# Enzymatic formation of prostamide $F_{2\alpha}$ from anandamide involves a newly identified intermediate metabolite, prostamide $H_2$

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**Abstract** **Prostaglandin $F_{2\alpha}$ 1-ethanolamide (prostamide $F_{2\alpha}$) is a potent ocular hypotensive agent in animals and represents a new class of fatty acid amide compounds. Accumulated evidence indicated that anandamide, an endogenous bioactive ligand for cannabinoid receptors, may serve as a common substrate to produce all prostamides, including prostamide $F_{2\alpha}$. After incubation of anandamide with cyclooxygenase 2 (COX-2), the reaction mixture was profiled by HPLC and an intermediate metabolite was discovered and characterized as a cyclic endoperoxide ethanolamide using HPLC-tandem mass spectrometry. Formation of prostamide $F_{2\alpha}$ was also demonstrated when the intermediate metabolite was isolated and incubated with prostaglandin F synthase (PGF synthase).** **These results suggest that the biosynthesis of prostamide $F_{2\alpha}$ proceeds in two consecutive steps: oxidation of anandamide to form an endoperoxide intermediate by COX-2, and reduction of the endoperoxide intermediate to form prostamide $F_{2\alpha}$ by PGF synthase. This endoperoxide ethanolamide intermediate has been proposed as prostamide $H_2$.**—Yang, W., J. Ni, D. F. Woodward, D. D-S. Tang-Liu, and K-H. J. Ling. **Enzymatic formation of prostamide $F_{2\alpha}$ from anandamide involves a newly identified intermediate metabolite, prostamide $H_2$.** ***J. Lipid Res.*** **2005.** 46: **2745–2751.**

**Supplementary key words** cyclooxygenase 2 • prostaglandin $F_{2\alpha}$ 1-ethanolamide • prostaglandin F synthase

Prostaglandins are unsaturated fatty acid metabolites with remarkably potent and diversified biological functions. They are generated from the oxidation of arachidonic acid by cyclooxygenases (1). Prostaglandin $H_2$, the hydroxy-endoperoxide, serves as a common intermediate product, through which prostaglandins are produced in specific tissues or cells (2). Because of the structural similarity, arachidonyl ethanolamide (anandamide), an endogenous agonist of cannabinoid receptors (3), was indicated as a selective substrate for cyclooxygenase 2 (COX-2) (4). Recent evidence had demonstrated that anandamide was effectively oxygenated by COX-2 to form prostamides, a new class of prostaglandin analogs (5–7), and the hydroxyl moiety of anandamide, as a critical determinant in the ability of COX-2 to effect robust endocannabinoid oxygenation (8).

Although the physiological functions of the prostamides are not well defined, prostaglandin $F_{2\alpha}$ 1-ethanolamide (prostamide $F_{2\alpha}$) was reported to be potent in the contraction of the cat iris sphincter (9) and to behave as an effective ocular hypotensive agent in monkeys (10). In addition, the antiglaucoma drug bimatoprost (Lumigan™) is similar to prostamide $F_{2\alpha}$ in structure and behaves as a prostamide analog (9, 10). To identify the potential biosynthetic pathway of prostamide $F_{2\alpha}$, we conducted a series of metabolic studies of anandamide using recombinant human COX-2 and prostaglandin F synthase (PGF synthase) and analyzed the enzymatic metabolites using HPLC-radiometric detection (HPLC-RAD) and HPLC tandem mass spectrometry (HPLC-MS/MS). The results indicated that a prostamide congener of prostaglandin $H_2$ serves as an intermediate metabolite of anandamide in prostamide $F_{2\alpha}$ synthesis and demonstrated two consecutive enzymatic reactions in prostamide $F_{2\alpha}$ formation.

## MATERIALS AND METHODS

### Chemicals

Prostamide $F_{2\alpha}$ [*N*-(2-hydroxyethyl)-9α,11α,15*S*-trihydroxy-prosta-5*Z*,13*E*-dien-1-amide] was synthesized at Allergan, Inc. Anandamide and prostamides $D_2$ and $E_2$ were purchased from Cayman Chemical Co. (Ann Arbor, MI). [$^3$H]anandamide (208 Ci/mmol, 99.7% purity), [$^3$H]prostamide $D_2$ (162 Ci/mmol, 98.1% purity), [$^3$H]prostamide $E_2$ (169 Ci/mmol, 97% purity), [$^3$H]prostamide $F_{2\alpha}$ (194 Ci/mmol, 99.7% purity), and [$^3$H]prostamide 11β-$F_{2\alpha}$ (20 Ci/mmol, 99.7% purity) were custom synthesized from Amersham Pharmacia Biotech (Piscataway, NJ). All chemicals and reagents were of reagent grade or better.

