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# The Enteropathy of Prostaglandin Deficiency

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## Abstract

**Purpose—**Small intestinal ulcers are frequent complications of therapy with non-steroidal anti-inflammatory drugs (NSAIDs). We present here a genetic deficiency of eicosanoid biosynthesis that illuminates the mechanism of NSAID-induced ulcers of the small intestine.

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**Methods—**Eicosanoids and metabolites were measured by isotope-dilution with mass spectrometry. cDNA was obtained by reverse transcription and sequenced following amplification with RT-PCR.

**Results—**We investigated the cause of chronic recurrent small intestinal ulcers, small bowel perforations, and gastrointestinal blood loss in a 45 year old male who was not taking any cyclooxygenase inhibitor. Prostaglandin metabolites in urine were significantly depressed. Serum thromboxane $B_2$ ($TxB_2$) production was 4.6% of normal controls ($p<0.006$) and serum 12-HETE was 1.3% of controls ($p<0.005$). Optical platelet aggregation with simultaneous monitoring of ATP release demonstrated absent granule secretion in response to ADP and a blunted aggregation response to ADP and collagen, but normal response to arachidonic acid (AA). $LTB_4$ biosynthesis by ionophore activated leukocytes was only 3% of controls and urinary $LTE_4$ was undetectable. These findings suggested deficient AA release from membrane phospholipids by cytosolic phospholipase $A_2$-α ($cPLA_2$-α) which regulates cyclooxygenase and lipoxygenase mediated eicosanoid production by catalyzing the release of their substrate, AA. Sequencing of $cPLA_2$-α cDNA demonstrated 2 heterozygous non-synonymous single base pair mutations: Ser111Pro (S111P) and Arg485His (R485H), as well as a known SNP: Lys651Arg (K651R).

**Conclusion—**Characterization of this $cPLA_2$-α deficiency provides support for the importance of prostaglandins in protecting small intestinal integrity, and indicates that loss of prostaglandin biosynthesis is sufficient to produce small intestinal ulcers.

## Introduction

Small intestinal ulcers are a well established consequence of the use of non-steroidal anti-inflammatory drugs (NSAIDS)[1–5]. The pharmacological property shared by all of the NSAIDs is inhibition of the cyclooxygenase enzymes that synthesize prostaglandins[6]. Considerable evidence indicates that inhibition of prostaglandin biosynthesis contributes to the small intestinal ulcers caused by NSAIDs. Other studies, however, have suggested that cyclooxygenase-independent effects of NSAIDS on the small intestine also are required for ulcer formation[7–8]. One postulated off-target mechanism has been topical injury to enterocytes by the NSAIDs during absorption of the drugs. The extent to which such topical cyclooxygenase-independent actions are required to produce small intestinal ulcers, however, has been unclear. To address whether inhibition of prostaglandin biosynthesis alone is sufficient to cause small intestinal ulcers, we present here the investigation of an inherited deficiency of prostaglandin biosynthesis that is associated with severe small intestinal ulcer disease with attendant diaphragm-like constrictions.

## Methods

### Study conduct

Informed consent was obtained from all participants after study approval by the Vanderbilt University Institutional Review Board. The patient was admitted to the Clinical Research Center at Vanderbilt University Medical Center (VUMC) for seven days, during which all medications were held. Consecutive 24 hour urine collections were obtained prior to phlebotomy, which was subsequently performed daily after an overnight fast. Blood specimens from family members were obtained remotely and shipped to our facility. Healthy male volunteers who reported no use of aspirin or NSAIDs in the preceding two weeks served as controls.

### Urinary metabolites of $PGE_2$, $PGD_2$, $PGI_2$, and $TxA_2$

Urinary metabolites of thromboxane $A_2$ (11-dehydrothromboxane $B_2$; 11-d$TxB_2$ ), prostacyclin (2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$; PGI-M), prostaglandin $D_2$ (9α,11β-

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dihydroxy-15-oxo-2,3,18,19- tetranorprost-5-ene-1,20-dioic acid; PGD-M), and prostaglandin $E_2$ (11α-hydroxy-9,15- dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid; PGE-M) were assayed using mass spectrometry as described previously[9–12] from separate 24 hour urine collections on different days.

