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Original Contribution

Characterization of free radicals formed from COX-catalyzed DGLA peroxidation

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ABSTRACT

Like arachidonic acid (AA), dihomo-γ-linolenic acid (DGLA) is a 20-carbon ω-6 polyunsaturated fatty acid and a substrate of cyclooxygenase (COX). Through free radical reactions, COX metabolizes DGLA and AA to form well-known bioactive metabolites, namely, the 1 and 2 series of prostaglandins (PGs1 and PGs2), respectively. Unlike PGs2, which are viewed as proinflammatory, PGs1 possess anti-inflammatory and anticancer activities. However, the mechanisms linking the PGs to their bioactivities are still unclear, and radicals generated in COX–DGLA have not been detected. To better understand PG biology and determine whether different reactions occur in COX–DGLA and COX–AA, we have used LC/ESR/MS with a spin trap, α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl nitrone (POBN), to characterize the carbon-centered radicals formed from COX–DGLA in vitro, including cellular peroxidation. A total of five types of DGLA-derived radicals were characterized as POBN adducts: *m/z* 266, *m/z* 296, and *m/z* 550 (same as or similar to COX–AA) and *m/z* 324 and *m/z* 354 (exclusively from COX–DGLA). Our results suggest that C-15 oxygenation to form PGGs occurs in both COX–DGLA and COX–AA; however, C-8 oxygenation occurs exclusively in COX–DGLA. This new finding will be further investigated for its association with various bioactivities of PGs, with potential implications for inflammatory diseases.

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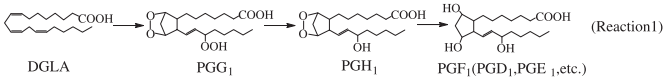
Dihomo-γ-linolenic acid (DGLA) is a 20-carbon ω-6 polyunsaturated fatty acid (PUFA) derived in vivo from linolenic acid, an essential fatty acid. DGLA can then be converted to arachidonic acid (AA), another 20-carbon ω-6 PUFA [1,2]. Both DGLA and AA are substrates of the lipid-peroxidizing enzyme COX. Through a series of free radical reactions, COX metabolizes DGLA and AA to form various bioactive metabolites, namely, the 1 and the 2 series of prostaglandins (PGs1 and PGs2), respectively. Unlike PGs2, which are generally viewed as proinflammatory [3–5], PGs1 actually possess anti-inflammatory and anticancer activities. For example, PGE1, one form of PGs1, could inhibit vascular smooth muscle cell proliferation, reduce vascular cell adhesion, and attenuate the development of atherosclerosis [6–10].

Although much research attention has been focused on the study of PG bioactivity [3–12], the mechanism linking the structure of PGs to their bioactivity is still unclear, and the free radicals generated in COX–DGLA have never been detected because of the lack of appropriate methodologies. As in COX–AA peroxidation, several free radical reactions are involved in the formation of PGG1 from DGLA, e.g., formation of the C-13 radical (the initial step), introduction of O_2 on C-11 to form the C-9/C11 endoperoxide, C-8/C-12 cyclization (creation of a carbon bond between C-8 and C-12), and 15-C peroxidation (addition of the second O_2 to the DGLA molecule) [13–16]. PGG1 can then be readily transformed into PGH1, PGE1, and PGF1α (Reaction 1) [15,16]:



However, it is still unclear whether different free radical reactions occur in COX–AA and COX–DGLA peroxidation and whether these differences are associated with the bioactivity of PGs and fatty acids.

Recently, we have successfully used the combination of LC/ESR and LC/MS to identify the radicals formed from COX–AA peroxidation and have observed that free radicals were formed from a special β-scission during COX–AA peroxidation [17]. In this study, to determine whether different free radical reactions occur in COX-catalyzed DGLA compared to AA peroxidation, a combination of LC/ESR and LC/MS was again applied to characterize DGLA-derived radicals formed from COX–DGLA in vitro, including cellular (human colon cancer cell line HCA-7 colony 29) peroxidation, in the presence of the spin trap α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl nitrone (POBN).

