# Formation of prostamides from anandamide in FAAH knockout mice analyzed by HPLC with tandem mass spectrometry

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**Abstract** **We investigated the formation of $PGF_{2\alpha}$ 1-ethanolamide, $PGE_2$ 1-ethanolamide, and $PGD_2$ 1-ethanolamide (prostamides $F_{2\alpha}$, $E_2$, and $D_2$, respectively) in liver, lung, kidney, and small intestine after a single intravenous bolus administration of 50 mg/kg of anandamide to normal and fatty acid amide hydrolase knockout (FAAH −/−) male mice. One group of three normal mice was not dosed (naïve) while another group of three normal mice received a bolus intravenous injection of 50 mg/kg of anandamide. Three FAAH −/− mice also received an intravenous injection of 50 mg/kg of anandamide. After 30 min, the lung, liver, kidney, and small intestine were harvested and processed by liquid-liquid extraction. The concentrations of prostamide $F_{2\alpha}$, prostamide $E_2$, prostamide $D_2$, and anandamide were determined by HPLC-tandem mass spectrometry. Prostamide $F_{2\alpha}$ was detected in tissues in FAAH −/− mice after administration of anandamide. Concentrations of anandamide, prostamide $E_2$, and prostamide $D_2$ in liver, kidney, lung, and small intestine were much higher in the anandamide-treated FAAH −/− mice than those of the anandamide-treated control mice. This report demonstrates that prostamides, including prostamide $F_{2\alpha}$, were formed in vivo from anandamide, potentially by the cyclooxygenase-2 pathway when the competing FAAH pathway is lacking.**—Weber, A., J. Ni, K-H. J. Ling, A. Acheampong, D. D-S. Tang-Liu, R. Burk, B. F. Cravatt, and D. Woodward. **Formation of prostamides from anandamide in FAAH knockout mice analyzed by HPLC with tandem mass spectrometry. *J. Lipid Res.* 2004.** 45: **757–763.**

**Supplementary key words** anandamide • cyclooxygenase-2 • fatty acid amide hydrolase • high-performance liquid chromatography • $PGD_2$ 1-ethanolamide • $PGE_2$ 1-ethanolamide • $PGF_{2\alpha}$ 1-ethanolamide • prostamide $D_2$ • prostamide $E_2$ • prostamide $F_{2\alpha}$

Anandamide (arachidonyl ethanolamide) is a potent endogenous ligand of central and peripheral cannabinoid receptors (1). It possesses various cannabimimetic activities in vitro and in vivo, including antinociception, hypotension, hypothermia, hypomotility, and catalepsy (2–6). One major enzymatic pathway responsible for the regulation of anandamide is hydrolysis of anandamide to arachidonic acid and ethanolamine via fatty acid amide hydrolase (FAAH) (7–9). Studies have indicated that anandamide could be converted to $PGE_2$ 1-ethanolamide (prostamide $E_2$) (10, 11) in the presence of cyclooxygenase-2 (COX-2) in vitro. COX-2 has been known to convert arachidonic acid to various prostaglandins (12) that possess potent biological activity. COX-2 may be a potential key enzyme in anandamide conversion to prostamides (prostanoid ethanolamides) in vivo. These prostamides possess biological activity (13, 14), including contraction of feline cat iris and the reduction of intraocular pressure in primates. Furthermore, these prostamides have longer plasma elimination half-life when compared with prostaglandins (15). The longer plasma half-life may allow them to exert biological effects remote from the site of synthesis.

If the FAAH pathway in rodents was absent or disrupted by knocking out the FAAH gene, then substantial concentrations of anandamide after exogenous administration of anandamide may be available to the COX-2 pathway for the potential production of prostamides in vivo. This may

Abbreviations: COX-2, cyclooxygenase-2; FAAH, fatty acid amide hydrolase; FAAH −/−, FAAH knockout; HPLC-MS/MS, HPLC tandem mass spectrometry; LC-MS/MS, liquid chromatography-MS/MS; MRM, multiple-reaction monitoring; prostamide $D_2$, $PGD_2$ 1-ethanolamide; prostamide $E_2$, $PGE_2$ 1-ethanolamide; prostamide $F_{2\alpha}$, $PGF_{2\alpha}$ 1-ethanolamide.

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more closely model higher species where anandamide and 2-arachidonylglycerol exhibit more resistance to ester or amide enzymatic hydrolysis (15). In this study, we investigated levels of biosynthesized prostamides by a sensitive and specific method involving HPLC tandem mass spectrometry (HPLC-MS/MS).