### Urinary $LTE_4$

$LTE_4$ was analyzed by a previously published LC/MS/MS method[13] with minor modifications.

### Platelet-derived (Serum) Thromboxane $B_2$ ($TxB_2$) and 12-hydroxyeicosatetraenoic acid (12-HETE)

Blood from the patient on multiple days was drawn into a glass container and allowed to clot by incubating at 37°C for 45 minutes. The serum layer was collected after centrifugation and $TxB_2$ was assayed by GC/ECI/MS as described previously[14–15]. 12-HETE was assayed by LC/APCI/MS/MS using a silica HPLC column as described previously[16]

### $TxB_2$ production in washed platelets

Platelets were gel-filtered as described previously[17]. 100 μl of the platelet suspension (600,000 cells/μl) was incubated with [$^2H_8$] AA 2 μM at 37°C for 15 minutes. After stopping the reaction, $TxB_2$ was purified, derivatized and analyzed by GC/ECI/MS.

### Immunoblotting of $cPLA_2$ -α protein

Western blot analysis of protein was performed using rabbit anti-$cPLA_2$ (Cell-Signaling), goat anti-COX-1 (Santa Cruz Biotechnology) or goat anti-Actin (Santa Cruz) primary antibodies and horseradish peroxidase-coupled secondary antibodies (Santa Cruz). Band intensity was quantified using image analysis software (Quantity One v4.3.1, Bio Rad).

### Platelet aggregation

Citrated platelet rich plasma from peripheral venous blood from the patient and control volunteers was used to assess turbidimetric platelet aggregation induced by adenosine diphosphate (ADP), collagen, or AA. Platelet counts were adjusted to 2.6–2.8 × $10^8$ cells/ml prior to aggregation. Simultaneous adenosine triphosphate (ATP) secretion was quantified by monitoring luminescence produced by luciferase using an optical platelet aggregometer (Chronolog).

### Lymphoblast separation and culture

Lymphoblasts were separated from whole blood using Lymphosep™ lymphocyte separation medium (MP Biochemicals) and cultured after addition of cyclosporine as described by the manufacturer.

### $PLA_2$ activity

Platelets obtained on two different days from the patient and from two volunteers were frozen as PRP in citrate at −80°C. Thawed platelets or fresh cultured lymphoblasts were washed and resuspended in buffer (Hepes 10mM, Sucrose.34M Glycerol 10%, EDTA 1mM, pH 7.75) with a protease inhibitor cocktail. Whole cell lysate was obtained by sonication of cell suspension followed by brief centrifugation at 10,000 × *g*. Dithiothreitol 1 mM was added to prevent oxidative enzyme degradation. $PLA_2$ activity was measured from whole cell lysate (50 μg protein) with a radiolabeled substrate, l-3- phosphatidylcholine, 1-stearoyl-2-[1-14 C] arachidonyl (14 C-SAPC; 450 pmol, 55,000 dpm), under conditions described by Leslie and Gelb18 (NaCl 150mM, BSA 1mg/ml, Hepes 42mM, $CaCl_2$ 10mM, pH 7.5). Substrate hydrolysis was measured over a time period demonstrating linear kinetics. Reactions were

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stopped by addition of chloroform:methanol 2:1 and the extracted lipids were separated by thin layer chromatography. Residual substrate and hydrolyzed arachidonic acid were quantified by analyzing radioactivity using a Bioscan AR-200 imaging scanner.

### cPLA$_2$ -α cDNA sequencing

Oligonucleotide primer sequences were those previously published[19]. cDNA was obtained by reverse transcription (Invitrogen Superscript III and Elongase Kits) using total RNA isolated with a whole blood RNA collection system (QIAGEN PAXgene) as a template and *oligo dT* as a primer. Following RT-PCR amplification using *Primer 1* and *Primer 2* oligonucleotide primers, DNA sequencing was done using *Primers 1–14* with Applied Biosystems Big Dye v3.1. All of the variants detected in RT-PCR products were confirmed by direct sequencing of the corresponding segments of genomic DNA.

