Synthesis of Prostaglandin E2 Ethanolamide from Anandamide by Cyclooxygenase-2*

(Received for publication, April 23, 1997, and in revised form, June 12, 1997)

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Because of its structural similarity to polyunsaturated fatty acids, anandamide could serve as substrate for enzymes such as lipoxygenases and cyclooxygenases, which metabolize polyunsaturated fatty acids to potent bioactive metabolites. Here the ability of recombinant human cyclooxygenase-1 (hCOX-1) and cyclooxygenase-2 (hCOX-2) to metabolize anandamide was studied. Baculovirus-expressed and -purified hCOX-2, but not hCOX-1, effectively oxygenated anandamide. Reverse phase high pressure liquid chromatography analysis of the products derived from 1-14C-labeled anandamide showed that the products formed are similar to those formed with arachidonic acid as substrate. The major prostaglandin product derived from anandamide was determined by mass spectrometry to be prostaglandin E2 ethanolamide. Incubation of anandamide with lysates and the intact cell line expressing COX-2 but not that of COX-1 produced prostaglandin E2 ethanolamide. These results demonstrate the existence of a COX-2-mediated pathway for anandamide metabolism, and the metabolites formed represent a novel class of prostaglandins.

Anandamide (arachidonoyl ethanolamide, AEA) is a polyunsaturated fatty acyl amide that was identified from porcine brain lipids as an endogenous ligand for brain cannabinoid receptor (1). Although structurally different from cannabinoids, AEA by its ability to activate the central CB1 receptor displays pharmacological properties similar to cannabinoids (2, 3). In addition to its central action via the CB1 receptor, AEA displays potent immunomodulatory and anti-inflammatory activities by interacting with peripheral CB1 and/or CB2 receptors (4–6).

Free AEA is present in both central and peripheral tissues (see Ref. 7 for a review) and could interact with CB receptors to display some of its immunomodulatory and anti-inflammatory activities. In addition, AEA is also stored esterified to phosphatidylethanolamines and is released by the action of phospholipase D in response to various stimuli (7). The AEA thus released inside the cell could participate in signal transduction as a second messenger and display some of its immunomodulatory and anti-inflammatory activities independent of its interaction with the CB receptors. In fact, AEA has been shown to antagonize CB2 receptors, and it is not clear how this antagonism results in immunomodulatory activities observed in cells only expressing CB2 receptors (8). It is possible that a metabolite of AEA rather than AEA itself could account for all or some of these properties. Furthermore, because of its structural similarities to polyunsaturated fatty acids, endogenously released AEA could serve as substrate for lipoxygenases and cyclooxygenases (COX) that metabolize polyunsaturated fatty acids to potent bioactive molecules. It has been demonstrated that lipoxygenase could metabolize AEA, and the metabolites have potent biological activities (9, 10). However, it is not known whether COX can metabolize AEA. Arachidonic acid (AA) is the substrate for both COX-1 and COX-2. AEA is structurally identical to AA except that the carboxylic acid group of AA is replaced by an ethanolamide group in AEA.

Here, we report for the first time the ability of hCOX-2 but not hCOX-1 to bind and oxidize AEA. We further demonstrate that the products of AEA metabolism by COX-2 are unique and represent a novel class of eicosanoids.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled AA, PGE2, AEA, and PGE2 ethanolamide were purchased from Cayman Chemicals (Ann Arbor, MI). 1-14C Arachidonic acid (specific radioactivity, 55–56 mCi/mmol) was purchased from Amersham Corp. 1-14C Arachidonoyl ethanolamide (specific radioactivity, 55–56 mCi/mmol) was a kind gift from Dr. M. Masjedianzadeh (Roche Bioscience, Radiochemistry Laboratory, Palo Alto, CA).

Recombinant COX-1 and COX-2 Enzymes—Recombinant human COX-1 and COX-2 enzymes were expressed in a baculovirus system and partially purified as described previously (17). The specific activity of the enzymes used were between 13,000 and 21,000 units (1 unit = 1 nmol of oxygen consumed/mg of protein/min) with a purity of ~60% as judged by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining.