The small intestine and kidney, which constitutively expressed COX-2 (16, 17) and other key tissues (i.e., liver and lung), were examined for anandamide remaining and for prostamide formation after intravenous bolus administration of 50 mg/kg anandamide to normal mice and FAAH knockout (FAAH −/−) mice.

## MATERIALS AND METHODS

### Materials

Anandamide was purchased from Cayman Chemicals (Ann Arbor, MI) with 98% purity as determined by HPLC. An IV formulation of anandamide (20 mg/ml) in 1:1:18 (ethanol:Incrocas 30:0.9% saline; v/v/v) was used in the study. Incrocas-30 (PEG-30:castor oil) was a gift from Croda, Inc. (Parsippany, NY). Reference standards $PGF_{2\alpha}$ 1-ethanolamide (prostamide $F_{2\alpha}$), $PGE_2$ 1-ethanolamide (prostamide $E_2$), and $PGD_2$ 1-ethanolamide (prostamide $D_2$), as well as the internal standard $d_8$-anandamide, were purchased from Cayman Chemicals. The internal standard, $d_4$-prostamide $F_{2\alpha}$, was synthesized by Allergan. All other chemicals used in the study were of reagent grade or better.

### Animals

Nine male Swiss Webster mice, 5–6 months old and weighing 20–30 g, were used in the study with six normal mice purchased from Charles River Laboratories (Portage, MI), and three FAAH −/− mice supplied from the Scripps Institute (San Diego, CA). The animal procedures that were used have been approved by the Allergan's Animal Care and Use Committee (AACUC). The FAAH −/− mice have been previously characterized, demonstrating the absence of the FAAH protein and related activity (18).

### Dosing and sample collection

Three groups of mice (two groups of normal mice and one group of FAAH −/− mice; n = 3/group) were cannulated with 0.3 mm silastic tubing (Dow Chemical, Midland, MI) in the jugular vein under isoflurane anesthesia the day before anandamide administration. One group of normal mice did not receive anandamide and another group of normal mice received intravenous administration of anandamide at 50 mg/kg. This dose of anandamide is similar to the dose that produced behavioral effects in rats and mice (3, 9). The FAAH −/− group also received intravenous administration of anandamide at 50 mg/kg. Thirty minutes after the bolus intravenous administration of anandamide, the treated control and the treated FAAH −/− mice were euthanized by $CO_2$ inhalation. Liver, lung, kidney, and small intestine were surgically removed from all the animals and were kept on ice until processing.

### Sample preparation

On the day tissues were harvested, samples were minced into 1 $mm^2$ sections and extracted with 5 ml of acetonitrile overnight at 4°C. The mixture was centrifuged at 2,500 *g* for 10 min at 4°C.

TABLE 1. HPLC gradient used for analysis

| Time | A[a] | B[a] | Gradient |
|---|---|---|---|
| *min* | % | % | |
| 0 | 90 | 10 | Initial conditions |
| 0–0.1 | 90 → 70 | 10 → 30 | Step |
| 0.1–5.0 | 70 → 40 | 30 → 60 | Linear |
| 5.0–5.1 | 40 → 0 | 60 → 100 | Step |
| 5.1–9.1 | 0 | 100 | Isocratic |
| 9.1–9.2 | 0 → 90 | 100 → 10 | Step |
| 9.2–13.0 | 90 | 10 | Isocratic |

Prostamide $D_2$, $PGD_2$ 1-ethanolamide; prostamide $E_2$, $PGE_2$ 1-ethanolamide; prostamide $F_{2\alpha}$, $PGF_{2\alpha}$ 1-ethanolamide.

[a] A = 0.5% formic acid in acetonitrile; B = 0.5% formic acid in water.

The supernatant was evaporated to dryness under nitrogen. The resulting dry residue was stored at −70°C until analysis. For liquid chromatography-MS/MS (LC-MS/MS) analysis, dried residues were spiked with the 10 ng of deuterated internal standards, $d_4$-prostamide $F_{2\alpha}$, and $d_8$-anandamide. The internal standards were added post extraction to compensate for the variability of the mass spectrometric response. The samples were evaporated to dryness at 40°C and reconstituted with 150 μl of 1:1 (v/v) mixture of 0.5% formic acid in water and acetonitrile. Standards of prostamide $F_{2\alpha}$, prostamide $E_2$, prostamide $D_2$, and anandamide were prepared in mobile phase at concentrations from 0.05 ng/ml to 100 ng/ml and were processed in the same fashion as the samples.