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### Enzyme preparation

Human recombinant COX-2 (rHCOX-2) was a gift from Dr. W. L. Smith at Michigan State University. The enzyme preparation contains 18,180 U/mg protein (1 unit of enzyme consumes 1 nmol of oxygen per minute at 37°C in a 0.1 M Tris-HCl buffer, pH 8.0, containing 100 μM arachidonate, 5 mM EDTA, 2 mM phenol, and 1 μM hematin).

Human recombinant PGF synthase (rHPGF synthase) was a gift from Dr. K. Watanabe at The University of East Asia. The expressed enzyme was partially purified to yield a protein concentration of 15 mg/ml. The same expression vector carrying no PGF synthase DNA insert was prepared as a negative control.

### rHCOX-2 enzymatic reaction and HPLC-RAD profiling

Twenty microliters of 0.5 mM anandamide solution (containing 0.43 μCi of [$^3$H]anandamide) was added to 960 μl of rHCOX-2 reaction buffer (100 mM Tris-HCl, pH 8.0, containing 2 mM phenol, 5 μM hematin, and 1 mM EDTA) to result in a final anandamide concentration of 10 μM. One hundred units of rHCOX-2 in 20 μl volume was added to start the enzymatic reaction. After incubation at 37°C for 2 min, the reaction was stopped by adding 1 ml of dry ice-cooled solution (ether-methanol-1 M acetic acid at 30:4:1, v/v/v). The samples were extracted with 3 ml of ethyl acetate, and the organic phase was collected and dried at room temperature under nitrogen. The samples were reconstituted into 150 μl of HPLC mobile phase (acetonitrile-10 mM ammonium formate, pH 2.8, at 28:72, v/v) for HPLC-RAD profiling. A Hewlett-Packard (Palo Alto, CA) 1100 HPLC system coupled with a Packard (Meriden, CT) radiometric detector was used to profile the samples, and a 5 μm, 4.6 × 250 mm Inertsil ODS-2 column (GL Sciences, Inc.) was used in the analysis. Mobile phase A was 10 mM ammonium formate, pH 2.8, in water, and mobile phase B was acetonitrile. The injection volume was 50 μl, and the flow rate was set at 1 ml/min with the gradients listed in **Table 1**.

The reconstituted sample was also used for structural elucidation by LC-MS/MS analysis. Reversed-phase HPLC-MS/MS and radiometric detection were used to characterize the reaction products. The HPLC conditions were the same as for profiling. The effluent from the HPLC column was split: one portion with a flow rate of ~0.2 ml/min was introduced into a mass spectrometer, whereas the other portion with a flow rate of 0.3 ml/min was directed into the flow cell of a β-RAM (IN/US System, Tampa, FL). The β-RAM response was recorded in real time by the mass spectrometer computer, which provided simultaneous recording of radioactivity and mass spectral data. The delay in response between the two detectors was ~0.4 min, with the mass spectrometric response being recorded first. The β-RAM was operated in homogeneous liquid scintillation counting mode with the addition of 3.2 ml/min Flo-scint III scintillation cocktail to the HPLC effluent. The radioactivity detector residence time was ~16 s.

Analysis of the metabolic products from the anandamide-rHCOX-2 reaction was carried out with a PE-Sciex API 3000 tandem mass spectrometer (Sciex, Toronto, Canada). The experiment was performed in positive ion turbo ion spray mode, and the LC effluent was sprayed into the mass spectrometer with a voltage of 5,000 V applied to the spray needle. The declustering voltage was 20 V, and the turbo ion spray temperature was set at 350°C. In the tandem mass spectrometric experiments, collision-activated dissociation in Q2 was induced by nitrogen as the collision gas at a collision energy of −30 eV.

