Evidence that the E_2-isoprostane, 15-E_{2t}-isoprostane (8-iso-prostaglandin E_2) is formed in vivo

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Abstract **D_2/E_2-isoprostanes are prostaglandin D_2/E_2-like compounds that are produced in vivo as non-enzymatic products of free radical catalyzed peroxidation of arachidonic acid. One E_2-isoprostane that should be formed is 15-E_{2t}-isoprostane (8-iso-prostaglandin E_2). 15-E_{2t}-isoprostane has been shown to exert potent biological activity but proof that it is formed in vivo is lacking. Evidence is now presented that 15-E_{2t}-isoprostane is formed in vivo by demonstrating that an endogenous E_2-isoprostane with a retention time on capillary GC identical with that of 15-E_{2t}-isoprostane co-chromatographs through four high resolving HPLC purification procedures with authentic radiolabeled 15-E_{2t}-isoprostane.—**Morrow, J. D., J. Scruggs, Y. Chan, W. E. Zackert, and L. J. Roberts II. **Evidence that the E_2-isoprostane, 15-E_{2t}-isoprostane (8-iso-prostaglandin E_2) is formed in vivo. *J. Lipid Res.* 1998.** 39: **1589–1593.**

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Free radical catalyzed lipid peroxidation has been implicated in the pathogenesis of a wide variety of human disorders (1–4). Nonetheless, much remains to be understood about mechanisms of oxidant injury in vivo. Autoxidation of fatty acids in vitro results in the formation of a number of products, including prostaglandin (PG)-like compounds (5). In the early 1990s, we reported that a series of PGF_2-like compounds, termed F_2-isoprostanes (F_2-IsoPs), is produced in vivo in humans as products of the free radical-catalyzed peroxidation of arachidonic acid independent of the cyclooxygenase enzyme (6). Formation of F_2-IsoPs proceeds through intermediates comprised of four positional peroxyl radical isomers of arachidonic acid to yield PGG_2-like bicycloendoperoxides. The endoperoxides are then reduced to F-ring IsoPs (7). F_2-IsoPs are primarily formed in situ from arachidonic acid esterified in phospholipids and subsequently released preformed into the circulation presumably by a phospholipase(s) (8, 9). Levels of F_2-IsoPs increase dramatically in animal models of free radical injury and quantification of these compounds has been useful to assess oxidant stress status in vivo (10). In addition, we have previously reported that the biologically active F_2-IsoP, 15-F_{2t}-IsoP (8-iso-$PGF_{2\alpha}$) is formed in vivo (11).

More recently, we have reported that in addition to F-ring IsoPs, IsoPs containing D- and E-prostane rings, termed D_2/E_2-IsoPs, are formed in vitro and in vivo in significant quantities from the free radical-catalyzed peroxidation of arachidonate (12). In contradistinction to F_2-IsoPs, however, D_2/E_2-IsoPs result from isomerization of IsoP endoperoxide intermediates rather than from reduction. We have also found that one E_2-IsoP, 15-E_{2t}-IsoP (8-iso-PGE_2), possesses potent biological activity as a vasoconstrictor at concentrations in the low nanomolar range and thus may mediate some of the adverse effects associated with oxidant stress (12–14). Interestingly, the vasoconstricting properties of 15-E_{2t}-IsoP appear to occur by interaction with a unique receptor (10).

Despite the fact that considerable work has been performed investigating the biological activity of 15-E_{2t}-IsoP, definitive evidence that it is formed in vivo is lacking. Previous studies suggested that 15-E_{2t}-IsoP might be formed in vivo in that D_2/E_2-IsoP-containing extracts of biological fluids and tissues contain compounds with similar properties on thin-layer chromatography and gas chromatography (GC) as authentic 15-E_{2t}-IsoP (12). However, as many as 128 different D_2/E_2-IsoPs can theoretically be formed and purification of D_2/E_2-IsoPs by thin-layer chromatography incompletely separates a mixture of these compounds (6). For obvious reasons, information as to whether 15-E_2-IsoP is produced in vivo is essential to the relevance of studies examining its biological activity. Therefore, to determine whether 15-E_{2t}-IsoP is generated in vivo, we assessed whether a D_2/E_2-IsoP was present in a biological extract that has a retention time on capillary GC identical to that of chemically synthesized 15-E_{2t}-IsoP and co-chromatographs with 15-E_{2t}-IsoP through four different high resolving high pressure liquid chromatography (HPLC) purification procedures.

Abbreviations: PG, prostaglandin; IsoP, isoprostane; GC, gas chromatography; PFB, pentafluorobenzyl; NICI, negative ion chemical ionization; MS, mass spectrometry.

