Archives of Biochemistry and Biophysics 546 (2014) 33–40

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Cyclooxygenase-2 catalysis and inhibition in lipid bilayer nanodiscs

Benjamin J. Orlando [a], Daniel R. McDougle [b,c,d], Michael J. Lucido [a], Edward T. Eng [e], Leigh Ann Graham [f], Claus Schneider [f], David L. Stokes [e], Aditi Das [b,c,d], Michael G. Malkowski [a,g,*]

[a] Department of Structural Biology, The State University of New York at Buffalo, Buffalo, NY 14203, USA
[b] Department of Comparative Biosciences, University of Illinois Urbana-Champaign, Urbana, IL 61802, USA
[c] Department of Biochemistry, University of Illinois Urbana-Champaign, Urbana, IL 61802, USA
[d] Department of Bioengineering and Beckman Institute for Advanced Science and Technology, University of Illinois Urbana-Champaign, Urbana, IL 61802, USA
[e] New York Structural Biology Center, New York, NY 10027, USA
[f] Division of Clinical Pharmacology, Department of Pharmacology, Vanderbilt University Medical School, Nashville, TN 37232, USA
[g] Hauptman-Woodward Medical Research Institute, Buffalo, NY 14203, USA

ARTICLE INFO

Article history:
Received 30 October 2013
and in revised form 15 January 2014
Available online 3 February 2014

Keywords:
Cyclooxygenase
Nanodisc
Arachidonic acid
Nonsteroidal anti-inflammatory drugs
Phospholipid
Aspirin

ABSTRACT

Cyclooxygenases (COX-1 and COX-2) oxygenate arachidonic acid (AA) to generate prostaglandins. The enzymes associate with one leaflet of the membrane bilayer. We utilized nanodisc technology to investigate the function of human (hu) COX-2 and murine (mu) COX-2 in a lipid bilayer environment. huCOX-2 and muCOX-2 were incorporated into nanodiscs composed of POPC, POPS, DOPC, or DOPS phospholipids. Size-exclusion chromatography and negative stain electron microscopy confirm that a single COX-2 homodimer is incorporated into the nanodisc scaffold. Nanodisc-reconstituted COX-2 exhibited similar kinetic profiles for the oxygenation of AA, eicosapentaenoic acid, and 1-arachidonoyl glycerol compared to those derived using detergent solubilized enzyme. Moreover, changing the phospholipid composition of the nanodisc did not alter the ability of COX-2 to oxygenate AA or to be inhibited by various nonselective NSAIDs or celecoxib. The cyclooxygenase activity of nanodisc-reconstituted COX-2 was reduced by aspirin acetylation and potentiated by the nonsubstrate fatty acid palmitic acid to the same extent as detergent solubilized enzyme, independent of phospholipid composition. The stabilization and maintenance of activity afforded by the incorporation of the enzyme into nanodiscs generates a native-like lipid bilayer environment to pursue studies of COX utilizing solution-based techniques that are otherwise not tractable in the presence of detergents.

Introduction

Cyclooxygenases (COX-1 and COX-2) oxygenate arachidonic acid (AA) to generate potent lipid signaling molecules collectively known as prostaglandins [1]. Aspirin, nonselective nonsteroidal anti-inflammatory drugs (NSAIDs)[1] and COX-2 selective diaryl-heterocycle-based drugs (coxibs) inhibit COX-2 [2,3]. COX-1 and COX-2 are sequence homodimers composed of tightly associated monomers. The tertiary structure of each monomer consists of an N-terminal epidermal growth factor-like domain involved in dimerization, a membrane-binding domain (MBD) and a large catalytic domain at the C-terminus [4]. The catalytic domain houses physically distinct cyclooxygenase and peroxidase active sites that are functionally linked via a heme moiety. Recent biochemical studies utilizing various fatty acids and COX inhibitors indicate that COX functions as a conformational heterodimer during catalysis and inhibition, such that only one monomer of the dimer can function at a given time [5]. COX also functions as an allosteric/catalytic couple, in which the cyclooxygenase activity in one monomer is modulated by the nature of the ligand bound in the cyclooxygenase active site of the opposite monomer [6–8]. For example, the nonsubstrate fatty acid palmitic acid (PA) binds to one monomer and stimulates the cyclooxygenase activity of COX-2 in the partner monomer [6]. However, the mechanism controlling the crosstalk between monomers has yet to be identified.