### Sample analysis

Fifty microliters of extract were injected into the LC-MS/MS for analysis. The Shimadzu HPLC system (Shimadzu Scientific Instruments, Columbia, MD) consisted of SCL-10A vp system controller, LC-10AD VP liquid chromatogram, and SIL-10AD VP autoinjector. A Luna C8 3 μm (100 × 2.0 mm) column maintained at ambient temperature was used in the analysis. The mobile phase A consisted of 0.5% formic acid in water and the mobile phase B consisted of 0.5% of formic acid in acetonitrile. The flow rate was set at 0.2 ml/min with a gradient depicted in **Table 1**.

LC-MS/MS analysis was performed using Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The mass spectrometry system was operated under positive

TABLE 2. MRM transitions for LC-MS/MS quantitation

| Compound | Retention Time | MRM Ion Pair Transition |
|---|---|---|
| First Set | | |
| | *min* | *m/z* |
| Anandamide | 7.8 | 348.1–62.2 |
| Prostamide $F_{2\alpha}$ | 4.0 | 398.2–380.0 |
| Prostamides $D_2$+$E_2$[a] | 4.4 | 396.2–378.0 |
| Second Set | | |
| Anandamide | 7.8 | 348.1–62.2 |
| Prostamide $F_{2\alpha}$ | 4.0 | 398.2–62.2 |
| Prostamides $D_2$+$E_2$[a] | 4.4 | 396.2–62.2 |

LC-MS/MS, liquid chromatography tandem mass spectrometry; MRM, multiple-reaction monitoring.

[a] Prostamide $D_2$ and prostamide $E_2$ coeluted.

ion turbo-ionspray ionization mode. The entire LC eluent was analyzed using a turbo ionspray ionization source and a tandem mass spectrometer with 5,000 V applied to the spray needle. The turbo ionspray temperature was set at 350°C, with the declustering potential set at 60 V. Previous studies (15, 19) have used LC-MS with single-ion monitoring for analysis of anandamide. For our study, samples were analyzed by using a product ion scan for characterization of metabolites or enzymatic products and by using multiple-reaction monitoring (MRM) for quantitation of anandamide and prostamides. Quantitation was performed using two sets of MRM transitions: one MRM set involved monitoring parent ions $(MH^+) \rightarrow$ water loss $(MH - H_2O)^+$ daughter ions; and the second set of MRM monitored parent ions $(MH^+) \rightarrow$ ethanolamide selective m/62 daughter ions. The first and second experiments utilized MRM ion pair transitions for quantitation (**Table 2**).

### Data calculation

The peak areas, peak area ratios, linear regression, assayed concentrations, and other quantitative analysis calculations were generated using the Sciex Analyst 1.1 quantitation software (Applied Biosystems, Foster City, CA). The Analyst software was used to construct weighted linear regression curves relating to peak area ratios of the analyte/internal standard to the concentration of analyte in the calibration standard. The assayed concentrations from extracted samples were determined from the calibration curve using the Analyst software. For each compound, the assayed concentration was multiplied by the volume of the extract then divided by the tissue weight. Thus, the results were expressed as ng/g of tissue.

## RESULTS AND DISCUSSION

### LC-MS/MS chromatograms and quantitation

Chromatograms of reference standards containing anandamide, prostamide $F_{2\alpha}$, prostamide $D_2$, and prostamide $E_2$, with the internal standards depicted in **Fig. 1**. Typical MRM chromatograms of extracted lung from an FAAH −/− mouse dosed with anandamide with the internal standards $d_4$-prostamide $F_{2\alpha}$ and $d_8$-anandamide are displayed in **Fig. 2**. Prostamide $E_2$ and prostamide $D_2$, which have identical molecular weights, were not separated chromatographically in samples; therefore, the concentrations of these two prostamides were reported as the sum, prostamides $E_2$ and $D_2$. The linearity of the calibration curves for anandamide, prostamide $F_{2\alpha}$, and prostamides $D_2+E_2$ was assessed by analyzing eight calibration standards of each compound over the assay range: 0.05, 0.125, 0.25, 0.5, 2.5, 5, 25, and 50 ng/ml. The calibration curve was determined by least-square linear regression analysis of peak area ratios of the analyte/internal standard. The