TABLE 1. HPLC gradient used for analysis

| Time | Mobile Phase A | Mobile Phase B | Gradient |
|---|---|---|---|
| *min* | % | | |
| 0 → 22.5 | 72 | 28 | Isocratic |
| 22.5 → 32 | 72 → 20 | 28 → 80 | Linear |
| 32 → 57 | 20 | 80 | Isocratic |
| 57 → 60 | 20 → 72 | 80 → 28 | Linear |
| 60 → 65 | 72 | 28 | Isocratic |

Mobile phase A = 10 mM ammonium formate, pH 2.8, in water; mobile phase B = acetonitrile.

### Isolation of anandamide intermediate

After incubation of anadamide with rHCOX-2 for 2 min, the enzymatic product with a retention time at 31.6 min from HPLC-RAD profiling was fractionally collected, dried under nitrogen, and stored at −80°C until use. The sample was used both for structural elucidation and for the PGF synthase reaction. In the PGF synthase reaction, the sample was reconstituted into 0.6 ml of PGF synthase reaction buffer (100 mM Tris-HCl, pH 8.0, 0.5 mM NADP, 5 mM glucose-6-phosphate, and 2 units/ml glucose-6-phosphate dehydrogenase). The concentration of the intermediate metabolite was determined by a radiolabeled standard calibration curve (range of 100–20,000 dpm/ml).

### PGF synthase reaction

Aliquots of 0, 10, and 100 μl of PGF synthase (1.5 μg protein/μl) were added to the reconstituted solution containing anandamide intermediate at a concentration of ~4.5 μM to initiate the PGF synthase reaction. The reaction was carried out at 37°C for 2 and 10 min, separately, and stopped by adding 1 ml of dry ice-cooled stop solution (ether-methanol-1M acetic acid at 30:4:1, v/v/v). The sample was extracted with 3 ml of ethyl acetate, and the organic phase was collected and dried at room temperature under nitrogen. The residue was reconstituted using HPLC mobile phase (acetonitrile-10 mM ammonium formate, pH 2.8, at 28:72, v/v) and used for both HPLC-RAD profiling and LC-MS/MS analysis.

## RESULTS

### Incubation of anandamide with rHCOX-2

After 2 min of incubation of anandamide with rHCOX-2, at least three major products with retention times of 31.6, 32.9, and 35.3 min were detected (**Fig. 1A**). Unlike the other two products, the peak with retention time at 31.6 min rapidly decreased with incubation time and completely disappeared after 20 min of incubation, suggesting that this product was a short-lived intermediate in anandamide metabolism. Reference standards of anandamide and its potential metabolites, including prostamide $D_2$, prostamide $E_2$, prostamide $F_{2\alpha}$, and 11β-prostamide $F_{2\alpha}$, were eluted at 40.9, 20.9, 16.9, 15.1, and 12.1 min, respectively (Fig. 1B).

Kinetic study of this intermediate formation was conducted in the presence of 100 units of rHCOX-2 and increasing concentrations of anandamide from 0.5 to 400 μM after a 2 min incubation at room temperature. The observed $K_m$ and $V_{max}$ values for the conversion of anandamide to this intermediate were ~15 μM and 1.3 μmol/min/mg protein, respectively.

### HPLC-RAD and LC-MS/MS analysis of rHCOX-2-anandamide intermediate product

The product ion spectrum of anandamide produced a major fragment ion at *m/z* 62, which was interpreted as the protonated 2-amino ethanol moiety (**Fig. 2**). Because