* Corresponding author at: Hauptman-Woodward Medical Research Institute, 700 Ellicott Street, Buffalo, NY 14203, USA. Fax: +1 (716) 898 8660.
E-mail address: malkowski@hwi.buffalo.edu (M.G. Malkowski).

[1] *Abbreviations used:* NSAIDs, nonsteroidal anti-inflammatory drugs; PG, prostaglandin; AA, arachidonic acid; PA, palmitic acid; EPA, eicosapentaenoic acid; 1-AG, 1-arachidonoyl glycerol; mu, murine; hu, human; C_{10}M, decyl maltoside; βOG, N-octyl β-D-glucopyranoside; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; SEC, size-exclusion chromatography; EM, electron microscopy; IMAC, immobilized metal affinity chromatography; FLAG muCOX-2, detergent solubilized FLAG-tagged mouse COX-2; FLAG huCOX-2, detergent solubilized FLAG-tagged human COX-2; POPC:muCOX-2, muCOX-2 incorporated into POPC nanodiscs; POPC:huCOX-2, huCOX-2 incorporated into POPC nanodiscs; MW, molecular weight; MSP, membrane scaffold protein.

http://dx.doi.org/10.1016/j.abb.2014.01.026
0003-9861/

COX catalysis and inhibition takes place in the context of a membrane environment. The enzymes are localized to the lumenal membrane of the ER and the nuclear membrane, where they associate with one leaflet of the membrane bilayer [9]. Bilayer association occurs via interactions with amphipathic α-helices located in the MBD of COX [10–12]. Analysis of the COX-1 and COX-2 crystal structures reveals an opening between the α-helices of the MBD that leads to the entrance of the cyclooxygenase channel [13–16]. It is hypothesized that substrates and inhibitors, which are hydrophobic and readily partition into membranes, travel from the lipid bilayer into the cyclooxygenase channel through the opening in the MBD [4]. Indeed, detergent molecules, derived from the solubilization and purification of COX, are resolved within the opening of the MBD in some COX crystal structures, lending support to the substrate access hypothesis [14,17].

There has been limited focus on studying COX catalysis and inhibition in a more natural lipid environment. Rand Doyen and colleagues characterized the kinetics of COX-1 and COX-2 in the presence of phosphatidylcholine molecules of varying acyl chain lengths and physical state [18]. These studies revealed that monomeric, micelle, and bilayer forms of phospholipids exhibit an inhibitory effect on cyclooxygenase catalysis, predominantly due to sequestration of substrate [18]. MirAfzali and colleagues successfully incorporated COX-1 and COX-2 into large unilamellar vesicles produced from a mixture of DOPC:DOPS that had been doped with oleic acid [19]. While incorporation of COX into the DOPS:DOPC:oleic acid vesicles did not significantly affect cyclooxygenase activity, no subsequent studies were performed to characterize the kinetics of COX catalysis and inhibition in these vesicles [11].

Nanodisc technology has proven to be a valuable tool in the study of membrane protein structure and function [20–23]. Nanodiscs are comprised of a small circular patch of lipid bilayer that is rendered soluble by two amphipathic α-helical membrane scaffold proteins (MSP) that encircle the circumference of the bilayer [24]. The surface area of the lipid bilayer is defined by the particular MSP used to create the nanodiscs. Various MSP constructs, derived from apo-lipoprotein A1, have been utilized to create nanodiscs with diameters typically ranging from ~9 to 15 nm [25]. Additionally, lipids with different head groups and varying acyl chain lengths can be used in nanodisc formulation. The ability to tailor the dimensions and bilayer composition of the discs makes the technology highly versatile and amenable to various membrane protein targets and experimental techniques.

We present here studies designed to further investigate COX catalysis and inhibition in a natural lipid bilayer. We reconstituted huCOX-2 and muCOX-2 into nanodiscs containing different phospholipids and isolated pure COX-2:nanodisc complexes using a dual affinity purification protocol. Incorporation of a single homodimer of COX-2 into the nanodisc was validated using size-exclusion chromatography (SEC) and negative-stain electron microscopy (EM). We subsequently used the system to investigate COX-2 catalysis and inhibition utilizing the COX-2 substrates AA, eicosapentaenoic acid (EPA), and 1-arachidonoyl glycerol (1-AG) and a variety of nonselective NSAIDs, aspirin, and COX-2 selective celecoxib. Finally, we characterized the ability of PA to potentiate the cyclooxygenase activity of COX-2. Our results indicate that nanodisc-reconstituted COX-2 exhibits the same catalytic, inhibitory, and activation properties as detergent solubilized COX-2. As such, the use of nanodisc-reconstituted COX-2 provides access to solution-based techniques to investigate COX catalysis and inhibition that have otherwise been elusive due to the presence of detergent or the large size and polydispersity of liposomes.

