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A rapid oxygen exchange on prostaglandins in plasma represents plasma esterase activity that is inhibited by diethylumbelliferyl phosphate with high affinity

Stephen A. Brose and Mikhail Y. Golovko¹

Department of Pharmacology, Physiology, and Therapeutics, University of North Dakota, Grand Forks, ND 58202-9037

Abstract

RATIONAL—Fatty acids labeled with ^{18}O at the carboxyl group, including oxidized species (FA^{18}O), are a useful, low cost, and easy to prepare tool for quantitative and qualitative mass spectrometry (MS) analysis in biological systems. In addition, they are used to trace FA fate in metabolic pathways including FA re-esterification and lipid remodeling pathways. Although a rapid ^{18}O exchange on FA^{18}O in biological systems has been reported, the mechanism contributing to ^{18}O exchange has not been fully evaluated. This gap of knowledge limits the use of FA^{18}O as a standard for MS and complicate data interpretation for FA metabolism in biological systems.

METHODS—In the present study we have addressed a number of possible mechanisms for a rapid ^{18}O exchange on prostaglandin E_2 (PGE_2) using rat plasma as a model. High performance liquid chromatography coupled with electro-spray ionization triple-quadrupole MS in the multiple reaction monitoring mode was used for quantification.

RESULTS—The major mechanism for a rapid ^{18}O exchange on $\text{PGE}_2^{18}\text{O}$ in rat plasma is PGE_2 processing with esterases, while FA re-esterification and non-enzymatic mechanisms do not significantly contribute to this phenomenon. In addition, we report a highly effective inhibition of ^{18}O exchange with diethylumbelliferyl phosphate that can be used to stabilize FA^{18}O in biological samples.

CONCLUSIONS—These data indicate the necessity to consider esterase activity when FA^{18}O are used to study FA metabolism, and importance of esterase activity inhibition when FA^{18}O are used as internal standards for MS analysis in biological systems. In addition, the results provide a rationale for the development of new approaches to study esterase activities and affinity towards modified FA.

Keywords

Fatty acid metabolism; re-esterification; prostaglandins; mass spectrometry; HPLC

Introduction

Stable isotope labeled substrates are a powerful tool that allow for utilizing mass spectrometry (MS) in quantitative and qualitative analysis in biological systems. One of the tracers that has been used in MS are fatty acids, including oxidized species (FA), labeled

¹Corresponding author: Mikhail Y. Golovko, Department of Pharmacology, Physiology, and Therapeutics, School of Medicine and Health Sciences, University of North Dakota, 501 N. Columbia Rd. Grand Forks, ND 58202-9037, mikhail.golovko@med.und.edu, 701-777-2305 phone, 701-777-4490 fax.

with heavy ^{18}O on carboxyl group (FA^{18}O). The major advantage of FA^{18}O is the ease and low cost synthesis in an ordinary laboratory setup that allows for a fast preparation of large quantities of different FA^{18}O , including modified FA^{18}O . One of the applications is utilizing FA^{18}O as an internal or external standard for FA quantification including oxidized species [1–7] and structural identification of fatty acids [8–10]. In addition, FA^{18}O might be used to trace FA fate in metabolic pathways, and to study kinetics of FA re-esterification and lipid remodeling pathways [11, 12]. Although a rapid ^{18}O exchange on FA^{18}O in biological systems has been reported [3, 11, 12], the mechanism contributing to ^{18}O exchange has not been fully evaluated. This gap of knowledge might limit the use of FA^{18}O as a standard for MS and complicate data interpretation for FA metabolism in biological systems. In the present study we have addressed a number of possible mechanisms for a rapid ^{18}O exchange on prostaglandin E_2 (PGE_2) using rat plasma as a model. Our results indicate that the major mechanism for a rapid ^{18}O exchange on $\text{PGE}_2^{18}\text{O}$ in rat plasma is PGE_2 processing with esterases, while PGE_2 re-esterification and non-enzymatic mechanisms do not significantly contribute to this phenomenon. This data provide important information for the development of new approaches to study esterase activities, especially if the esterase affinity towards modified FA is addressed. In addition, our data indicate that esterase activity should be considered when FA^{18}O are used to study FA metabolism, and the necessity to inhibit esterase activity when FA^{18}O are used as internal standards for MS analysis of biological matrix.

Methods

Animal Protocol

This study was performed according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publication 80-23) and under an animal protocol approved by the IACUC at the University of North Dakota (Protocol #0806-1). The rat plasma was a pooled sample collected from Sprague Dawley rats using sodium heparin as an anticoagulant and was purchased commercially from Bioreclamation, LLC (Westbury, NY).