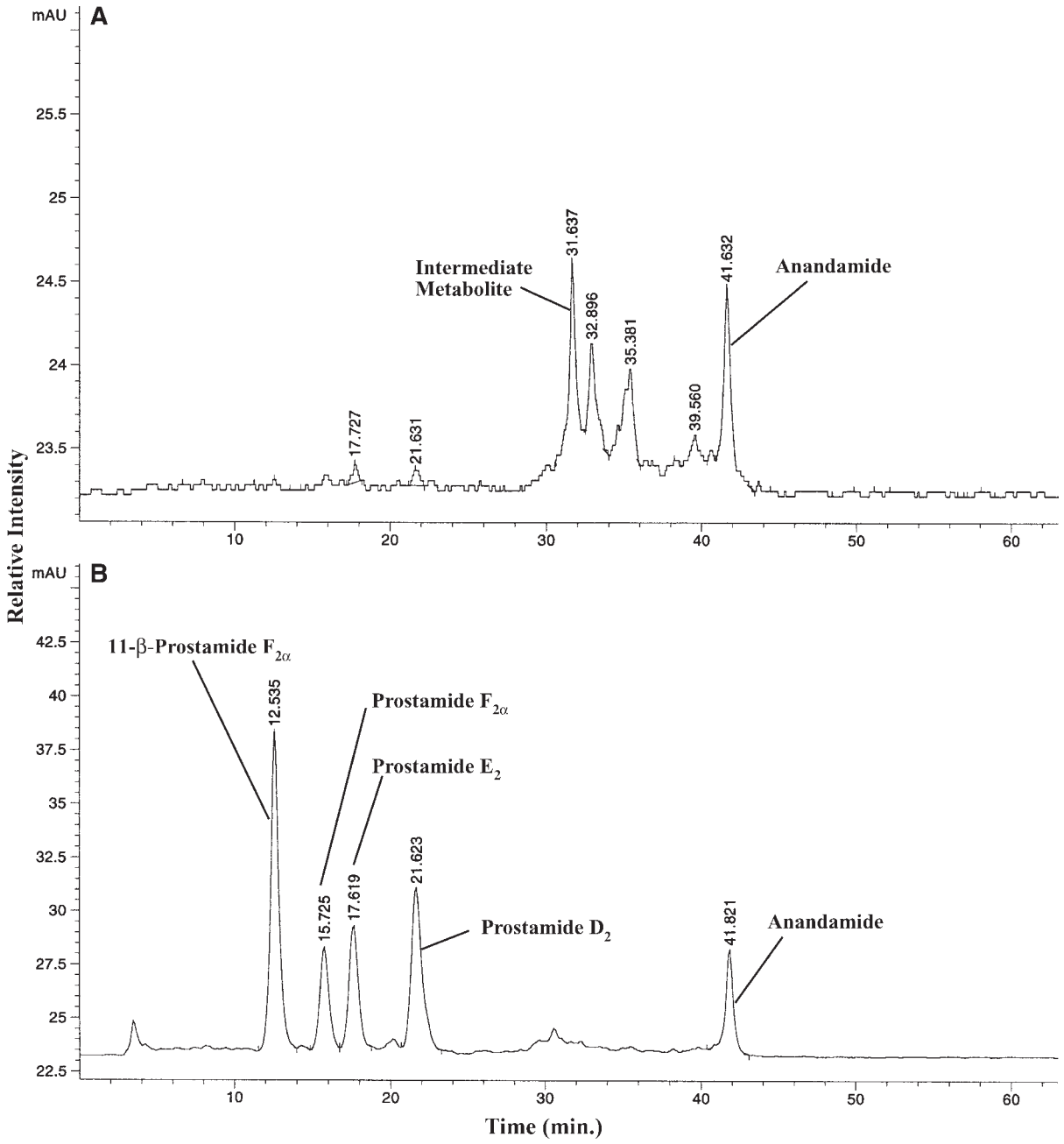


**Fig. 1.** Conversion of anandamide to the intermediate metabolite in the presence of human recombinant cyclooxygenase 2 (A) and five prostamide standards (B). AU, absorbance units.

the protonated 2-amino ethanol ion was a characteristic fragment ion in the product ion spectra of ethanolamide-containing compounds such as anandamide and prostamides, a precursor ion scan of *m/z* 62 was conducted to monitor compounds with a prostamide structure. Prostamides $E_2$ and $D_2$ and the intermediate, in the precursor ion spectrum of *m/z* 62, all had the same molecular weight of 395 but different retention times (Fig. 1B). LC-MS/MS product ion spectra of *m/z* 396.4 for prostamides $D_2$ and $E_2$ and the intermediate metabolite showed different fragmentation patterns (**Fig. 3**). The product ion spectra of prostamides $D_2$ and $E_2$ were similar and displayed characteristic fragment ions at *m/z* 378, 360, and 342 via three consecutive water losses, at *m/z* 281 after further loss of neutral 2-amino ethanol from *m/z* 342, and at *m/z* 62 as protonated 2-amino ethanol ion (Fig. 3A, B). The product ion spectrum of the unique intermediate metabolite also showed a fragment ion at *m/z* 62, the protonated 2-amino ethanol ion. However, unlike those of prostamides $D_2$ and $E_2$, the product ion spectrum of this intermediate metabolite showed fragment ions at *m/z* 344 and 283 (Fig. 3C). The displayed characteristic fragment ion at *m/z* 344 could be interpreted as the loss of one water molecule and one neutral $H_2O_2$ moiety from the parent ion, and the fragment ion at *m/z* 283 could be interpreted as a further loss of neutral 2-amino ethanol from *m/z* 344.

