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Differential Sensitivity and Mechanism of Inhibition of COX-2 Oxygenation of Arachidonic Acid and 2-Arachidonoylglycerol by Ibuprofen and Mefenamic Acid†

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ABSTRACT: Ibuprofen and mefenamic acid are weak, competitive inhibitors of cyclooxygenase-2 (COX-2) oxygenation of arachidonic acid (AA) but potent, noncompetitive inhibitors of 2-arachidonoylglycerol (2-AG) oxygenation. The slow, tight-binding inhibitor, indomethacin, is a potent inhibitor of 2-AG and AA oxygenation whereas the rapidly reversible inhibitor, 2′-*des*-methylindomethacin, is a potent inhibitor of 2-AG oxygenation but a poor inhibitor of AA oxygenation. These observations are consistent with a model in which inhibitors bind in one subunit of COX-2 and inhibit 2-AG binding in the other subunit of the homodimeric protein. In contrast, ibuprofen and mefenamate must bind in both subunits to inhibit AA binding.

Cyclooxygenase (COX)[1] enzymes oxygenate polyunsaturated fatty acids to prostaglandin endoperoxides in the first step of a metabolic cascade that leads to the generation of prostaglandins and thromboxanes. Inhibition of COX enzymes, especially COX-2, is a major contributor to the pharmacological effects of nonsteroidal anti-inflammatory drugs (NSAIDs). COXs are homodimers of 70 kDa subunits that are comprised of membrane-binding and catalytic domains (*1*). The cyclooxygenase active site is located deep inside the catalytic domain separated by a gate from a channel that leads through the membrane-binding domain to the exterior of the protein. Recent work indicates that the two monomers of each COX enzyme are functionally interdependent and that binding of a substrate or inhibitor at one active site alters the properties of the other active site (*2*). The communication between subunits occurs through the dimer interface (*3*).

COX-2 oxygenates a range of fatty acyl substrates including fatty acids, esters, and amides. Arachidonic acid (AA) and 2-arachidonoylglycerol (2-AG) are the best acid and ester substrates and display comparable k_{cat}/K_m's for oxygenation (*4*). Despite this similarity, we report here that COX-2 oxygenation of 2-AG is dramatically more sensitive to inhibition by a series of acidic NSAIDs than is oxygenation of AA. In fact, these compounds, which have been considered relatively weak COX inhibitors (i.e., ibuprofen and mefenamic acid), inhibit 2-AG oxygenation at concentrations that are orders of magnitude lower than the concentrations required for inhibition of AA oxygenation.

The potency of individual inhibitors toward AA and 2-AG oxygenation was determined in instantaneous inhibition assays in which the substrate and inhibitor were incubated in an O_2 monitor followed by addition of murine COX-2 (mCOX-2) purified as previously described (*5*). Substrate oxygenation velocities were measured at multiple concentrations of substrate and inhibitor. A plot of V vs [S] for AA oxygenation at increasing ibuprofen concentrations demonstrated increased K_m^{app}'s but comparable V_{max}'s consistent with competitive inhibition (Figure 1A). Similar results were obtained with mefenamic acid (Supporting Information, Figure S1). The K_I's were calculated from a secondary plot of the dependence of K_m^{app} on inhibitor concentration (Supporting Information, Figure S2). Ibuprofen exhibited a K_I of 80 ± 20 μM, whereas mefenamic acid exhibited a K_I of 10 ± 5 μM.

Oxygenation of 2-AG was completely inhibited by the lowest concentrations of inhibitors used in the experiments with AA (ibuprofen-25 μM, mefenamate-5 μM), so a much lower concentration range was evaluated. In contrast to the results with AA, increasing concentrations of inhibitor did not increase the K_m^{app} but rather decreased V_{max} (Figure 1B for ibuprofen and Supporting Information, Figure S1 for mefenamate). The general appearance of the V vs [2-AG] plots at different inhibitor concentrations was not consistent with competitive inhibition but was suggestive of noncompetitive inhibition. Thus, the K_I for ibuprofen was calculated from a secondary plot of $1/V_{max}^{app}$ vs [ibuprofen] and found to be 1.2 μM (Supporting Information, Figure S3).