Chemicals

Water- ^{18}O , diethylumbelliferyl phosphate (DEUP) and SigmaCote® were purchased from Sigma Chemical Co. (St. Louis, Mo). Porcine liver esterase was purchased from Lee Biosolutions (St. Louis, Mo). Deuterium labeled prostaglandin D_2 -17,17,18,18,19,19,20,20,20- d_9 (PGD_2d_9), deuterium labeled prostaglandin E_2 -3,3,4,4- d_4 (PGE_2d_4), prostaglandin E_2 -1-glycerol ester ($\text{PGE}_2\text{-Gly}$), and methyl arachidonyl fluorophosphonate (MAFP) were purchased from Cayman Chemical (Ann Arbor, MI). LC/MS/MS grade acetonitrile, water, methanol, and chloroform were from Fisher Scientific. All other chemicals of analytical or higher quality were purchased from VWR International (West Chester, PA).

Synthesis of Oxygen ^{18}O labeled PGE_2d_4

PGE_2d_4 was labeled with ^{18}O using porcine liver esterase to exchange the two oxygen atoms on the carboxylic acid with ^{18}O from water- ^{18}O as previously described [4] with slight modifications. Briefly, porcine esterase (130 Units, 1mg) was dissolved in 250 μL water- ^{18}O (97% atom enrichment) and combined with 25 μg of PGE_2d_4 dissolved in 25 μL of methanol. The mixture was incubated at 37 °C for 1 hr. Acetone (0.5 mL) was added to precipitate the protein. Sodium chloride (2.5 mg) was added and the solution was acidified to pH 3.5 with formic acid. The labeled prostaglandin was then extracted using 0.5 mL CHCl_3 . The extract was dried under a stream of nitrogen and dissolved in ethanol. The percent of ^{18}O into PGE_2d_4 ($\text{PGE}_2^{18}\text{Od}_4$) incorporation was determined using LC/MS/MS.

and was 91% for dual ^{18}O labeled, 8% for mono ^{18}O labeled, and below 1% for zero ^{18}O labeled PGE₂d₄.

Kinetics studies

Stock solutions of each inhibitor and substrate were prepared in either DMSO (DEUP) or ethanol (MAFP and substrates). Rat plasma was diluted in phosphate buffered saline (PBS, pH 7.4). Before each experiment, the inhibitor (DEUP or MAFP) was added to the plasma-PBS solution and pre-incubated for ten minutes. PGE₂-Gly and PGE₂¹⁸O_d₄ were diluted in PBS and the reaction was started by adding the substrate-PBS solution to the rat plasma-PBS solution so the final concentrations were 20 ng/mL PGE₂-Gly, 10 ng/mL PGE₂¹⁸O_d₄, and 10% rat plasma. The final volume of each reaction was 250 μL with less than 0.1% organic solvent. The mixture was incubated at 37 $^{\circ}\text{C}$. The rate of ^{18}O back exchange was calculated as the mass of PGE₂ that lost one and two ^{18}O labels per unit of time per plasma volume. The enzyme activity (ng/min/mL) towards PGE₂-Gly was determined by measuring the amount of PGE₂ released during the incubation time and dividing by incubation time and by the amount of plasma in the sample. For the IC₅₀ calculation, the reaction rates were plotted against the decimal logarithm of inhibitor concentrations, and IC₅₀ were determined from non-linear regression using GraphPad Prism 5 (Graphpad, San Diego, CA). The half life was calculated as ln2 divided by reaction constant.

Prostaglandin extraction and analysis

Prostaglandins were quantified using LC/MS/MS as previously described [13]. The prostaglandins were separated on a Luna C-18(2) column (3 μm , 100 $\text{\AA}$ pore diameter, 150 $\times$ 2.0 mm, Phenomenex, Torrance, CA) with a 0.5 μm stainless steel frit filter and a C-18 security guard cartridge (Phenomenex). The HPLC system consisted of an Agilent 1100 series LC pump with a wellplate autosampler (Agilent Technologies, Santa Clara, CA) set at 4 $^{\circ}\text{C}$. The injection volume was 25 μL . The flow rate was 0.2 mL/min and the solvents used in the separation were 0.1 % formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Solvent B was increased from 20% to 42.5% over 50 min and was increased to 90% over the next 10.5 min. At 65.5 min solvent B was returned to 20% over 1 min to equilibrate the column for 14 min.

A quadrupole mass spectrometer (API3000; Applied Biosystems, Foster City, CA) with a TurbolonSpray ionization source was used for MS analysis. Instrument control, data acquisition, and data analysis were performed with Analyst software version 1.4.2 (Applied Biosystems). The mass spectrometer was operated in multiple reaction monitoring mode (MRM). The source was in negative ion electrospray mode at 350 $^{\circ}\text{C}$, the electrospray voltage was -4250 V, the nebulizer gas was zero grade air at 8 L/min, and the curtain gas was ultrapure nitrogen at 11 L/min. The declustering potential was optimized for each analyte. The focusing potential was 2200 V, the entrance potential was 210 V, and the collision gas was 12 arbitrary units. The mass spectrometer was operated at unit resolution.