In addition to forming similar free radicals via the same pathway reported in COX–AA [17], unique radical reactions were also observed in COX–DGLA. A total of five types of DGLA-derived radicals were characterized as POBN adducts, including three types that were the same as or similar to those formed in COX–AA, *m/z* 266 (POBN/$^{\bullet}C_5H_{11}$),

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; DGLA, dihomo-γ-linolenic acid; EIC, extracted ion current; ESR, electron spin resonance; HPLC, high-performance liquid chromatography; MS, mass spectrometry; PG, prostaglandin; POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone; t_R, retention time; TIC, total ion current.

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m/z 296 (POBN/$^{\bullet}C_6H_{13}O$), and *m/z* 550 (POBN/$^{\bullet}C_{20}H_{35}O_5$), and two types formed exclusively in COX–DGLA, *m/z* 354 (POBN/$^{\bullet}C_8H_{15}O_3$) and *m/z* 324 (POBN/$^{\bullet}C_7H_{13}O_2$). The results of our studies suggested that the C-15 oxygenation to form PGGs (Reaction 1) seems to occur in both COX-mediated DGLA and AA peroxidation, whereas C-8 oxygenation forming related peroxides occurs exclusively in COX–DGLA. We will further investigate the association of these new products with PG bioactivity and assess their potential implications for inflammatory diseases.

Materials and methods

Reagents

Ethyl alcohol, glacial acetic acid (HOAc), hydroquinone, and porcine hematin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile (ACN; HPLC grade) and Tris-(hydroxymethyl) aminomethane were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). LC/MS-grade water (H_2O) was purchased from EMD Chemicals (Gibbstown, NJ, USA). COX-2 enzyme (ovine) and DGLA were purchased from Cayman Chemical (Ann Arbor, MI, USA). Chelex 100 (200–400 mesh sodium form) was bought from Bio-Rad Laboratories (Hercules, CA, USA). High-purity POBN was purchased from Alexis Biochemicals (San Diego, CA, USA), and deuterated POBN (D_9-POBN) was obtained from CDN Isotopes (Pointe-Claire, QC, Canada). The human colon cancer cell line HCA-7 colony 29 was purchased from the European Collection of Cell Cultures (Porton Down, Salisbury, UK). HyClone DMEM high-glucose medium was obtained from Thermo Fisher Scientific (Logan, UT, USA). Fetal bovine serum (FBS) and trypsin–EDTA were purchased from GIBCO BRL (Grand Island, NY, USA).

Reaction conditions

The reactions of COX catalysis of DGLA were performed in 0.1 M (pH 8.0) "metal-free" Tris–Cl buffer solutions. Metal ions in the Tris–Cl buffer solution were chelated by treatment with Chelex 100 resin to provide a virtually metal-free buffer solution, which passed an ascorbic acid test [18]. A reaction mixture containing 5 kUnit/ml COX enzyme, 100 mM POBN, and 50 μM hematin was preincubated for 5 min at 37 °C and 600 rpm on a Thermo-Shaker (Boekel Scientific, Feasterville, PA, USA). Five millimolar hydroquinone and 2 mM DGLA (in ethanol) were then added to start the reaction. This complete reaction mixture (~1% ethanol, v/v, from a DGLA stock solution) was then incubated at 37 °C and 600 rpm on a Thermo-Shaker in the absence of light. After a 30-min incubation, the COX-catalyzed peroxidation was immediately stopped by mixing with the same amount of ACN and was centrifuged and condensed for later LC/ESR and LC/MS analysis as described elsewhere [17,19–21].

Free radicals from cellular COX-mediated peroxidation were generated with HCA-7 colony 29 cells grown in HyClone DMEM high-glucose medium supplemented with 10% FBS in an incubator containing a humidified atmosphere of 5% CO_2 at 37 °C. At 70–80% confluence, the cells were trypsinized, harvested, and suspended in phosphate-buffered saline (PBS) at ~10^7 cells/ml. POBN and DGLA at final concentrations of 50 and 0.1–1.0 mM, respectively, were then added to the cell suspension to start the DGLA peroxidation and POBN spin trapping reaction. After a 30-min incubation, the peroxidation of the cell suspension was stopped with ACN (1:1 v/v), and then the supernatant was collected, centrifuged, and condensed for LC/ESR and LC/MS analysis.