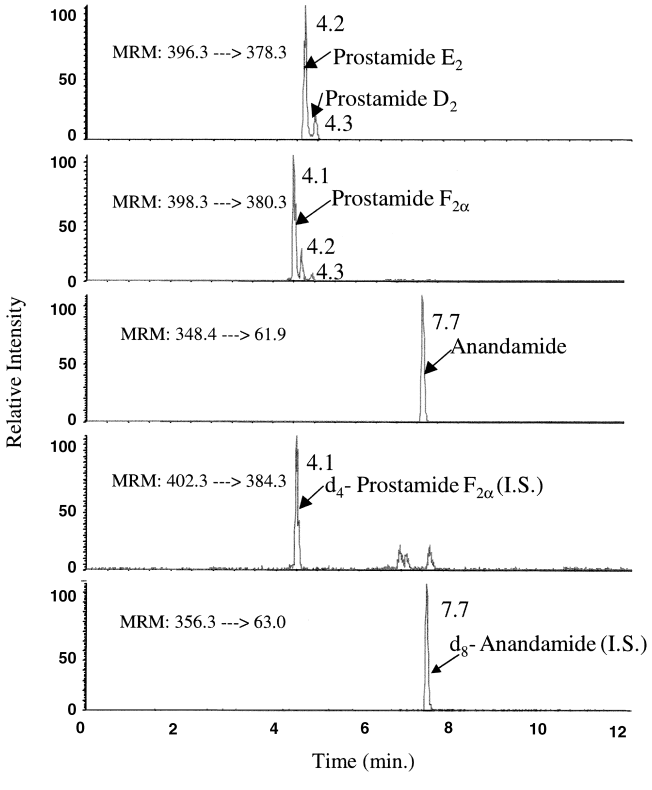


**Fig. 1.** Liquid chromatography tandem mass spectrometry (LC-MS/MS) chromatograms of reference standards containing 1.0 ng of anandamide, $PGF_{2\alpha}$ 1-ethanolamide (prostamide $F_{2\alpha}$), $PGE_2$ 1-ethanolamide (prostamide $E_2$), and $PGD_2$ 1-ethanolamide (prostamide $D_2$), and 0.2 ng of d4-prostamide $F_{2\alpha}$ and d8-anandamide.

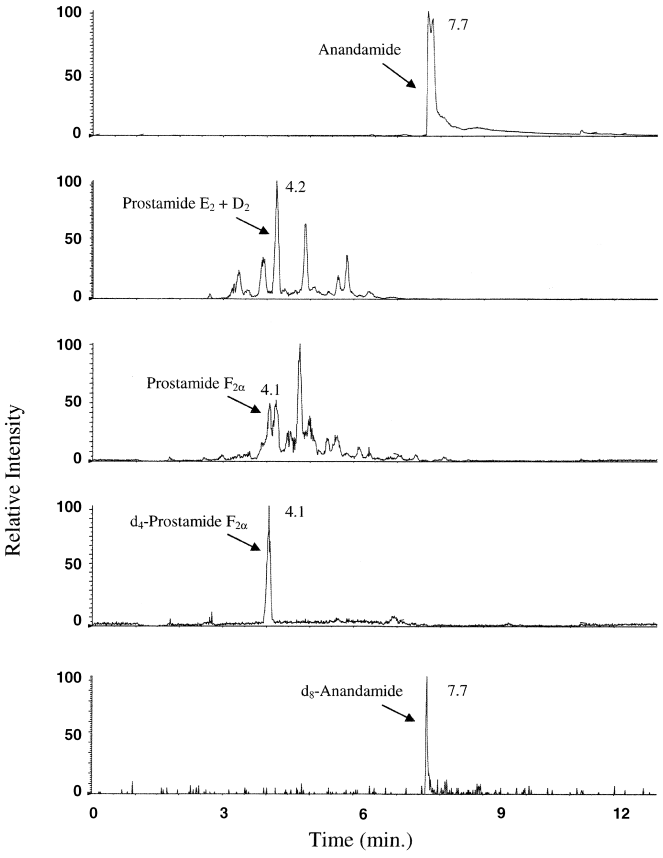


**Fig. 2.** LC-MS/MS chromatograms of anandamide, prostamide $F_{2\alpha}$, prostamide $E_2$, and prostamide $D_2$ with $d_4$-prostamide $F_{2\alpha}$ and $d_8$-anandamide as internal standards in the lung of fatty acid amide hydrolase knockout (FAAH −/−) mouse dosed with anandamide.

$d_4$-prostamide $F_{2\alpha}$ was used as an internal standard for quantifying prostamide $E_2$+$D_2$ and prostamide $F_{2\alpha}$. Likewise, the $d_8$-anandamide was used as an internal standard to quantify anandamide. The calibration range for anandamide was from 0.05 ng/ml to 50 ng/ml, and correlation coefficient was at 0.9973. The calibration range for prostamide $F_{2\alpha}$ was from 0.125 ng/ml to 50 ng/ml, and correlation coefficient was at 0.9927. The calibration range for prostamide $E_2$+$D_2$ was from 0.05 ng/ml to 50 ng/ml, and correlation coefficient was at 0.9894. The accuracy of the back-calculated concentrations of standards was within 80–120%. The precision (CV%) of the assays was ±20%.