### Statistics

Normally distributed continuous variables were compared using t-test with Welch's correction applied when variances were different. Mann-Whitney U test was used for comparison of continuous variables without normal distribution. $PLA_2$ activity was compared using two-way ANOVA. All analyses were performed using Graphpad Prism 4.0 software for MS Windows. Means are reported ± standard deviation unless otherwise specified.

## Results

### Clinical findings

A 45 year old white American male of Italian descent presented with a life-long history of occult gastrointestinal blood loss, chronic anemia, iron deficiency, and frequent bouts of abdominal pain as a child and young adult. Repeated episodes of acute gross gastrointestinal bleeding late in his fourth decade and multiple episodes of small bowel perforation required five surgical interventions between 38–45 years of age. Surgical exploration of the small intestine and intra-operative endoscopy revealed multiple recurrent small intestinal ulcerations. Histologic specimens of the ileum and jejunum revealed multiple small, well-demarcated ulcers with minimal surrounding inflammation. Stenotic ileal web formations were also described proximal to several ulcers. Misoprostol (800 mcg daily), an oral prostaglandin $E_1$ analog, had been initiated with subsequent resolution of anemia for a period of 27 months until GI bleeding recurred again.

Colonoscopy and esophagogastroduodenoscopy had been performed on multiple occasions and always had been normal. Mesenteric arteriograms, gastrin secretion and laboratory and histologic evaluation for celiac sprue and inflammatory bowel disease were all normal. He denied having had diarrhea, nausea, vomiting or constipation. He had no history of bruising or non-gastrointestinal bleeding symptoms or severe or recurrent infections.

A clear cell renal carcinoma (Furman Grade II) was resected at age 40. A brief period of mild wheezing in early childhood was reported without subsequent respiratory complaints. A shellfish allergy causing urticaria was reported. Medications included iron sulfate and a multivitamin in addition to misoprostol. Use of nonsteroidal anti-inflammatory and corticosteroid medications was specifically denied.

The patient's father had died with multiple myeloma, and varying malignancies were reported in second and third degree relatives. No renal malignancy was reported in family members. There was no family history of ulcers.

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Physical examination revealed a well-developed male. Blood pressure was normal. A 2/6 systolic murmur was heard at the left sternal border. His abdomen was notable for multiple well-healed surgical scars. Physical exam was otherwise unremarkable.

An electrocardiogram and an echocardiogram were normal. Ultrasonography demonstrated normal kidneys with evidence of prior partial resection. Blood cell counts, electrolytes, renal and liver function values were normal.

### Urinary metabolites of $PGE_2$, $PGD_2$, $PGI_2$, and $TxA_2$

Quantification of the patient's urinary prostanoid metabolites demonstrated reduced levels compared with reference values (Table 1). The patient's mother and sister did not have reduced urinary prostanoid metabolite levels.

### Platelet-derived (Serum) $TxB_2$ and 12-HETE

The finding that 11d-$TxB_2$ excretion was reduced by 84.1% led to the hypothesis that $TxA_2$ biosynthesis by the platelet was impaired. This was assessed by measurement of $TxB_2$ released into the serum during blood clotting, which is derived almost entirely from platelets[20]. The patient's mean serum $TxB_2$ was reduced by 95.9% (Table 2). This could reflect pharmacologic inhibition of platelet COX-1, COX-1 deficiency or lack of the COX-1 substrate, AA (Figure 1). A genetic deficiency of thromboxane synthase seemed unlikely due to the depressed biosynthesis of other prostanoids. To distinguish between these possibilities, the product of the platelet 12-lipoxygenase, 12-HETE, was measured in serum; 12-HETE should be unchanged or increased with pharmacologic or genetic impairment of COX-1 catalytic activity. The level of 12-HETE in the patient's serum was decreased by 97.8% (Table 2), a finding consistent with an almost complete absence of the release of AA during platelet activation. The patient's mother and sister exhibited intermediate values for platelet-derived $TxB_2$ and 12-HETE that were below or at the lower end of the normal range.