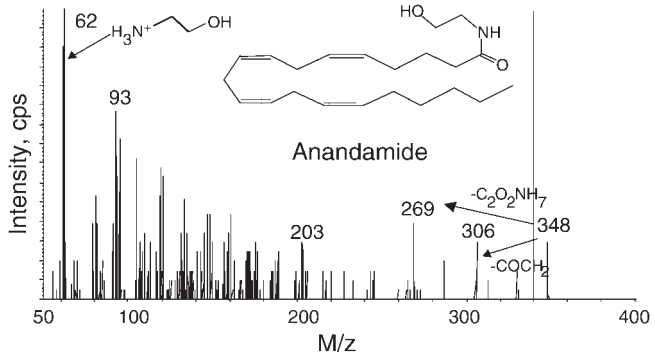


**Fig. 2.** Liquid chromatography-tandem mass spectrometry (LC-MS/MS) product ion spectrum of anandamide.

### Identification of the product from purified anandamide intermediate metabolite-PGF synthase incubation and confirmation by LC-MS/MS

To investigate whether the intermediate metabolite is a precursor of prostamide $F_{2\alpha}$, the intermediate with retention time at 31.6 min was isolated via fraction collection. The sample was reconstituted in PGF synthase reaction buffer, and the purity of the intermediate metabolite was estimated to be >90% by HPLC-RAD.

After incubation of 15 μg of rHPGF synthase with purified intermediate metabolite of anandamide at a concentration of 4.5 μM at 37°C for 2 min, 69% of the isolated intermediate was converted to prostamide $F_{2\alpha}$ (**Fig. 4C**), whereas no conversion occurred after incubation of same amount of enzyme negative control with purified intermediate metabolite at the same concentration at 37°C for 2 min (Fig. 4B). The conversion of the intermediate to prostamide $F_{2\alpha}$ was nearly completed after 10 min of incubation (Fig. 4D). The newly converted prostamide $F_{2\alpha}$ (**Fig. 5A**) was further confirmed by comparing its retention time and fragmentation pattern with those of prostamide $F_{2\alpha}$ standard using LC-MS/MS (Fig. 5B).

## DISCUSSION

Prostamide $F_{2\alpha}$ is a potent ocular hypotensive agent in animals and represents a potentially important metabolite