Enzymatic Assays—The purified COX enzymes were reconstituted with 2 mM phenol and 1 mM hematin (Sigma), and the cyclooxygenase activity was measured either by the oxygen uptake assay or by the radiometric assay exactly as described previously (17). Km values were determined radiometrically using 2–200 μM 1-14C-labeled AA or AEA (specific activity, 3–4 × 106 cpm/nmol). For characterization of the metabolites by RP-HPLC, reconstituted hCOX-1 and hCOX-2 enzymes were incubated in a total volume of 100 μl with 40 μM [1-14C]AA or [1-14C]AEA (specific activity, 3–4 × 106 cpm/nmol) for 5 min at room temperature. The reaction was stopped by the addition of 10 μl of 2 N HCl and 1 volume of methanol. An aliquot (50–75 μl) was directly injected and analyzed by HPLC as described below. For characterization of the metabolites by HPLC-MS, unlabeled AA and AEA were used and processed as above for RP-HPLC studies.

Cell Culture—Human promonocytic THP cells were grown in RPMI medium (Life Technologies, Inc.), washed, and suspended in fresh RPMI medium. COX-1 was induced by treating cells with 0.1 μM phorbol 12-myristate 13-acetate (Sigma) for 40–48 h at 37 °C. Primary cultures of human foreskin fibroblasts (HFF) were obtained from ATCC.

The abbreviations used are: AEA, arachidonoyl ethanolamide; HPLC, high pressure liquid chromatography; RP-HPLC, reverse phase HPLC; MS, mass spectrometry; COX, cyclooxygenase; COX-1, cyclooxygenase-1 or prostaglandin H synthase isoenzyme-1; COX-2, cyclooxygenase-2 or prostaglandin H synthase isoenzyme-2; hCOX-1, human COX-1; hCOX-2, human COX-2; AA, arachidonic acid; PGE2, prostaglandin E2; PGD2, prostaglandin D2; 15-HETE, 15-hydroxyeicosatetraenoic acid; 12-HHT, 12-hydroxyheptadecatrienoic acid; HFF, human foreskin fibroblasts; LC, liquid chromatography.

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and grown to about 70% confluency in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1% penicillin, and 1% streptomycin (Life Technologies, Inc.). Cells were then treated with 0.1 μM phorbol 12-myristate 13-acetate (Calbiochem) and 10 ng/ml interleukin-1α (Sigma) to induce COX-2 (18).

**Metabolism of AA and AEA by THP and HFF Cells**—COX-1-expressing THP cells and COX-2-expressing HFF cells were suspended in 50 mM Tris-HCl buffer containing 2 mM EDTA. Cells (5–8 × 10⁵ cells) were incubated with 50–100 μM of either AA (open bar) or AEA (filled bar). Oxygen consumption was measured using an O₂ electrode, and the initial rate of O₂ consumption was determined. The rate of O₂ consumption by hCOX-1 and hCOX-2 with AA substrate was normalized to 100% and compared with that with AEA as substrate. Each value represents mean ± S.D. of five or more independent determinations.

**Metabolism of AEA by Cell Lysate**—Following COX-2 induction, HFF cells were detached by treating with trypsin and washing with phosphate-buffered saline. COX-1-expressing THP cells and COX-2-expressing HFF cells were suspended in 50 mM Tris-HCl buffer containing 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 20 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 10 μg/ml pepstatin. Cells were lysed by homogenization, and the cell lysate was stored on ice until used. Lysates (200–400 μg) from COX-1- or COX-2-expressing cells were incubated with either [1-14C]AA or [1-14C]AEA (specific activity, 3–4 × 10⁶ cpm/nmol) for 30 min at 30 °C. The reaction was stopped and 1 volume of isopropanol:acetic acid (100:1.2, v/v) and 1 volume of chloroform. The tubes were mixed by vortexing and centrifuged (1000 g, 10 min); the lower chloroform layer was aspirated and concentrated to dryness, and the residue was dissolved in 200 μl of methanol and analyzed by HPLC as described below.

**Metabolism of AEA by Cell Lysate**—Following COX-2 induction, HFF cells were detached by treating with trypsin and washing with phosphate-buffered saline. COX-1-expressing THP cells and COX-2-expressing HFF cells were suspended in 50 mM Tris-HCl buffer containing 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 20 μg/ml leupeptin, 10 μg/ml trypsin inhibitor, and 10 μg/ml pepstatin. Cells were lysed by homogenization, and the cell lysate was stored on ice until used. Lysates (200–400 μg) from COX-1- or COX-2-expressing cells were incubated with either [1-14C]AA or [1-14C]AEA (40 μM) for 20 min at 30 °C. The reaction was stopped and processed as described above for intact cells.