Online LC/ESR measurements

The online LC/ESR system consisted of an Agilent 1200 series HPLC system and a Bruker EMX ESR system. The outlet of the Agilent UV detector was connected to a highly sensitive Aquax ESR cell with red PEEK HPLC tubing (0.005 i.d.). The POBN radical adducts were monitored via UV absorption at 265 nm [22,23] followed by ESR detection. There was a delay of about 9 s between the UV and the ESR detection in our online LC/ESR settings. LC separations were performed on a C18 column (Zorbax Eclipse-XDB, 4.6×75 mm, 3.5 μm) and a guard column (Zorbax Eclipse-XDB, 4.6×12.5 mm, 5 μm) equilibrated with 90% A (H_2O–0.1% HOAc) and 10% B (ACN–0.1% HOAc). Forty microliters of enzyme-free condensed sample was injected into the HPLC system by autosampler and eluted at a 0.8 ml/min flow rate with a combination of gradient and isocratic elution: (1) 0–6 min, 100 to 75% A and 0 to 25% B; (2) 6–18 min (isocratic), 75% A and 25% B; (3) 18–40 min, 75 to 30% A and 25 to 70% B; (4) 40–43 min, 30 to 5% A and 70 to 95% B; and (5) 43–50 min (isocratic), 5% A and 95% B. Online ESR measurements were performed using a time scan mode with the magnetic field (~3498 G) fixed on the maximum of the first line of the six-line spectrum of the POBN adduct as described elsewhere [17,19–21]. Other ESR settings were modulation frequency, 100 kHz; modulation amplitude, 3.0 G; microwave power, 20 mW; receiver gain, 4×10^5; and time constant, 2.6 s.

Online LC/MS and LC/MS^2 measurements

The LC/MS system consisted of an Agilent 1200 series HPLC system and an Agilent LC/MSD SL ion trap mass system. The outlet of the UV detector in LC was connected to the MS system with red PEEK HPLC tubing as well. Chromatographic conditions were identical to those used for online LC/ESR. However, the LC flow rate (0.8 ml/min) into the MS inlet was adjusted to 30–40 μl/min via a splitter. There was a delay of ~35 s between the UV and the MS detection in our online LC/MS settings. Electrospray ionization in positive mode was used for all LC/MS and LC/MS^2 measurements unless otherwise specified. Total ion current (TIC) chromatograms in full mass scan mode (*m/z* 50 to *m/z* 600) were performed to profile all products formed in the reaction of COX-catalyzed DGLA in vitro in the presence of POBN. Other MS settings were capillary voltage, −4500 V; nebulizer press, 20 psi; dry gas flow rate, 8 L/min; dry temperature, 60 °C; compound stability, 20%; and number of scans, 50. Extracted ion current (EIC) chromatograms of ions of interest were projected from TIC to acquire MS chromatograms that could match well with ESR chromatograms, in which all POBN radical adducts were monitored as structure-nonspecific ESR-active peaks. EIC was also performed to determine the number of isomers of given ions. Normally an isolation width of ±0.5 Da was selected for EIC. The multiple reaction monitoring mode of LC/MS^2 was conducted to confirm structural assignments of POBN adducts. A width of ±2.0 Da was typically selected to isolate parent ions of interest.

Results

When offline ESR (magnetic field scan) was used to measure free radical adducts in the COX–DGLA complete reaction mixture, we obtained a mixed spectrum (data not shown) composed of a six-line ESR signal of POBN radical adducts ($a^N\approx15.33$ G and $a^H\approx2.39$ G) and an overlapping five-line ESR signal in the center from benzosemiquinone radicals generated from hydroquinone oxidation as described elsewhere [17]. However, the offline ESR measurement can provide only an overall signal intensity of radical adducts, not specific information in terms of types, numbers, and structures, because many of the POBN spin adducts tend to have the same or similar hyperfine couplings of a^N and a^H [24].

When we used the combined techniques of online LC/ESR and LC/MS to further examine more specific radical information from the COX–DGLA complete reaction mixture, we observed a total of 13 ESR-active peaks corresponding to different POBN radical adducts (include isomers) (Fig. 1B). Among these ESR-active peaks, some (peaks 2, 3, 4, 7, 12, and 13) were matched with their corresponding UV peaks (Fig. 1A, peaks marked by asterisks), whereas for others