### Prostamide quantitation in animal tissue

Mice were fully recovered from the cannulation prior to anandamide administration. FAAH −/− mice receiving anandamide became unconscious for 10–15 min but gradually recovered prior to euthanasia. The sample preparation for the LC-MS/MS quantitation was effective as demonstrated by the ⩾80% recovery of radioactivity using liquid-liquid extraction with acetonitrile from mouse plasma, human plasma, and buffered solutions spiked with [$^3$H]prostamide $F_{2\alpha}$ (data not shown). Concentrations of prostamides $F_{2\alpha}$, $E_2$, and $D_2$ and anandamide in the liver, kidney, lung, and small intestine are depicted in **Table 3**.

There were strong correlations between the tissue concentrations obtained from parent ion → $(MH\text{-}H_2O)^+$ MRM (reported in Table 1) and those obtained from parent ion → *m/z* 62. Although the former MRM method was more sensitive than the latter one, the latter was more selective as shown by less interfering peaks (**Figs. 2**, **3**).

The limited sample size did not allow for meaningful statistical analysis. The variability for tissue concentration of the endogenous compounds in this study had approximate CV values approaching 100%; however, the observed concentrations in tissues between the different groups (n = 3) increased by several orders of magnitude.

Anandamide was found in all tissues collected from all mice. This is consistent with published results that anandamide was found in rodent plasma and brain (19, 20), porcine ocular tissues (21), and our finding that anandamide was found in ocular tissues (22), indicating that endogenous anandamide may be ubiquitous throughout the body. Concentrations of anandamide increased over 100× in liver, kidney, and lung in normal mice receiving exoge-

TABLE 3. The concentrations of prostamide $F_{2\alpha}$, prostamide $E_2$+$D_2$, and anandamide in the liver, kidney, lung, and small intestine of naïve control mice, treated control mice, and treated FAAH −/− mice (n = 3)[a]

| Anandamide (ng/g) | | | | | | |
|---|---|---|---|---|---|---|
| Tissues | Naïve | | Treated Controls | | Treated FAAH −/− | |
| | *Mean* | *SD* | *Mean* | *SD* | *Mean* | *SD* |
| Liver | 0.14 | 0.04 | 54.9 | 42.7 | 239 | 169 |
| Kidney | 0.63 | 0.28 | 88.0 | 25.8 | 347 | 314 |
| Lung | 0.32 | 0.19 | 85.6 | 31.2 | 286 | 184 |
| Small intestine | 3.22 | 5.47 | 5.22 | 6.71 | 85.4 | 77.8 |
| **Prostamide $F_{2\alpha}$ (ng/g)** | | | | | | |
| Tissues | Naïve | | Treated Controls | | Treated FAAH −/− | |
| Liver | BLQ[b] | — | BLQ | — | 14.1 | 2.9 |
| Kidney | BLQ | — | BLQ | — | 6.15 | 4.88 |
| Lung | BLQ | — | BLQ | — | 12.5 | 14.7 |
| Small intestine | BLQ | — | BLQ | — | 0.45 | 0.51 |
| **Prostamide $E_2$+$D_2$ (ng/g)[c]** | | | | | | |
| Tissues | Naïve | | Treated Controls | | Treated FAAH −/− | |
| Liver | BLQ | — | BLQ | — | 0.12 | 0.21 |
| Kidney | BLQ | — | 0.36 | 0.08 | 3.72 | 6.44 |
| Lung | BLQ | — | 3.17 | 1.95 | 13.4 | 12.2 |
| Small intestine | BLQ | — | BLQ | — | 0.45 | 0.51 |

FAAH, fatty acid amide hydrolase; FAAH −/−, FAAH knockout.

[a] → Naïve—Naïve normal mice. Treated controls—normal mice receiving 50 mg/kg of intravenous anandamide. Treated FAAH knockouts—FAAH knockout mice receiving 50 mg/kg of intravenous anandamide.

[b] → BLQ is "below the limit of quantitation" defined as less than 50 pg/ml for each compound.

[c] → Prostamide $E_2$ and prostamide $D_2$ were not always separated chromatographically; therefore, the sum of prostamide $E_2$ and $D_2$ was reported as prostamide $E_2$+$D_2$.