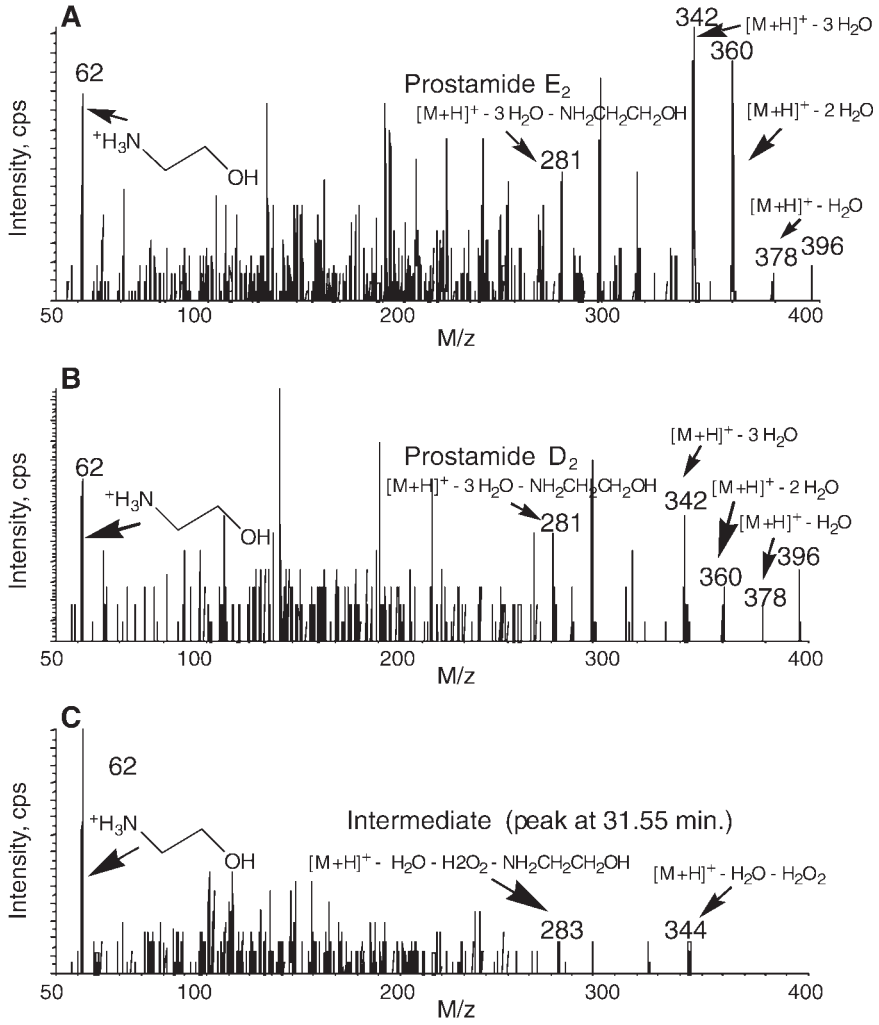


**Fig. 3.** LC-MS/MS product ion spectrum of prostamide $E_2$ (A), prostamide $D_2$ (B), and the intermediate metabolite with retention time of 31.55 min (C).