Experimental procedures

Materials

The fatty acids AA (5*Z*, 8*Z*, 11*Z*, 14*Z*-eicosatetraenoic acid), eicosapentaenoic acid (EPA; 5*Z*, 8*Z*,11*Z*, 14*Z*, 17*Z*)-eicosapentaenoic acid), and palmitic acid (PA; hexadecanoic acid), and the inhibitors ibuprofen, flurbiprofen, meclofenamic acid, naproxen, indomethacin, and aspirin were purchased from Cayman Chemical Company (Ann Arbor, MI). Celebrex® (celecoxib) was from a physician sample. Fe^{3+}-protoporphyrin IX was purchased from Frontier Scientific (Logan, UT). Decyl maltoside (C_{10}M) was purchased from Affymetrix (Santa Clara, CA). N-octyl β-D-glucopyranoside (βOG) was purchased from Inalco Pharmaceuticals (San Luis Obispo, CA). The QuikChange™ Mutagenesis kit II was purchased from Agilent Technologies (Santa Clara, CA). The Bac-to-Bac® baculovirus expression kit, and associated reagents, including *Spodoptera frugiperda* 21 (Sf21) insect cells, fetal bovine serum, fungizone, penicillin–streptomycin, and sf-900 III serum free media were purchased from Invitrogen (Carlsbad, CA). HiTrap™ HP Chelating and HiPrep™ Superdex 200 10/300-GL chromatography columns were purchased from GE Healthcare (Piscataway, NJ). Oligos used for site-directed mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA). The phospholipids 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) were purchased from Avanti Polar Lipids (Alabaster, AL). Sodium cholate hydrate from ox or sheep bile ⩾99%, Triton-X-100, anti-FLAG M2 affinity resin, and FLAG peptide were from Sigma–Aldrich (St. Louis, MO). BioBeads SM-2 were purchased from Bio-Rad (Hercules, MA).

Construction of human and mouse FLAG-COX-2

His_6 N580A murine (mu) COX-2 in pFastbac1 from [16] was used as a template to engineer FLAG N580A muCOX-2 (FLAG muCOX-2). We utilized the QuikChange™ mutagenesis kit II to replace the His_6 sequence, which was inserted between Asn-19 and Pro-20 in muCOX-2 [26], with the sequence DYKDDDDK, using the following primer (note: forward primers are listed only; inserted amino acids are boldface and underlined): 5′-GCCCTG GGGCTCAGCCAGGCAGCAAAT**GATTACAAGGATG ACGACGATAAG**CC TTGCTGTTCCAATCCATGTCAAAACC-3′. The N580A mutation prevents variable glycosylation at Asn-580 during expression [27]. The resulting FLAG muCOX-2 construct was cloned into the Sma1 and Nhe1 restriction sites of pFastbac Dual so that expression was driven from the p10 promoter of the vector. The equivalent FLAG N580A human (hu) COX-2 construct (FLAG huCOX-2) was generated in the same manner using His_6 N580A huCOX-2 in pFastbac1 as the template and the following primer: 5′-GCT CAGCCATACAGCA**GACTACAAGGACGACGATGACAAG**AATCCTTGCTG TTCCC-3′. The resulting FLAG huCOX-2 construct in pFastbac1 was used for expression, with protein expressed from the polH promoter of the vector. Each construct was verified by DNA sequence analysis at the Roswell Park Cancer Institute DNA Sequencing Laboratory.

Expression and purification of FLAG-COX-2 constructs

The expression of FLAG huCOX-2 and FLAG muCOX-2 was carried out in insect cells as described in [16]. Cell pellet from a 2 L culture was resuspended in 50 mM TRIS, pH 7.4, 300 mM NaCl followed by the addition of a protease inhibitor cocktail tablet (Roche, Indianapolis, IN). The cells were then lysed using a microfluidizer