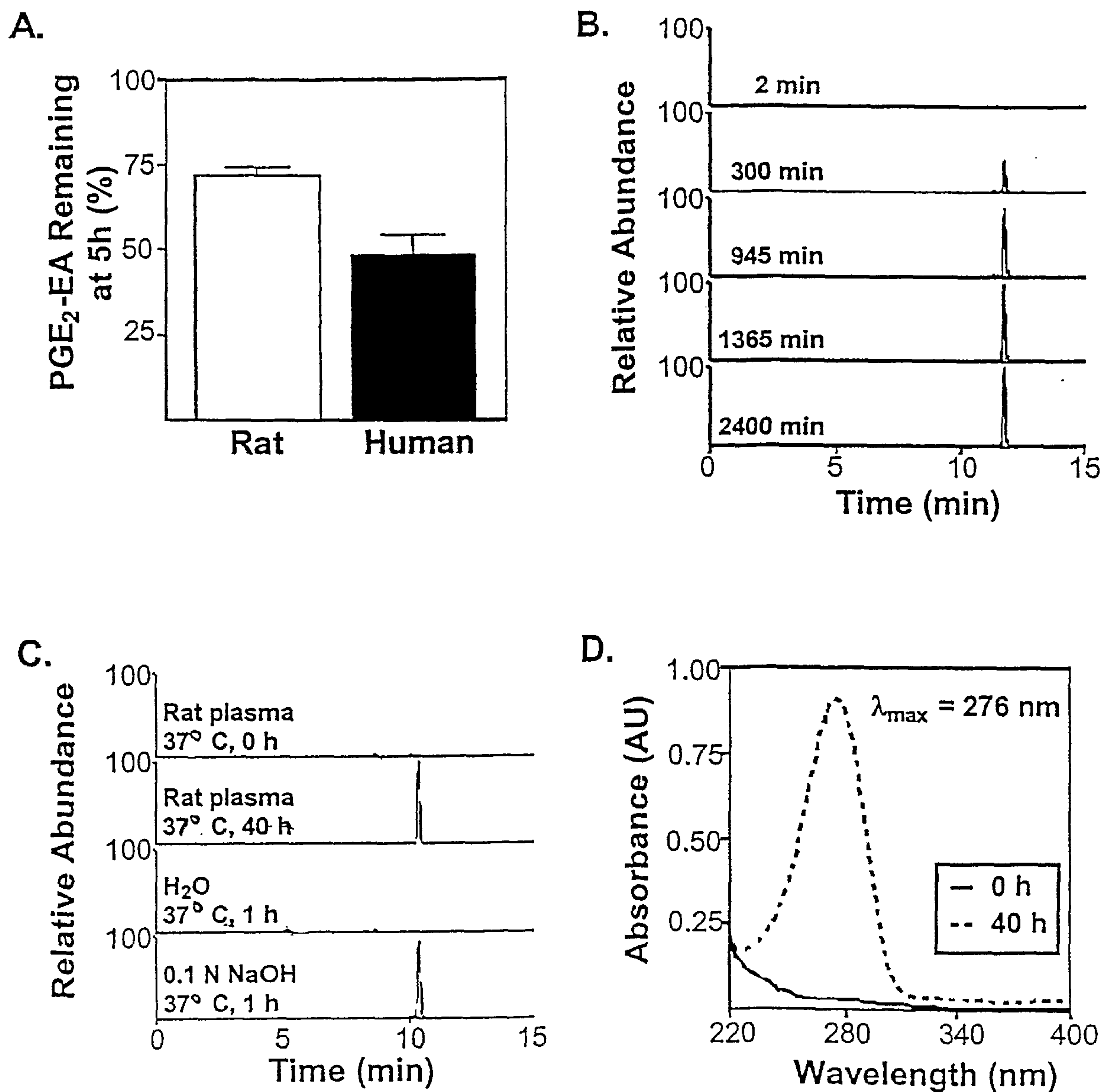
PGE<sub>2</sub>-EA STABILITY IN PLASMA

FIG. 12