### Urinary Leukotriene $E_4$ ($LTE_4$)

$LTE_4$ was below the limit of detection of 1 pg/ml in the patient's urine whereas the range of normal values was 19–60 pg/ml.

### Whole Blood Leukotriene $B_4$ ($LTB_4$)

$LTB_4$ produced by whole blood activated by calcium ionophore from the patient measured 7.2 ± 3.3 ng/ml whereas whole blood from healthy volunteers produced 243.7 ± 22.6 ng/ml $LTB_4$ (Table 2).

### $TxA_2$ Biosynthesis

COX-1 activity was assessed by measuring production of thromboxane $A_2$ by washed platelets after incubation with [$^2H_8$] AA. Both deuterated and non-deuterated $TxB_2$ were quantified, reflecting conversion by COX-1 of exogenous and endogenous substrate, respectively. [$^2H_8$] $TxB_2$ levels were similar between the patient and controls, reflecting normal COX-1 activity in the patient's platelets. Platelet activation by exogenous agonists, including arachidonic acid, induces release of endogenous arachidonic acid from cellular storage pools. Notably, non-deuterated $TxB_2$ levels from the patient's platelets were much lower than controls (0.04 vs. 0.51 ng/$10^3$ platelets; n=1), indicating an impaired release of endogenous AA.

### Platelet aggregation

Platelet function was assessed by monitoring platelet aggregation and dense granule secretion in response to stimulation by ADP or collagen. ADP 5μM and collagen 2 μg/ml were the lowest doses of these reagents inducing granule secretion in all controls. ADP 5 μM induced a

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diminished aggregation of the patient's platelets compared with controls (n=6), mean 73.0 ± 8.29 vs. 92.67 ± 11.91 maximum % aggregation ($p<0.005$). Collagen 2 μg/ml also induced less aggregation in the patient, inducing a mean % maximal aggregation of 61.0 ± 14.8 vs. 95.33 ± 15.2 in controls ($p<0.005$). Notably, ATP release, which is a measure of dense granule secretion, in response to ADP was completely absent in the patient's platelets as opposed to controls which released a mean 0.88 ± 0.33 nmol ATP ($p=0.001$). ATP release in response to collagen 2μg/ml also was decreased significantly in the patient vs. controls, mean 0.16 ± 0.032 vs. 1.1 ± 0.34 nmol ($p=0.002$). Normal aggregation and ATP release were observed in the patient's PRP with addition of AA 250 μM.

### $cPLA_{2\alpha}$ protein in platelets

Immunobloting for $cPLA_{2\alpha}$ in the patient's platelets detected protein of expected molecular weight but in diminished quantity (approximately 38% of control). COX-1 was detected in equal amounts in the patient and controls.

### $PLA_2$ activity

The patient's mean total $PLA_2$ activity in sonicated platelets represented 27.2 % of the activity in controls, with mean activity 0.77 ± 0.32 vs. 2.83 ± 0.47 pmol/min/50μg protein ($p<0.0005$) in controls.

### $cPLA_{2\alpha}$ DNA sequencing

Three transitions encoding non-synonymous codons in the patient's $cPLA_{2\alpha}$ alleles were found by sequencing $cPLA_{2\alpha}$ cDNA. These included a T to C transition (c.[331T>C]) resulting in a TCT to CCT substitution encoding a Ser to Pro change at residue 111 (p.[S111P]), a G to A transition (c.[ 1454G>A]) resulting in a CGT to CAT substitution encoding an Arg to His change at residue 485 (p.[R485H]) and an A to G transition (c.[ 1952A>G]) resulting in an AAG to AGG substitution encoding a Lys to Arg change at residue 651 (p.[K651R]). The patient was heterozygous for all three transitions and no changes were found in the 3′ or 5′ untranslated regions. Sequencing of $cPLA_{2\alpha}$ cDNA from the patient's mother and sister showed that the mother was heterozygous for the p.[S111P] variant but was homozygous for the p.[R485] and p.[K651] alleles. The sister was heterozygous for both the p.[R485H] and p.[K651R] variants but was homozygous for p.[S111] alleles. This inheritance pattern demonstrates that the patient was compound heterozygous with the p.[S111P] transition on one allele and both the p.[R4885H] and p.[K651R] transitions on the other allele. All of the variants detected in cDNA products were confirmed by direct sequencing of the corresponding segments of genomic DNA.