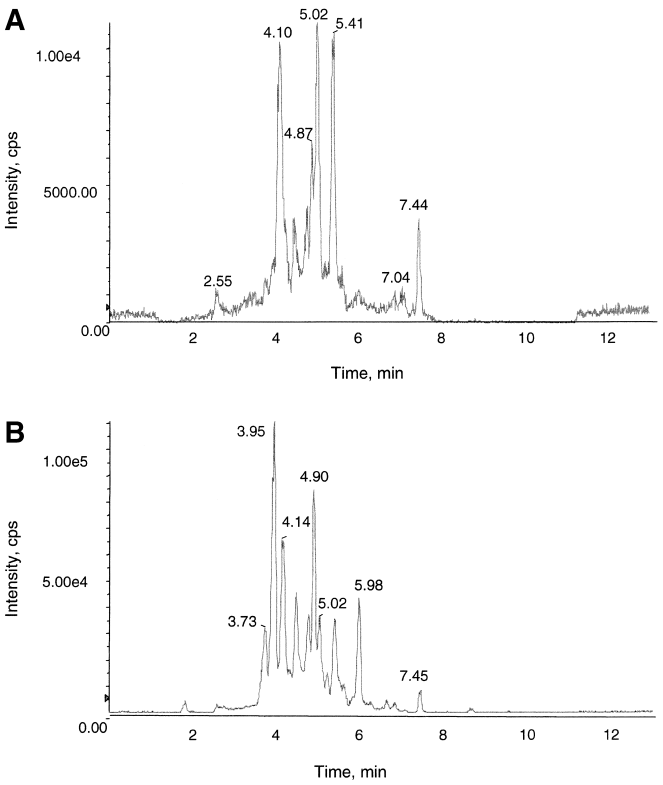


**Fig. 3.** Representative multiple-reaction monitoring chromatograms with a 398.2/380.2 transition pair for liver in normal mouse receiving anandamide (A), and an FAAH −/− mouse receiving anandamide (B). Note that the *y* axis of B is 10-fold higher than the *y* axis of A.

nous anandamide when compared with that in naïve animals. The small intestine showed only a modest increase in anandamide concentration after exogenous administration. Additional increases of 3–4× in concentration were found in the treated FAAH −/− mice when compared with normal mice receiving exogenous anandamide in liver, lung, and kidney. In the small intestine, the increase with treated FAAH −/− mice was more than 15× over treated normal mice.

Prostamide $F_{2\alpha}$ was not detected in the tissues from control mice but was found in all tissues from treated FAAH −/− mice, with the highest amount found in the liver (14.1 ± 2.9 ng/g) and the lowest amount in the small intestine (0.45 ± 0.51 ng/g). Prostamides $E_2$+$D_2$ were found only in animals receiving the exogenous anandamide but were highest in the FAAH −/− mice. The lung had the highest concentrations of prostamides $E_2$+$D_2$: 3.17 ± 1.95 ng/g in the normal mice receiving exogenous anandamide and 13.4 ± 12.2 ng/g in the treated FAAH −/− mice.

Multiple peaks eluted during the chromatography, which have the same transition periods as prostamide $F_{2\alpha}$ and prostamide $E_2$+$D_2$, suggest that unknown anandamide metabolites may exist with the same molecular weight as prostamide $F_{2\alpha}$ and prostamide $E_2$+$D_2$ but with different retention times. Figure 3 is a representative chromatogram with a *m/z* 398/380 transition pair for both liver samples in a normal mouse receiving anandamide and in an FAAH −/− mouse receiving anandamide. The peaks in the chromatogram from the FAAH −/− mouse were 10-fold higher than those in the chromatogram of the normal mouse.

Analysis of tissues, using the MRM ion pair of $MH^+$ > ion of *m/z* 62 and retention times, confirmed that these were prostamides containing the ethanolamide moiety, $HO\text{-}(CH_2)_2\text{-}NH_2$, and the peaks quantitated were indeed prostamides.