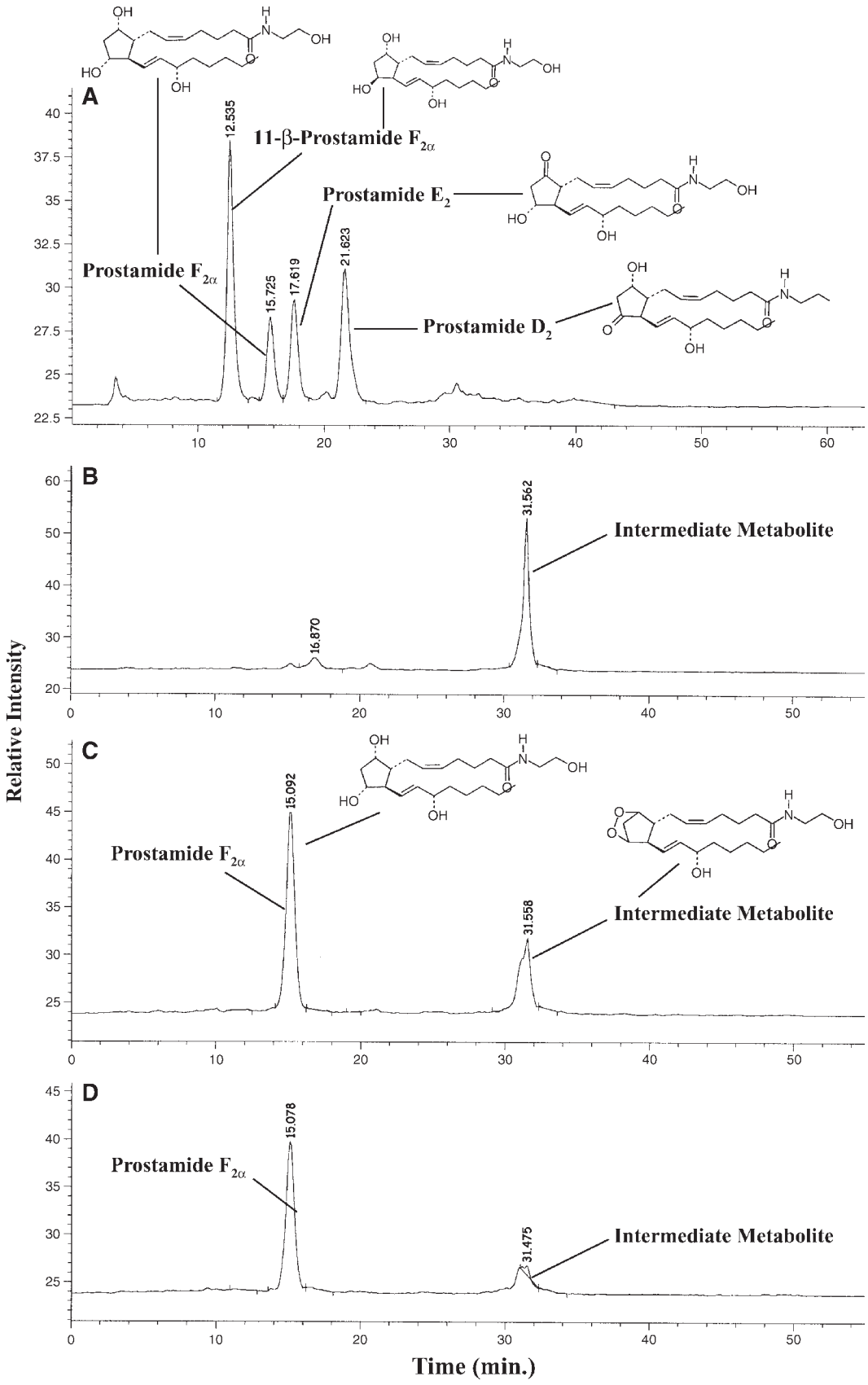


**Fig. 4.** Conversion of the intermediate metabolite to prostaglandin $F_{2\alpha}$ 1-ethanolamide (prostamide $F_{2\alpha}$). The reaction was carried out in the absence (B) or presence of 15 μg of human recombinant prostaglandin F synthase (rHPGF synthase) at 37°C for 2 min (C) or 10 min (D). Prostamide standards were presented as references (A).

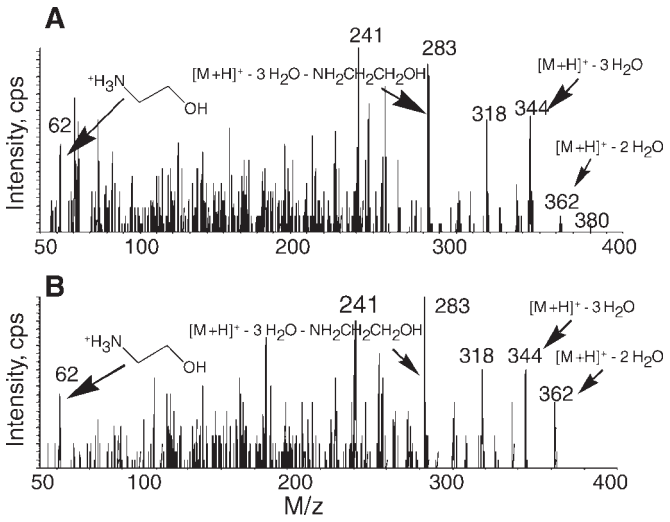


**Fig. 5.** LC-MS/MS product ion spectrum of the proposed prostamide $F_{2\alpha}$. Both the biosynthesized product from incubation of 4.5 μM of the intermediate metabolite and 15 μg of PGF synthase (A) and the standard prostamide $F_{2\alpha}$ (B) were analyzed using the LC-MS/MS product ion spectrum.