## Discussion

We have characterized a novel functional deficiency of $cPLA_2$-α resulting from compound heterozygosity for 2 rare variants(p.[S111P] and p.[R485H]) in a patient with ulcers and diaphragm-like strictures of the small intestine, platelet dysfunction, and globally decreased eicosanoid production. Our findings indicate that $cPLA_2$-α provides substrate for virtually all eicosanoid biosynthesis by human platelets, leukocytes and the cells from which cysteinyl leukotrienes are derived, and also indicate that products of this phospholipase contribute importantly to maintaining the integrity of the small intestine.

Concerted evidence supports a causal relationship between the observed mutations and the phenotype. Both the c.[331T>C] and the c.[1454 G>A] are missense mutations. Each of these mutations is rare; neither was found in the population of 418 DNA multiethnic samples that we analyzed, and neither was reported in the NCBI SNP database. These rare mutations are associated with a rare biochemical phenotype; no evidence for a block in eicosanoid

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biosynthesis at the level of cPLA$_2$-α has previously been reported in humans. The clinical phenotype of early onset of iron-deficiency anemia due to non–drug-related small bowel ulcers also is very uncommon. The biochemical phenotype of impaired biosynthesis of both TxB$_2$ and 12-HETE in platelets cosegregates with the mutations in the family, with the compound heterozygosity for the mutations in the patient yielding virtually complete absence of their biosynthesis and with the expected intermediate reduction in biosynthesis in the family members who are heterozygous for a single mutant allele. Each of the amino acids affected by the mutations is conserved across species, and molecular modeling implies that conformational changes should result from the mutations[19].

The mechanism of NSAID-induced small intestinal ulcers is informed by the finding that severe ulcers and diaphragmatic constrictions of the small intestine result from a genetic deficiency of prostaglandin biosynthesis. This supports a conclusion that loss of cyclooxygenase products is sufficient to cause complicated small intestinal ulcers. As the loss of prostaglandin biosynthesis is genetic, it is not possible to attribute this enteropathy to off-target effects of drugs.

The fact that reduced prostaglandin biosynthesis alone can cause small intestinal ulcers of course does not preclude modifying factors that could influence the development of small intestinal ulcers in a particular patient. Indeed, there is considerable inter-individual variation in the extent of small intestinal pathology produced by an NSAID. It is known, for example, that bile and intestinal microflora can modify the small intestinal response to NSAIDs. Pharmacokinetic differences would be predicted to affect the response to an NSAID, as would genetic differences in the prostaglandin biosynthetic enzymes and signaling molecules. Indeed, it could be hypothesized that heterozygous loss of a functional cPLA$_2$-α allele would exaggerate the response to an NSAID.

Although small intestinal ulcers unrelated to use of NSAIDs or enteric-coated drugs are uncommon, a number of such patients have been reported[2]. CPLA$_2$-α deficiency may be considered in the molecular differential diagnosis of such ulcers, particularly those with onset early in life. The finding of jejunoileal ulcers as a phenotype of cPLA$_2$-α deficiency identifies a biochemical pathway linked to this disease and affords a hypothesis that loss of function at other steps in the biosynthetic or signaling pathways for the relevant prostaglandin could yield a similar phenotype. The small intestinal sequelae of cPLA$_2$-α deficiency also become relevant in consideration of the consequences of employing selective cPLA$_2$-α inhibitors as therapeutic agents.