The concentrations of mefenamic acid that inhibited 2-AG oxygenation were in the range of the enzyme concentration so a K_I could not be calculated from the inhibitor data. Fortunately, mefenamic acid quenches the intrinsic protein fluorescence of apoCOX-2 (without heme) so experiments were conducted using fluorescence quenching to monitor inhibitor association (*6*). The equilibrium constant for dissociation (K_d) could be measured by titration of mCOX-2 with mefenamic acid, as previously described for darbufelone (*7*). Fitting the corrected data to a logarithmic plot of fluorescence vs inhibitor concentration yielded an EC_{50} value representing the apparent K_d of quenching (Supporting Information, Figure S4). The calculated EC_{50} was in the range of the protein concentration so it is only an approximation of the K_d. Therefore, the experiment was repeated at varying enzyme concentrations, and a plot of EC_{50} versus enzyme

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[1]Abbreviations: COX, cyclooxygenase; AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; NSAID, nonsteroidal anti-inflammatory drug.

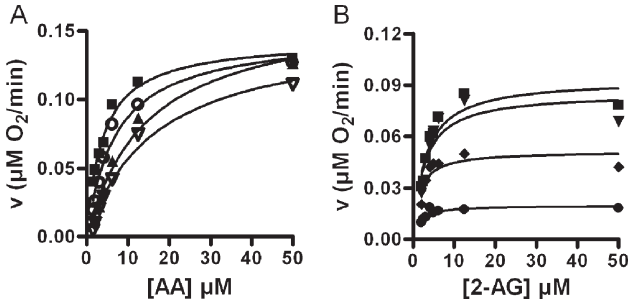

FIGURE 1: Inhibition of mCOX-2 oxygenation of AA and 2-AG by ibuprofen. Ibuprofen and substrate were mixed in an oxygraph cell, and the reaction was initiated by addition of COX-2. The initial velocity of O_2 uptake was determined from a tangent to the most rapidly descending portion of the curve. (A) Ibuprofen at 0 μM (■), 50 μM (○), 200 μM (▲), and 300 μM (●). (B) Instantaneous COX-2 inhibition of 2-AG oxidation by ibuprofen at 0 μM (■), 0.5 μM (▼), 1.25 μM (♦), and 2.5 μM (●).

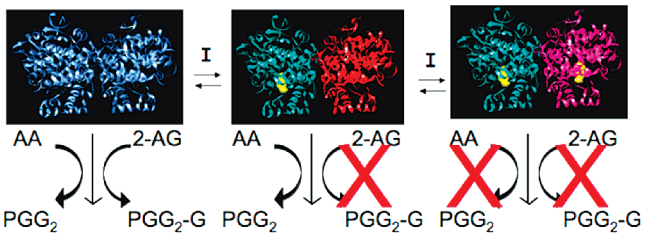

FIGURE 2: Model for differential inhibition 2-AG and AA oxygenation by COX-2. The uninhibited mCOX-2 homodimer (blue) is able to effectively metabolize both AA and 2-AG to form PGG_2 and PGG_2-G. Binding of an inhibitor (yellow) to a single monomer (green) precludes the productive binding of 2-AG in the partner monomer (red) but still allows for AA oxygenation. Metabolism of AA is inhibited only when an inhibitor occupies both active sites of the COX dimer as shown on the far right.

concentration was constructed. The *y*-intercept of this plot (equivalent to the EC_{50} at infinitely small enzyme concentration) provided an estimate of the true K_d (4 nM).

These data suggest that ibuprofen and mefenamic acid inhibit COX-2 oxygenation of AA and 2-AG by different mechanisms and with different potencies. An interpretation of the experimental findings can be made that is consistent with recent results establishing that the two subunits of the homodimeric COX-2 protein are not identical once substrate or inhibitor is bound (*2*). In the case of 2-AG oxygenation, binding of ibuprofen or mefenamate at one subunit prevents productive binding of 2-AG at the other subunit. Therefore, a single molecule of bound inhibitor suffices to inhibit 2-AG oxygenation (Figure 2). The K_I for inhibition by ibuprofen and the K_d for binding of mefenamate suggest high affinity binding at the first COX-2 subunit. The kinetics of ibuprofen and mefenamate inhibition of 2-AG oxygenation are typical of noncompetitive inhibition, consistent with the hypothesis that binding of the inhibitor at one subunit inhibits productive binding of 2-AG at the other subunit. We cannot rule out the possibility that a separate, high affinity allosteric binding site exists for these compounds outside of the active site, but it seems unlikely given (a) the high affinity of the inhibitors for the protein, (b) the existence of crystal structures demonstrating the presence of arylcarboxylic acid inhibitors in the active sites of both subunits, and (c) the likelihood that the active site residue, Trp-387, is responsible for the fluorescence quenched by mefenamic acid.