**HPLC**—Products from the reaction of [14C]AA and [14C]AEA with purified hCOX-1 and hCOX-2, intact cells, and the cell lysates were separated using a Shimadzu LC-6A HPLC system. RP-HPLC was carried out on a Waters μBondapak C18 column (300 × 3.9 mm, 10-μm particle size) using solvent A (water/trifluoroacetic acid, 100:0.05, v/v) and solvent B (acetonitrile/trifluoroacetic acid, 100:0.05, v/v). The initial settings were 35% solvent B from 0 to 5 min, which was linearly increased to 60% solvent B in the next 30 min, held there for an additional 5 min, linearly increased to 100% solvent B in the next 10 min, and held there for 9 min. The flow rate was 1.5 ml/min, and the total run time was 60 min. The radioactivity in the column effluent was detected using an on-line Beckman 171 radioactivity detector. The signals from the detector were acquired, stored, and analyzed using

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**Fig. 1. Utilization of AEA as substrate by hCOX-2.** Purified hCOX-1 (PGHS-1) and hCOX-2 (PGHS-2) enzymes were reconstituted with phenol and heme as described under “Experimental Procedures” and incubated with 100 μM of either AA (open bar) or AEA (filled bar). Oxygen consumption was measured using an O₂ electrode, and the initial rate of O₂ consumption was determined. The rate of O₂ consumption by hCOX-1 and hCOX-2 with AA substrate was normalized to 100% and compared with that with AEA as substrate. Each value represents mean ± S.D. of five or more independent determinations.

**Fig. 2. Metabolism of AA and AEA by hCOX-1 and hCOX-2.** Purified hCOX-1 (panels A and B) and hCOX-2 (panels C and D) enzymes were reconstituted with phenol and heme as described under “Experimental Procedures” and incubated with 40 μM of either [1-14C]AA (panels A and C) or [1-14C]AEA (panels B and D) for 5 min. The reaction was stopped and analyzed by RP-HPLC as described under “Experimental Procedures.” Peak 3 (panels A and C) has the same retention time as [3H]PGE₂ standard. Peak 9 is AA and peak 9a is AEA.

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**Table I**

Kinetic parameters of hCOX-1 and hCOX-2 for AA and AEA substrates

| Substrate | hCOX-1 | hCOX-2 |
|-----------|--------|--------|
| AA        | 5.1 ± 1.2 | 8.2 ± 1.3 |
| AEA       | ND*    | 23.7 ± 5.7 |

* Specific activity is the initial rate expressed as μmol of oxygen consumed/mg of protein/min and is the average of two determinations (COX-1) or mean ± S.D. of three determinations (COX-2).

* Not determined.
RESULTS AND DISCUSSION

There is considerable interest in recent years in understanding the metabolism of AEA as a mechanism of termination of its potent neuromodulatory and immunomodulatory activities. The principal mode of metabolism of AEA appears to be an amidohydrolase-mediated hydrolysis to AA and ethanolamine (19, 20). Here we test the ability of COX enzymes to metabolize AEA. Fig. 1 shows the relative initial rates of oxygenation of AA and AEA by recombinant hCOX-1 and hCOX-2. Both enzymes effectively oxygenated AA. In contrast, COX-2 oxygenated AEA at 60–85% of the rates observed with AA, whereas COX-1 displayed no detectable activity against this substrate. Using the more sensitive radiometric method (17) no activity was detected for COX-1 with AEA as substrate (data not shown). Furthermore, when 2–5 molar excess unlabeled AEA was added along with [1-14C]AA, no inhibition of AA metabolism by COX-1 was observed suggesting that not only is AEA not a substrate for COX-1 but it also fails to compete with AA.

Fig. 3. Reconstituted LC-MS profiles of metabolites of AA and AEA by hCOX-2. Details of the experiment are as in Fig. 2, except unlabeled AA and AEA were used, and following the reaction the samples were separated and analyzed by LC-MS as described under "Experimental Procedures." Panel A is the base peak profiles of metabolites of AA with hCOX-2, and panel B is the base peak profiles of metabolites of AEA with hCOX-2. Peak numbers correspond to those in Fig. 2. Peaks eluting between 36 and 40 min are due to detergents in the enzyme preparations.
for binding to the enzyme active site (data not shown).

The affinity of hCOX-2 for AEA was consistently lower than that for AA as evident from the mean $K_m$ values for the two substrates (Table I). The initial rates of oxygenation of AEA by COX-2 were also consistently lower than that of AA but did not achieve statistical significance because of batch to batch variations in the specific activities of COX-2. Furthermore, as with AA, COX-2 underwent inactivation following oxygenation of AEA (data not shown). With both AA and AEA as substrate, the COX-2 enzyme turned over $\sim$250–400 times before it inactivated. Thus, it appears that the mechanism involved in the oxidation of AEA by COX-2 is similar to that of AA.