Nonselective COX inhibition by NSAIDs causes gastroduodenal ulceration with high morbidity and mortality[21]. In contrast, this patient with cPLA$_2$-α deficiency suffered from severe ulcer disease exclusively of the ileum and jejunum; no gastroduodenal ulcers had been detected on repeated upper intestinal endoscopies. This restriction of ulcer disease to the jejunum and ileum has been characteristic of other reported patients with NSAID-independent small intestinal ulcers[2,4]. The selectivity for the jejunum and ileum could result from cPLA$_2$-α serving as the key phospholipase providing AA substrate in these portions of the small intestine, in contrast with a different phospholipase releasing AA for biosynthesis of the PGs that protect the stomach[22–23] and duodenum from ulceration. Alternatively, lipoxygenase derived products of AA may be more important contributors to ulcer formation in the stomach and duodenum than in the ileum and jejunum. LTB$_4$ concentrations were markedly increased in the fundic mucosa of rats that received indomethacin compared with controls, and 5-lipoxygenase inhibitors have been shown to mitigate NSAID-induced gastric and intestinal lesions[24]. Notably, this attenuation was more profound in gastric mucosa, which demonstrated 95.4% and 98.8% reductions in lesion number and size, respectively, compared with 72.5% and 86.9% reductions in the small intestine. No such increase in gastric leukotriene products

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would be expected in the absence of $cPLA_2$-α. Moreover, the findings in this patient with $cPLA_2$-α deficiency indicate that NSAID-induced increases in leukotrienes can not be implicated in the causality of the small intestinal ulcers produced by these drugs.

A concerted body of evidence indicates that $cPLA_2$-α is the principal and rate-limiting phospholipase responsible for initiating eicosanoid biosynthesis in the platelet[25–28]. Our findings strongly support the concept that in the intact platelet, $cPLA_2$-α initiates the signaling-induced release of virtually all of the AA that is substrate for both the COX-1 and 12-lipoxygenase–derived pathways. Analysis of eicosanoid biosynthesis in activated platelets, therefore, provides an optimal biochemical phenotype with which to detect heterozygosity. $LTB_4$ biosynthesis by blood leukocytes also is derived almost entirely via the $cPLA_2$-α pathway.

$cPLA_2$-α (−/−) mice[28–33] manifest platelet dysfunction and have small bowel ulcers that are much less severe than in the patient lacking this enzyme. These knockout mice have cardiac hypertrophy, which is not evident in the patient.

In conclusion, we have characterized compound heterozygous $CPLA_2$-α variants that cause platelet dysfunction and are associated with multiple recurrent small bowel ulcers. Characterization of this deficiency informs the mechanism of NSAID-induced small intestinal ulcers and has the potential for elucidating the biology and pathophysiology of $cPLA_2$-α. These findings also have significant implications for the safety and effectiveness of pharmacologic inhibition of the $cPLA_2$-α enzyme.