### PGE<sub>2</sub>-EA STABILITY *IN VIVO*

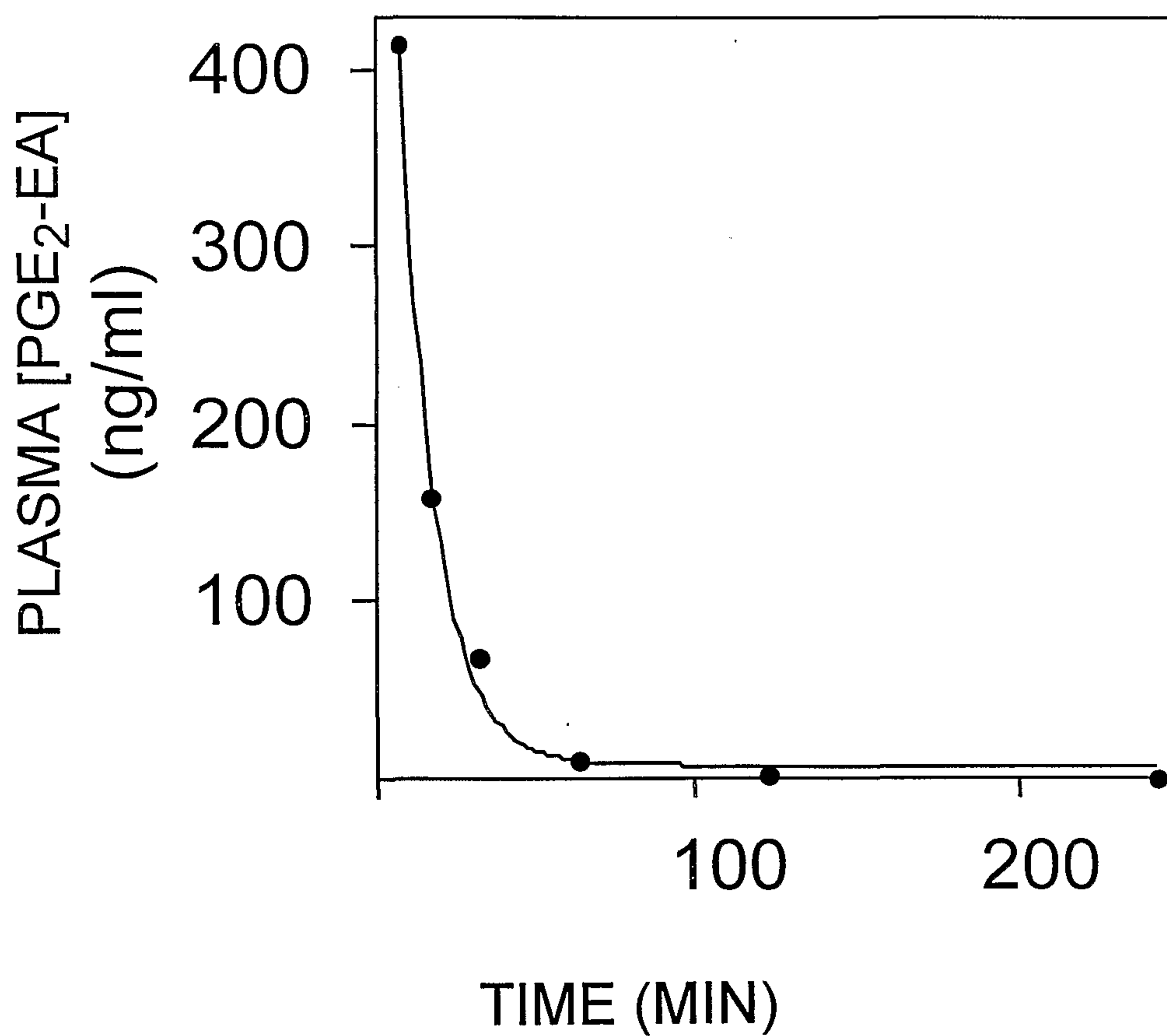


FIG. 13