Prostaglandins were quantified using PGD₂d₉ as the internal standard. The mass transition used for MRM for non-labeled PGE₂ was 351.2/189.1 that allows for its differentiation from endogenous isoprostanes [13], and for labeled PG we used more abundant 355.2/275.2 for PGE₂d₄, 360.3/280.2 for PGD₂d₉, 359.3/275.2 for PGE₂¹⁸O₂d₄, and 357.3/275.2 for PGE₂¹⁸O₁d₄.

ATP quantification

ATP was quantified using the ATP Determination Kit from Life Technologies (Carlsbad, CA) which is a firefly luciferase based assay with a limit of detection of 0.1 pmole ATP. To measure ATP in serum, 10 μL was added to 90 μL of the assay solution from the kit and the

assay was performed according to manufacturer instructions. Luminescence at 560 nm was measured using a FlexStation III (Molecular Devices) plate reader.

Statistics

All statistical comparisons were calculated using one-way ANOVA analysis with Tukey post-test using GraphPad Prism 5 (Graphpad, San Diego, CA). Statistical significance was defined as <0.05 . All values were expressed as mean $\pm$ SD.

Results and Discussion

A rapid ^{18}O exchange during prostaglandin (PG) $\text{F}_{2\alpha}$ ^{18}O incubation with human plasma has been previously reported [3]. Thus, we used plasma as a model system to address mechanisms contributing to ^{18}O back exchange on FA. In the first set of experiments, we measured kinetics for ^{18}O back exchange on PGE_2 ^{18}O in rat plasma. Because of a high rate for ^{18}O back exchange, we used a 10 fold dilution of rat serum with PBS (pH=7.4), and limited the incubation time to 60 min. In order to differentiate exogenous PGE_2 that loses both ^{18}O on carboxyl group during incubation from endogenous PGE_2 found in plasma, we used dual labeled PGE_2 with the ^{18}O label at the carboxyl group, and ^2H label on radical. The rate of ^{18}O back exchange of PGE_2 ^{18}O with ^{16}O from water was calculated as an amount of PGE_2 that lost one or two ^{18}O . Consistent with high rates reported for $\text{PGF}_{2\alpha}$ ^{18}O [3], the rate for PGE_2 ^{18}O exchange was very high (2.09 ± 0.08 ng/min/mL) with reaction rate constant $k=0.84$ min $^{-1}$. The calculated half life of oxygen on PGE_2 carboxyl group was relatively short and equals 0.82 ± 0.11 min.

Next, we tested if non-enzymatic mechanisms contribute for a rapid ^{18}O exchange on PGE_2 ^{18}O in rat plasma. To test non-enzymatic mechanism, 10% rat plasma in PBS (pH=7.4) was boiled for 10 min in a closed container to heat denature proteins. As indicated in the Figure 1, there was no detectable ^{18}O loss from PGE_2 ^{18}O in boiled plasma, indicating that non-enzymatic, including albumin-catalyzed, mechanisms do not contribute to the ^{18}O back exchange.

Because esterases are used for FA ^{18}O preparation by oxygen exchange with H_2 ^{18}O [1–12], we next thought that esterases present in rat plasma might catalyze ^{18}O exchange on PGE_2 ^{18}O . The mechanism for esterase catalyzed oxygen exchange has been previously discussed [3]. To test this hypothesis, we used two different substrates: PGE_2 ^{18}O and PGE_2 -1-glycerol ester (PGE_2Gly) and compared IC_{50} of esterase inhibitors for both substrates (Fig. 2). The calculated IC_{50} are presented in the Table 1. Both inhibitors tested, DEUP (esterase inhibitor [14–16]) and MAFP (serine dependent hydrolase inhibitor [17, 18]), completely inhibited ^{18}O exchange and PGE_2Gly with the same IC_{50} values, indicating that both reactions might be catalyzed by the same enzymes belonging to the esterase class.

Finally, we addressed the role of a re-esterification mechanism in ^{18}O exchange. According to the Fisher esterification mechanism, each cycle of esterification-hydrolysis (re-esterification) randomly exchanges one of the labeled oxygen atoms on carboxyl group of carboxylic acid on the oxygen from water. This mechanism has been previously applied to study re-esterification of FA and lipid remodeling [11, 12]. Because re-esterification cycle associated with oxygen exchange has been described for a number of different FA [19–22], we hypothesized that re-esterification contributes to the oxygen exchange on the PGE_2 ^{18}O in rat plasma. Because esterification of the released free FA requires the energy of ATP [23], we measured ATP levels in rat plasma. Consistent with previous reports [24, 25], we found low but detectable ATP levels in rat plasma equal to 21.5 ± 5.5 nM, that provides the possibility for a re-esterification mechanism. To further evaluate this mechanism, we measured the levels of labeled PGE_2 ^{18}O during incubation with plasma in the presence of