### Product ion spectra

The product ion spectra of anandamide and its metabolite prostamide $F_{2\alpha}$ detected in FAAH −/− mice were compared with those of anandamide and prostamide $F_{2\alpha}$ reference standards. The product ion spectra for standards of anandamide, prostamide $F_{2\alpha}$, prostamide $D_2$, and prostamide $E_2$ are depicted in **Fig. 4**. Anandamide observed in the liver of FAAH −/− mice dosed with anandamide showed the characteristic protonated ethanolamine ion at *m/z* 62 in the product ion spectrum of protonated anandamide at *m/z* 348 (**Fig. 5**). The retention time of the peak and the fragmentation pattern were consistent with those of the anandamide reference standard. Similarly, prostamide $F_{2\alpha}$ observed in the liver of FAAH −/− mice dosed with anandamide (Fig. 5) also shows the characteristic protonated ethanolamine ion at *m/z* 62, as well as the peaks corresponding to the sequential water loss from the

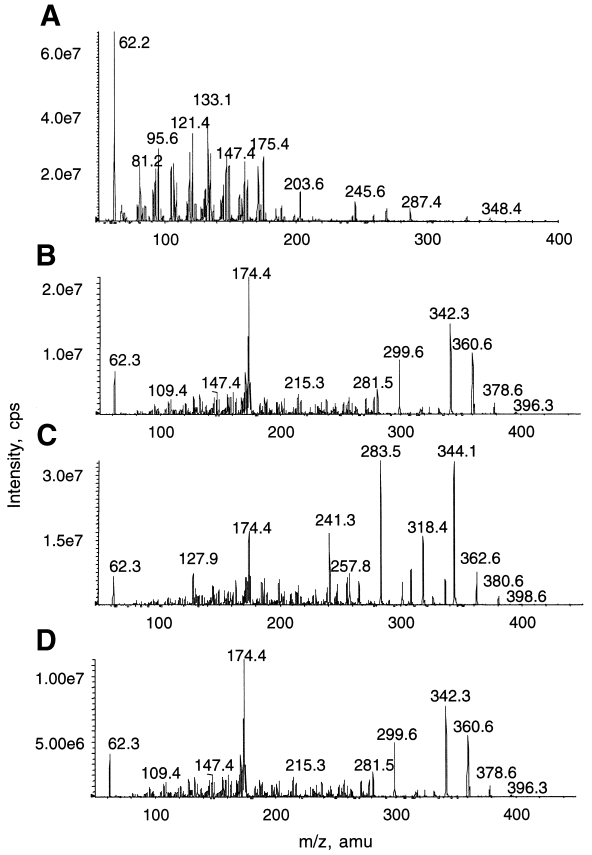


**Fig. 4.** Product ion spectra for standards of anandamide (*m/z* 348) (A), prostamide $E_2$ (*m/z* 396) (B), prostamide $F_{2\alpha}$ (*m/z* 398) (C), and prostamide $D_2$ (*m/z* 396) (D).

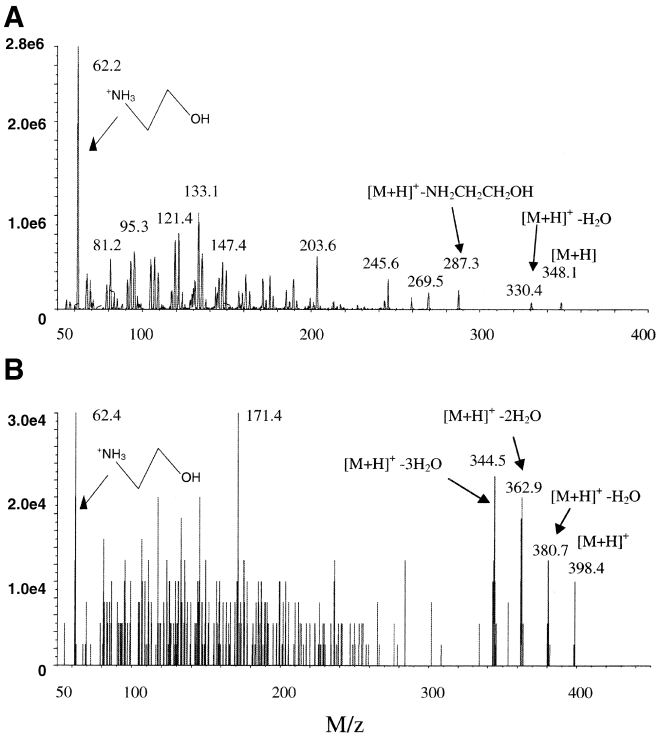


**Fig. 5.** Product ion spectrum of proposed anandamide (A) and prostamide $F_{2\alpha}$ (B) moieties observed in the liver of an FAAH −/− mouse dosed with anandamide.