Next, to characterize the metabolites, the reaction products of AEA with COX-2 were analyzed by HPLC. Fig. 2 shows the RP-HPLC profiles of the metabolites of the reaction of hCOX-1 and hCOX-2 with the $^{14}$C-labeled AA and AEA. Once again, it is clear that hCOX-1 was unable to use AEA as substrate (Fig. 2B), whereas it effectively converted AA into one major product (Fig. 2A, peak 3) that comigrated with the $[^3]$H]PGE$_2$ standard. Small amounts of minor metabolites (peaks 4–8) were also formed. The concentrations of these minor metabolites varied from assay to assay and represented $\leq$1–5% of the total radioactivity. In contrast to hCOX-1, hCOX-2 effectively oxygenated both AA and AEA as substrates (Fig. 2, C and D). The profiles of the products formed from these two substrates by hCOX-2 were similar except for their retention times (compare Fig. 2C with 2D). Here again, hCOX-2 produced one major metabolite with AA (Fig. 2C, peak 3) that comigrated with the $[^3]$H]PGE$_2$
standard. It also produced significant amounts of other metabolites (peaks 5–8), which is in agreement with previous reports (21, 22). These minor peaks displayed significant UV absorption at 235 nm, and two of these peaks (peak 6 and 8) had the same retention time as 12-HHT and 15-HETE, respectively. The metabolic profile of the products of hCOX-2 with AEA was very much identical to that with AA, except for shorter retention time because of the ethanolamide group and relative abundance of the individual peaks (Fig. 2D).

The major metabolite of AEA (Fig. 2D, peak 3a) did not display any UV absorption suggesting that it is unlikely to have conjugated double bonds. To get more structural information, the products of the reaction of hCOX-2 with AA and AEA were subsequently analyzed by LC-MS, and the base peak profiles are shown in Fig. 3. The mass spectra of major peaks of interest are shown in Figs. 4 and 5. Peak 3 (Fig. 3A) displayed a fragmentation ion pattern identical to the synthetic PGE2 standard (compare Fig. 4A with 4B). The base peak at m/z 317 was due to the loss of a –OH group and a molecule of H2O from the molecular ion (M − 35). Other diagnostic fragment ions included M + H2O (at m/z 370), the molecular ion (at m/z 352), and M − OH (at m/z 335). Similarly, peak 3a (Fig. 3B) displayed a fragmentation ion pattern identical to the synthetic PGE2 ethanolamide (compare Fig. 5A with 5B). The base peak at m/z 378 was due to the loss of a −OH group from the molecular ion. Other diagnostic ion fragments included MH+
expressing COX-1 or COX-2 and THP cells expressing COX-1 with \(^{14}\text{C}\)labeled AEA, and the products formed were analyzed by RP-HPLC (Fig. 6). Like the purified enzyme, HFF cells expressing COX-2 effectively oxygenated AEA. The profile of the products formed from AEA by the COX-2-expressing cells was identical to that formed by the purified enzyme (Fig. 6B). Similar results were also obtained when \(^{14}\text{C}\)AEA was incubated with cell-free preparations of COX-2-expressing HFF cells. On the other hand, neither the intact cells nor the lysates of cells expressing COX-1 oxygenated \(^{14}\text{C}\)AEA (data not shown). These data clearly demonstrate that AEA is also metabolized by COX-2 in a more physiological environment.

At the present time the physiological significance of the metabolism of AEA by COX-2 is not known. Several possible roles for this pathway can be speculated. First, metabolism of AEA by COX-2 is yet another physiological mechanism by which AEA is inactivated. Second, this could be a pathway for generating novel prostaglandins that in turn might have many important pathophysiological functions. Third, by its ability to compete with AA, AEA might modulate the local prostaglandin tone mediated by COX-2. Our future work should provide answers to some of these questions.

Acknowledgments—We thank Drs. David Swinney and Richard Eglen for helpful discussion and comments. M. Magjedizadeh for synthesizing \(^{14}\text{C}\)anandamide, Drs. B. Schwartz and J. Haung for LC-MS analysis, and L. A. Taylor for growing COX-1- and COX-2-expressing cells.