esterase inhibitors that inhibited ^{18}O exchange (Table 1). Esterase inhibition is expected to inhibit hydrolysis of FA esters without altering the esterification reaction. Thus, if re-esterification contributes to ^{18}O exchange, esterase inhibition should result in decreased free PG levels with increased esterified PG. As indicated in the Figure 3, the PGE_2d_4 levels were unchanged during incubation with or without esterase inhibitors, indicating that re-esterification does not contribute to oxygen exchange. This is important because this data indicate that oxygen exchange might not be associated with re-esterification cycle, and esterase activity should be considered in oxygen exchange experiments.

Together, our data provide evidence that oxygen exchange on FA^{18}O might represent plasma esterase activity, and provides a rationale for the development of new approaches to study esterase activities, especially if the esterase affinity towards modified FA is addressed. This might be of interest because of the ease and low cost for FA^{18}O synthesis. In addition, our data indicate that esterase activity should be considered when FA^{18}O are used to study FA metabolism, and the necessity to inhibit esterase activity when FA^{18}O are used as internal standards for MS analysis of biological matrix.

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List of Abbreviations

DEUP	diethylumbelliferyl phosphate
DMSO	dimethyl sulfoxide
FA^{18}O	fatty acids labeled with ^{18}O at the carboxyl
HPLC	high performance liquid chromatography
IC_{50}	half maximal inhibitory concentration
LC	liquid chromatography, MS, mass spectrometry
PBS	phosphate buffered saline
PGD_2d_9	prostaglandin D_2 -17,17,18,18,19,19,20,20,20- d_9
$\text{PGE}_2^{18}\text{Od}_4$	a mixture of prostaglandin E_2 -1,1- ^{18}O -3,3,4,4- d_4 (91%) and prostaglandin E_2 -1- ^{18}O -3,3,4,4- d_4 (9%)
PGE_2d_4	prostaglandin E_2 -3,3,4,4- d_4

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Figure 1.

Heat denaturing of plasma proteins prevents oxygen exchange on PGE₂ carboxyl group. Rat plasma (10% in PBS, pH=7.4) was boiled for 10 min in closed container to heat denature proteins. Two hundred fifty μ L of boiled or control (not-boiled) plasma was incubated with 2 ng PGE₂¹⁸O at 37°C, and PG were extracted and analyzed using LC-MS as described in the Methods section. Results are mean $\pm$ SD, n=3.

Figure 2.

Inhibition of oxygen exchange and PGE₂-glyceryl ester hydrolysis by DEUP and MAFP. Two ng of prostaglandin E₂ labeled with ¹⁸O at the carboxyl group (PGE₂¹⁸O₄, lower panel), and PGE₂-1-glyceryl ester (PGE₂Gly, upper panel) were incubated for 20 min at 37°C with 10% rat plasma in PBS (10%, pH=7.4, 250 mL total incubation volume) in the presence of different concentrations of diethylumbelliferyl phosphate (DEUP, esterase inhibitor) and methyl-arachidonoyl-fluorophosphate (MAFP, serine dependent hydrolase inhibitor). At the end of incubation, PG were extracted and analyzed using LC-MS as described in the Methods section. The rate of ¹⁸O back exchange from PGE₂¹⁸O₄ with ¹⁶O from water was calculated as an amount of PGE₂ that lost one or two ¹⁸O. Results are mean ± SD, n=3.

Figure 3.

Exogenous prostaglandin content is not changed during incubation with rat plasma. Deuterium labeled prostaglandin E₂ (PGE₂d₄, 2ng) was incubated with rat plasma (10% in PBS, pH=7.4, 250 mL total incubation volume) at 37°C in the presence of diethylumbelliferyl phosphate (DEUP, 5 μM) and methyl-arachidonoyl-fluorophosphate (MAFP, 6 μM). At the indicated incubation times, PG were extracted and analyzed using LC-MS as described in the Methods section. Results are mean ± SD, n=3. * indicates statistically different values as compared to boiled plasma.

Table 1IC₅₀ values for inhibitors of oxygen exchange and PGE₂-glyceryl ester hydrolysis

Substrate used	Inhibitor, IC ₅₀ , μ M	
	MAFP	DEUP
Oxygen exchange for PGE ₂ ¹⁸ Od ₄	0.334±0.081	0.078±0.023
PGE ₂ Gly	0.484±0.076	0.076±0.012

IC₅₀ values were calculated from data presented in the Fig. 2 using non-linear regression (GraphPad Prism5 software). Experimental conditions and abbreviations are as described in the Fig. 2. Results are mean (μ M) $\pm$ SD, n=3.