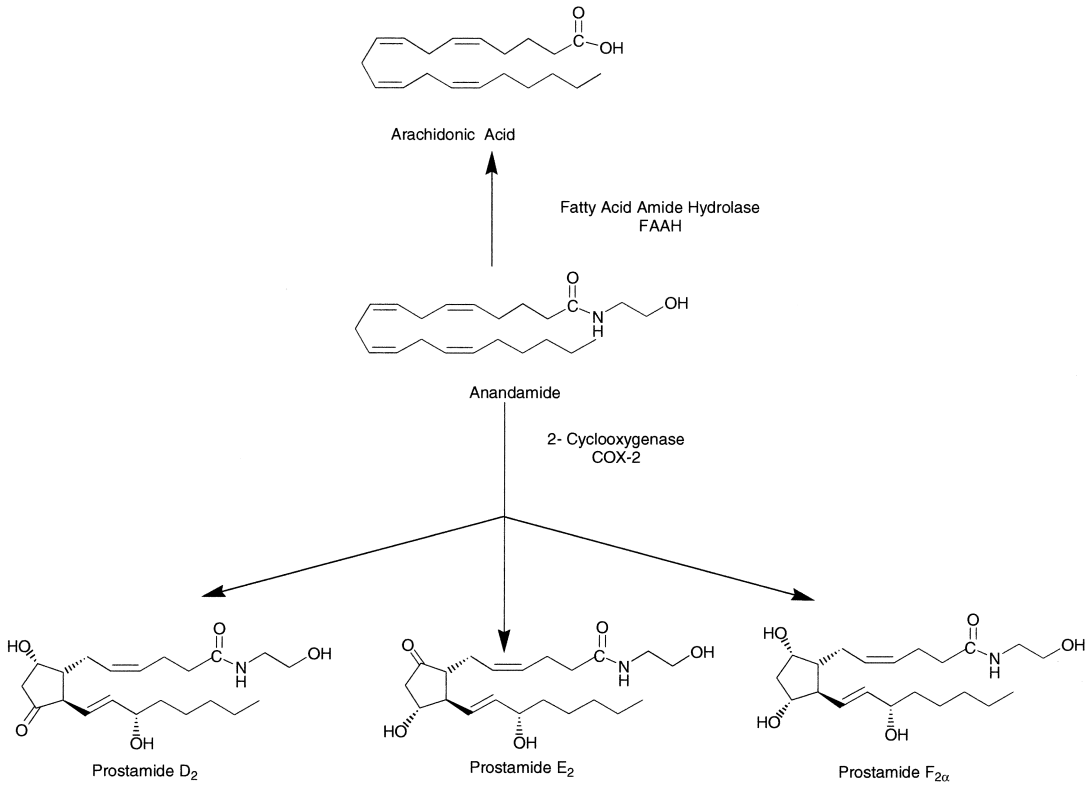


**Fig. 6.** The proposed biosynthetic pathway of prostamides $F_{2\alpha}$, $E_2$, and $D_2$ from anandamide.

parent drug in the product ion spectrum of *m/z* 398 (Figs. 4, 5). The retention times of the peak and fragmentation pattern were consistent with those of the prostamide $F_{2\alpha}$ reference standard.

## DISCUSSION

Both arachidonic acid and anandamide have been shown to be substrates of COX-2, an inducible COX isoform forming prostaglandins or prostamides, respectively. Prostaglandins are short-lived and yet can have substantial biological effects. In contrast, prostamides and glyceryl esters are not efficient substrates for 15-hydroxyprostaglandin dehydrogenase; therefore, endocannabinoid-derived COX-2 metabolites may be sufficiently stable to exert systemic activity (15). Furthermore, prostamides are not inhibitors of FAAH hydrolysis of anandamide (23), therefore, prostamides are not substrates of FAAH. Pharmacological investigation of prostamide activity is in its infancy, but studies to date indicate potent ocular hypotensive activity and a pharmacological activity profile that is distinct from known prostaglandin receptors (14, 24). The endogenous pool of anandamide is substantially less than that of arachidonic acid. Consequently, the detection of the prostamides is more difficult. Nevertheless, by using FAAH −/− mice we were able to measure prostamides and thereby demonstrate that prostamides can be formed in vivo by the proposed biosynthetic pathway from anandamide as depicted in **Fig. 6**. The major pathway of anandamide metabolism is the hydrolysis by FAAH; however, other pathways involving COX-2 can eventually lead to the formation of prostamides.

## CONCLUSION

Prostamides were detected in tissues of FAAH −/− mice after administration of anandamide using LC-MS/MS analysis. Concentrations of anandamide, prostamide $F_{2\alpha}$, and prostamide $E_2$+$D_2$ in liver, kidney, lung, and small intestine were much higher in the anandamide-treated FAAH −/− mice than those in anandamide-treated normal mice. This is the first report demonstrating that prostamides, including prostamide $F_{2\alpha}$, were formed from anandamide in vivo, possibly by the COX-2 pathway.

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