REFERENCES

1. Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, D., Mandelbaum, A., Eltinger, A., and Mechoulam, R. (1992) Science 258, 1946–1949
2. Crawley, J. N., Corwin, R. L., Robinson, J. K., Felder, C. C., Devane, W. A., and Axelrod, J. (1995) Pharmacol. Biochem. Behav. 50, 967–972
3. Smith, P. B., Compton, D. R., Welch, S. P., Radan, R. K., Mechoulam, R., and Martin B. R. (1994) J. Pharmacol. Exp. Ther. 270, 219–227
4. Schwartz, H., Bianco, F. J., and Lutz, M. (1994) J. Neuroimmunol. 55, 107–115
5. Stefano, G. B., Liu, Y., and Goligorsky, M. S. (1996) J. Biol. Chem. 271, 19238–19242
6. Bayewitch, M., Rhee, M.-H., Avidor-Reiss, T., Breuer, A., Mechoulam, R., and Vogel, Z. (1996) J. Biol. Chem. 271, 9902–9905
7. Schmid, H. O., Schmidt, P. C., and Natarajan, V. (1996) Chem. Phys. Lipids 80, 133–142
8. Barnette-Curley, D., and Cabral, G. A. (1995) Proc. Soc. Exp. Biol. Med. 210, 64–76
9. Hapson, A. J., Hill, W. A. G., Zan-Phillips, M., Makriyannis, A., Leung, E., Eglen, R. C., and Bornheim, L. M. (1986) Biochim. Biophys. Acta 859, 173–179
10. Ueda, N., Yamamoto, K., Kurahashi, Y., Yamamoto, S., Ogawa, M., Matsu, N., Kudo, I., Shinkai, H., Shiri-kawa, E., and Tokunaga, T. (1995) Adv. Prostaglandin Thromboxane Leukotriene Res. 31, 163–165
11. Laneville, O., Breuer, D. K., Xu, N., Huang, Z. H., Gage, D. A., Watson, J. T., Lagarde, M., DeWitt, D. L., and Smith, W. L. (1995) J. Biol. Chem. 270, 19330–19336
12. Lueng, C., Miller, A., Barnett, J., Chow, J., Ramesha, C., and Browner, M. F. (1996) Nat. Struct. Biol. 3, 927–933
13. Mancini, J. A., Rienteau, D., Falgueyret, J. P., Vickers, P. J., and O'Neill, G. P. (1995) J. Biol. Chem. 270, 29072–29077
14. Bhattacharaya, D. K., Leomonte, M., Rieke, C. J., Garavito, R. M., and Smith, W. L. (1996) J. Biol. Chem. 271, 2179–2184
15. Picot, D., Loll, P. J., and Geraviti, M. (1994) Nature 367, 243–249
16. Kurumah, R. G., Stevens, A. M., Giese, J. K., McDonald, J. J., Stegeman, R. A., Pak, J. Y., Gildehaus, D., Miyashiro, J. M., Penning, T. D., Seibert, K., Isakson, P. C., and Stallings, W. C. (1997) Nature 385, 555–560
17. Barnett, J., Chow, J., Ives, D., Choas, M., Mackenzie, R., Osen, O., Nguyen, B., Tsin, S., Bach, C., Freire, J., Chan, H., Sigal, E., and Ramesha, C. (1997) Biochim. Biophys. Acta 252, 130–139
18. Raz, A., Wyche, A., Siegel, N., and Needleman, P. (1988) J. Biol. Chem. 263, 3022–3028
19. Ueda, N., Kurahashi, Y., Yamamoto, S., and Tokunaga, T. (1995) J. Biol. Chem. 270, 23823–23827
20. Bisogno, T., Maurelli, S., Melck, D., De Petrocellis, L., and Di Marzo, V. (1997) Proc. Natl. Acad. Sci. USA 94, 17347–17351
21. Mancini, J. A., Rienteau, D., Falgueyret, J. P., Vickers, P. J., and O'Neill, G. P. (1995) J. Biol. Chem. 270, 29072–29077
22. Bhattacharaya, D. K., Leomonte, M., Rieke, C. J., Garavito, R. M., and Smith, W. L. (1996) J. Biol. Chem. 271, 2179–2184
23. Holtzman, M. J., Turk, J., and Shornick, L. P. (1992) J. Biol. Chem. 268, 6610–6614
24. Gottsch, M. J., Turk, J., and Shornick, L. P. (1992) J. Biol. Chem. 268, 21438–21445
25. Hamberg, M., and Samuelsson, B. (1967) J. Biol. Chem. 242, 5336–5343