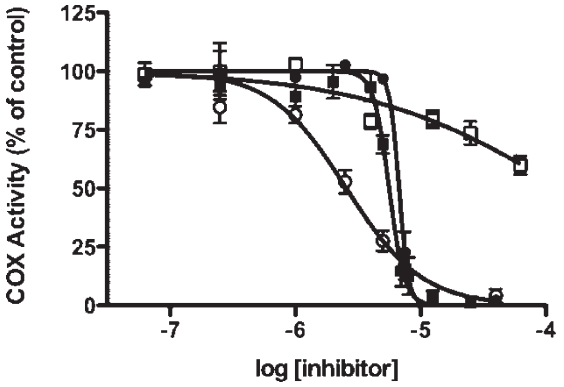

FIGURE 3: Determination of IC_{50} values for the inhibition of mCOX-2 oxygenation of AA and 2-AG by indomethacin and 2′-*des*-methylindomethacin. mCOX-2 was preincubated with indomethacin (●) or 2′-*des*-methylindomethacin (■) for 2 min before the addition of 2-AG. For AA, maximal inhibition was achieved following a 15 min preincubation with indomethacin (○) and a 2 min preinucation with 2′-*des*-methylindomethacin (□). Inhibitor concentrations ranged from 250 nM to 500 μM. Following the addition of 50 μM substrate, rates of oxygen uptake were determined and normalized to a DMSO control.

In contrast to the observations with 2-AG, inhibition of COX-2 oxygenation of AA by ibuprofen or mefenamate requires much higher concentrations of inhibitor and displays kinetic behavior typical of competitive inhibition. The most straightforward interpretation of these results is that inhibition of AA oxygenation requires inhibitor molecules to bind in both active sites (Figure 2). Binding in the first active site is necessary but not sufficient to inhibit AA oxygenation; inhibition is only observed when the second molecule of inhibitor binds. Binding in the first active site must decrease the affinity of the unoccupied active site for the second molecule of inhibitor so that higher concentrations are required and their binding is competitive with that of AA.

Several arylcarboxylic acids or diarylheterocycles are slow, tight-binding inhibitors of COX-2 (*8*). These compounds exhibit low K_d's for binding and potent inhibition but only after a lengthy preincubation period. The indoleacetic acid derivative, indomethacin, is a classic slow, tight-binding inhibitor of both COX-2 and COX-1 (*9*). Inhibition of AA oxygenation by COX-2 requires a preincubation period of up to 15 min, and its inhibition potency increases dramatically during this time. Binding of a single molecule of indomethacin to a COX homodimer is sufficient to inhibit AA oxygenation (*10*). Following a 15 min preincubation, indomethacin displayed an IC_{50} of 2 μM for inhibition of AA oxygenation and 5.5 μM for inhibition of 2-AG oxygenation (Figure 3). Thus, a single indomethacin molecule bound in one subunit is sufficient to inhibit the oxygenation of either a fatty acid or fatty acid ester substrate in the other subunit.

A major determinant of the slow, tight binding of indomethacin to COX-2 is insertion of the 2′-methyl group on the indole ring into a hydrophobic depression in the side of the COX-2 active site (*11*). Removal of the 2′-methyl group generates a molecule, 2′-*des*-methylindomethacin, which exhibits rapid reversible inhibition of AA oxygenation with a much higher IC_{50}. Figure 3 demonstrates that removal of the 2′-methyl group from indomethacin increases the IC_{50} for inhibition of AA oxygenation from 2 μM for indomethacin to ~500 μM for 2′-*des*-methylindomethacin. In contrast to the results with AA, removal of the 2′-methyl group from indomethacin has no effect on the

inhibition of 2-AG oxygenation; the IC_{50} for inhibition of 2-AG oxygenation by 2′-*des*-methylindomethacin is 6.8 μM, essentially the same as the IC_{50} of indomethacin (Figure 3).

Rome and Lands first demonstrated that some COX inhibitors display rapid, reversible inhibition, whereas others exhibit slow, tight-binding inhibition after the initial rapid, reversible interaction with the enzyme (*9*). The rapid, reversible inhibitors are relatively weak inhibitors of AA oxygenation, whereas the slow, tight-binding inhibitors are more potent. The slow, tight-binders exhibit very low dissociation rates so they are poorly reversible even in the presence of saturating concentrations of AA. The validity of the two-step mechanism of inhibition has been demonstrated repeatedly and in some cases extended as exemplified by certain diarylheterocycles, which demonstrate a second time-dependent step responsible for COX-2 selective inhibition (*12*−*14*). Subsequent work has revealed that for several slow, tight-binding inhibitors (e.g., indomethacin, flurbiprofen) association of only a single molecule of inhibitor is sufficient to inhibit the activity of both subunits, and this can be understood by the recent discovery that the two subunits communicate through the dimer interface (*2*).