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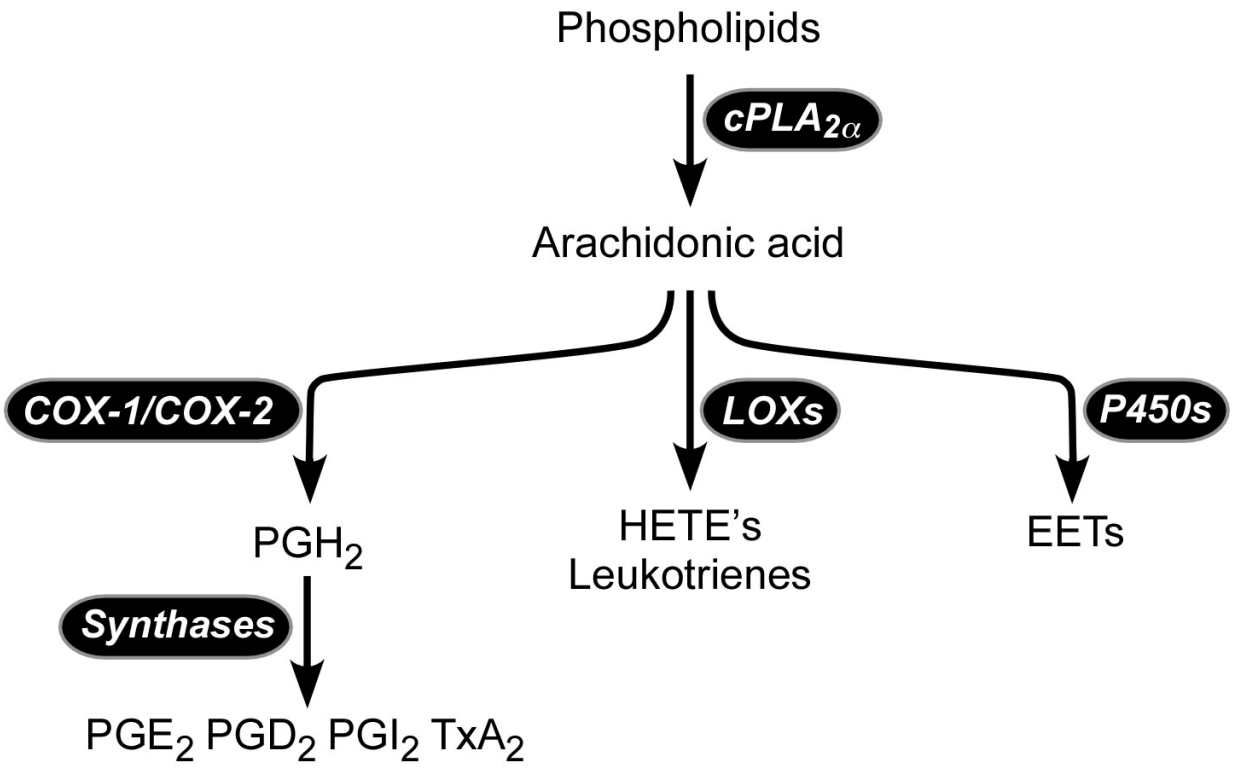


**Figure 1. Arachidonic acid cascade initiated by $cPLA_{2\alpha}$**

P450, cytochrome P450; LOX, lipoxygenase; COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid.

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## Table 1

| Urinary Eicosanoid Metabolites | | | |
|---|---|---|---|
| | **Patient** | **Reference range** | **patient vs. normal mean (%)** |
| 11-dTxB$_2$ (pg/mg creatinine) | 58.7 ± 15.1[A] | 96–644 | 15.90% |
| PGI-M (pg/mg creatinine) | 53.7 ± 39.3[A] | 65.1–291.9 | 30.0% |
| PGD-M (ng/mg creatinine) | 0.39 ± 0.35[A] | 0.74–1.64 (males) 0.72–1.2 (females) | 32.8 |
| PGE-M (ng/mg creatinine) | 4.48 ± 1.65[A] | 7.4–13.4 (males) 4.6–7.4 (females) | 43.1 |
| LTE$_4$ (pg/mg creatinine) | Undetectable | 19–60 | |

Urinary metabolites of TxA$_2$(11-dTxB$_2$), PGI$_2$ (PGI-M), PGD$_2$ (PGD-M), and PGE$_2$ (PGE-M) were measured by mass spectroscopy from 24-hour urine collections. Normal values for urinary prostanoid metabolites were published previously (6–9).

[A] Mean outside of reference range.

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## Table 2

Eicosanoids Derived From Platelets and Leukocytes

| Platelet-derived Eicosanoids | Patient | Reference Range † | Patient vs. Normal Mean (%) |
|---|---|---|---|
| $TxB_2$ (ng/ml) | 11.0 ± 6.9* | 40.2 – 415 | 4.82 % |
| 12-HETE (ng/ml) | 9.5 ± 9.6* | 44.2 – 1279 | 1.44 % |
| **Leukocyte-derived Eicosanoids** | | | |
| $LTB_4$ (ng/ml) | 7.2 ± 3.3* | 223 – 271 | 2.96% |

*Mean outside of reference range.

†Values reported are from male volunteers.