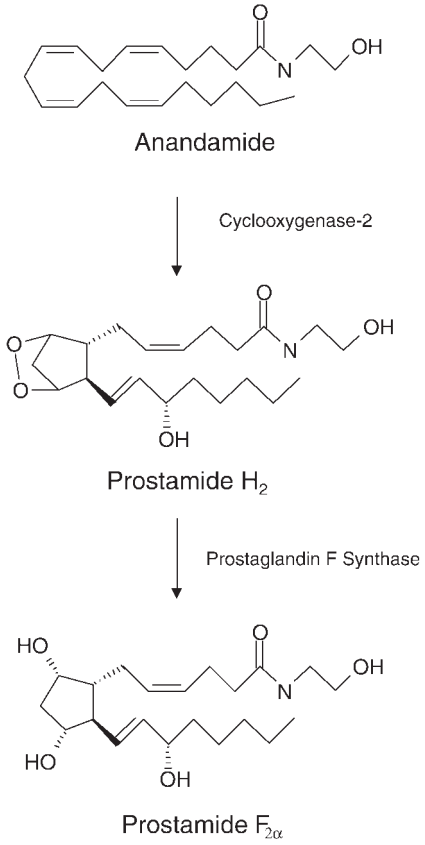


**Fig. 6.** Metabolic scheme of anandamide to prostamide $F_{2\alpha}$.

of anandamide (11). This study indicated that the biosynthesis of prostamide $F_{2\alpha}$ from anandamide, one of the natural ligands for the cannabinoid receptors (3), might include two consecutive steps, an oxidization of anandamide to form an endoperoxide intermediate by enzyme COX-2, and a reduction of the endoperoxide intermediate to form prostamide $F_{2\alpha}$ by PGF synthase (**Fig. 6**, proposed metabolic scheme of prostamide $F_{2\alpha}$). The observed $K_m$ value of COX-2 oxidation of anandamide was 15 μM, and the observed $V_{max}$ value for COX-2 oxidation of anandamide was ~1.3 μmol/mg protein/min. Our result for COX-2 oxygenation of anandamide is consistent with recent structure-activity studies by Kozak et al. (8), who had examined the structural requirements for COX-mediated anandamide oxygenation using a number of substrate analogs and site-directed mutants of COX-2. They concluded that the hydroxyl moiety of anandamide is a critical determinant in the ability of COX enzymes to effect robust endocannabinoid oxygenation, whereas anandamide binds within the COX-2 active site in a conformation roughly similar to that of arachidonic acid.

LC-MS/MS analysis indicated that the intermediate metabolite of anandamide might be a new member of the prostaglandin 1-ethanolamide class of compounds. Although this intermediate metabolite shares the same molecular weight of 395 with prostamides $D_2$ and $E_2$, the product ion spectra of prostamides $D_2$ and $E_2$ indicated that there were three consecutive losses of water molecules, resulting in fragment ions at *m/z* 378, 360, and 342 (Fig. 3A, B), whereas the intermediate metabolite showed no direct loss of water molecules (Fig. 3C). The fragment ion at *m/z* 344 could be interpreted as a loss of one water and one $H_2O_2$ with a combined mass of 52. However, the intermediate metabolite, like prostamide $D_2$ or $E_2$, also had the characteristic *m/z* 62 fragment ion (Fig. 3), suggesting that it contained an ethanolamide group. In addition, a fragment ion at *m/z* 283 could be interpreted as a breakdown of the ion at *m/z* 344 by losing a neutral species of 2-amino ethanol (Fig. 3C). Based on the similarity of its molecular weight to that of prostamides $D_2$ and $E_2$, the unique mass spectral fragmentation pattern, including the characteristic ions at *m/z* 344, 283, and 62, as well as its instability during incubation, we proposed this unique intermediate metabolite as prostamide $H_2$, with the chemical name 6-(6-hept-1-enyl-2,3,-dioxa-bicyclo[2.2.1]hept-5-yl)-hex-5-enoic acid (2-hydroxy-ethyl)-amide.

According to the prostaglandin biosynthesis pathway, PGF synthase, an aldo-keto reductase, reduces prostaglandin $H_2$ to $PGF_{2\alpha}$ (12). Because of the structural similarity between prostamide $F_{2\alpha}$ and prostaglandin $F_{2\alpha}$, the isolated intermediate metabolite was incubated with rHPGF synthase and the reaction mixture was profiled by HPLC-RAD. As demonstrated in Fig. 4, ~70% of the intermediate metabolite was converted to prostamide $F_{2\alpha}$ after 2 min of incubation at 37°C in the presence of 15 μg of rHPGF synthase (Fig. 4C), whereas no conversion occurred in the presence of the rHPGF synthase negative control (Fig. 4B). The conversion was time and enzyme concentration dependent. In the presence of the same amount of rHPGF for 10 min, >90% of the intermediate metabolite was converted to $PGF_{2\alpha}$ (Fig. 4D). When a higher amount of rHPGF synthase (150 μg) was used, ~90% of the intermediate metabolite was converted to $PGF_{2\alpha}$ after 2 min of incubation and was completely converted to prostamide $F_{2\alpha}$ after 10 min (data not shown). Finally, the converted prosta-

mide $F_{2\alpha}$ was confirmed using prostamide $F_{2\alpha}$ standard by LC-MS/MS (Fig. 5).

In conclusion, for the first time, we have demonstrated the biosynthesis of prostamide $F_{2\alpha}$ from anandamide and proposed prostamide $H_2$ as a possible intermediate of anandamide metabolism, similar to the role of prostaglandin $H_2$ in the metabolism of arachidonic acid.

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