The results of the present experiments demonstrate that both reversible and time-dependent inhibitors can potently inhibit oxygenation of the fatty acid ester substrate 2-AG and that some of the reversible inhibitors (e.g., ibuprofen and mefenamate) actually display much higher affinity for the enzyme than originally thought as judged by their K_I's for inhibition and K_d's for binding. The K_d for ibuprofen and the K_I for mefenamic acid establish these compounds as high affinity ligands for first subunit binding. The differential potency and mechanism of inhibition of 2-AG oxygenation compared to AA oxygenation displayed by ibuprofen and mefenamic acid adds a new dimension to our understanding of COX inhibition by this class of compounds. They actually associate much more tightly with COX-2 than previously appreciated and prevent the productive binding of 2-AG but not AA. Their status as relatively weak, rapidly reversible COX inhibitors derives from the fact that they have to bind to both subunits of COX-2 (and presumably COX-1) to inhibit productive binding of AA and that binding in the second subunit is competitive with AA.

Our data suggest that binding of a single molecule of ibuprofen, mefenamic acid, indomethacin, or 2′-*des*-methylindomethacin is sufficient to cause noncompetitive inhibition of 2-AG oxygenation but only indomethacin inhibits AA oxygenation under these conditions. These observations indicate that although all of the inhibitors induce conformational changes in the second subunit following binding in the first subunit, there are differences in the nature of the conformational changes induced as judged by the differential effects on 2-AG and AA binding in the second subunit. In addition, this differential sensitivity to inhibition reveals differences in the binding of 2-AG and AA that are not anticipated by the similarities in the k_{cat}/K_m for oxygenation of the two substrates (*4*).

Inhibition of 2-AG oxygenation provides a powerful tool with which to investigate the binding of NSAIDs to COX-2. It will be interesting to examine a broader range of inhibitors than described here for their ability to differentially inhibit the oxygenation of 2-AG and AA and to compare the molecular determinants for this differential inhibition. It is also intriguing to consider the possibility that the greater potency for inhibition of 2-AG oxygenation exhibited by certain NSAIDs against purified protein has implications for understanding the pharmacological properties of these compounds in vivo.

SUPPORTING INFORMATION AVAILABLE

Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Garavito, R. M., and Mulichak, A. M. (2003) *Annu. Rev. Biophys. Biomol. Struct. 32*, 183–206.
2. Yuan, C., Rieke, C. J., Rimon, G., Wingerd, B. A., and Smith, W. L. (2006) *Proc. Natl. Acad. Sci. U.S.A. 103*, 6142–6147.
3. Yuan, C., Sidhu, R. S., Kuklev, D. V., Kado, Y., Wada, M., Song, I., and Smith, W. L. (2009) *J. Biol. Chem. 284*, 10046–10055.
4. Kozak, K. R., Rowlinson, S. W., and Marnett, L. J. (2000) *J. Biol. Chem. 275*, 33744–33749.
5. Rowlinson, S. W., Crews, B. C., Lanzo, C. A., and Marnett, L. J. (1999) *J. Biol. Chem. 274*, 23305–23310.
6. Houtzager, V., Ouellet, M., Falgueyret, J. P., Passmore, L. A., Bayly, C., and Percival, M. D. (1996) *Biochemistry 35*, 10974–10984.
7. Johnson, A. R., Marletta, M. A., and Dyer, R. D. (2001) *Biochemistry 40*, 7736–7745.
8. Blobaum, A. L., and Marnett, L. J. (2007) *J. Med. Chem. 50*, 1425–1441.
9. Rome, L. H., and Lands, W. E. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4863–4865.
10. Kulmacz, R. J., and Lands, W. E. M. (1985) *J. Biol. Chem. 260*, 12572–12578.
11. Prusakiewicz, J. J., Felts, A. S., Mackenzie, B. S., and Marnett, L. J. (2004) *Biochemistry 43*, 15439–15445.
12. Lanzo, C. A., Sutin, J., Rowlinson, S. W., Talley, J., and Marnett, L. J. (2000) *Biochemistry 39*, 6228–6234.
13. Walker, M. C., Kurumbail, R. G., Kiefer, J. R., Moreland, K. T., Koboldt, C. M., Isakson, P. C., Seibert, K., and Gierse, J. K. (2001) *Biochem. J. 357*, 709–718.
14. Copeland, R. A., Williams, J. M., Giannaras, J., Nurnberg, S., Covington, M., Pinto, D., Pick, S., and Trzaskos, J. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 11202–11206.