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METHODS

Materials

CCl_4 was purchased from Fischer Scientific Corp. (Pittsburgh, PA). Methoxyamine HCl, pentafluorobenzyl (PFB) bromide, diisopropylethylamine, dimethylformamide, undecane, and *Apis mellifera* venom phospholipase A_2 were obtained from Sigma-Aldrich (St. Louis, MO). *N,O*-bis(trimethylsilyl)trifluoroacetamide was obtained from Supelco (Bellefonte, PA). All other organic reagents were obtained from Baxter Healthcare (Burdick and Jackson Brand, McGaw Park, IL). 15-E_{2t}-IsoP (8-iso-PGE_2) and [$^2H_4$]PGE_2 were obtained from Cayman Chemical (Ann Arbor, MI). [$^3H_6$]15-E_{2t}-IsoP (161 Ci/mmol) was commercially prepared from [$^3H_7$]PGE_2 by Amersham Radiochemicals (Arlington Heights, IL). To prepare [$^3H_6$]15-E_{2t}-IsoP, [$^3H_7$]PGE_2 was incubated for 12 h at room temperature with 0.15 M potassium acetate in ethanol (15). [$^3H_6$]15-E_{2t}-IsoP was separated from [$^3H_7$]PGE_2 by reversed phase HPLC. The yield of [$^3H_6$]15-E_{2t}-IsoP was approximately 5%.

Isolation of D_2/E_2-IsoPs

A mixture of D_2/E_2-IsoPs was isolated from the liver of a Sprague-Dawley rat 2 h after the intragastric administration of CCl_4 (2 ml/kg) in corn oil. The entire protocol was approved by the Vanderbilt University Animal Care Committee in accordance with IACUC protocol. CCl_4 induces marked lipid peroxidation in the livers of treated animals (12). Subsequently, the rat was anesthetized with pentobarbital (60 mg/kg) intraperitoneally, killed, and the liver was removed. Four grams of tissue was immediately extracted (16) to obtain a crude phospholipid extract containing D_2/E_2-IsoPs esterified to phospholipids. The lipid extract was then subjected to hydrolysis with *Apis mellifera* venom phospholipase A_2 as described (12, 17). Free D_2/E_2-IsoPs were extracted and partially purified using C18 and Silica Sep Pak cartridges (Waters Associates, Milford, MA) (12). Using these methods, generation of D_2/E_2-IsoPs in tissues ex vivo during sample workup does not occur (8, 12, 18).

HPLC separation of 15-E_{2t}-IsoP

To the partially purified hydrolysate containing a mixture of D_2/E_2-IsoPs was added 2 μCi of [$^3H_6$]15-E_{2t}-IsoP. The mixture was then subjected to 4 successive HPLC purification steps. To maximize purification and resolution of different D_2/E_2-IsoPs, HPLC procedures were utilized that yielded relatively long retention volumes for 15-E_{2t}-IsoP (approximately 20–35 ml) and each solvent was run isocratically. In addition, the solvent systems separate 15-E_{2t}-IsoP from cyclooxygenase-derived PGE_2 and PGD_2 by at least 3 ml. For each HPLC procedure, 0.5-ml fraction volumes were collected. In preliminary studies, radiolabeled 15-E_{2t}-IsoP separated to a significant degree (1–2.5 ml) from unlabeled 15-E_{2t}-IsoP due to the fact that it contains 6 tritium atoms. Thus, for each HPLC step, fractions corresponding to those containing both labeled and unlabeled 15-E_{2t}-IsoP were collected and pooled for further purification. The first HPLC system was normal phase using a 25 cm × 4.6 mm Econosil SI column with 5-μm particles (Alltech Associates, Deerfield, IL). The solvent system was hexane–isopropanol–acetic acid 87:13:0.1 (v/v/v) at a flow rate of 1 ml/min. The second HPLC system was reversed phase using an Econosil C18 column (25 cm × 4.6 mm, 5-μm particles (Alltech Associates)). The solvent system was acetonitrile–water–acetic acid 30:70:0.1 (v/v/v) at a flow rate of 1 ml/min. For the third and fourth HPLC purifications, D_2/E_2-IsoPs were converted to PFB esters by reaction with 10% pentafluorobenzyl bromide in acetonitrile for 20 min at 37°C (18) and rechromatographed on normal and reversed HPLC. A solvent system of hexane–isopropanol 90:10 (v/v) was used for the third HPLC step and acetonitrile–water 48:52 (v/v) was used for the 4th HPLC, both at a flow rate of 1 ml/min.

Analysis of D_2/E_2-IsoPs by gas chromatography

Quantification of D_2/E_2-IsoPs in the partially purified liver extract and throughout subsequent HPLC purification procedures was performed by analyzing aliquots by selected ion monitoring GC/negative ion chemical ionization (NICI) mass spectrometry (MS) using [$^2H_4$]PGE_2 as an internal standard (12). Compounds were analyzed as O-methyloxime, PFB ester, trimethylsilylether derivatives monitoring the M-PFB (M-181) ions *m/z* 524 for endogenous D_2/E_2-IsoPs and *m/z* 528 for [$^2H_4$]PGE_2.

RESULTS

Isolation of D_2/E_2-IsoPs from rat liver

Figure 1 shows the selected ion current chromatograms obtained from the analysis of the hydrolysate of the initial rat liver extract prior to HPLC purification. A number of *m/z* 524 peaks are present representing a mixture of different D_2/E_2-IsoPs. Synthetic 15-E_2-IsoP cochromatographs as syn and anti O-methyloxime isomers represented by the starred (*) peaks in the *m/z* 524 chromatogram. The quantity of D_2/E_2-IsoPs represented by the two starred peaks was 1.4 μg and the amount of

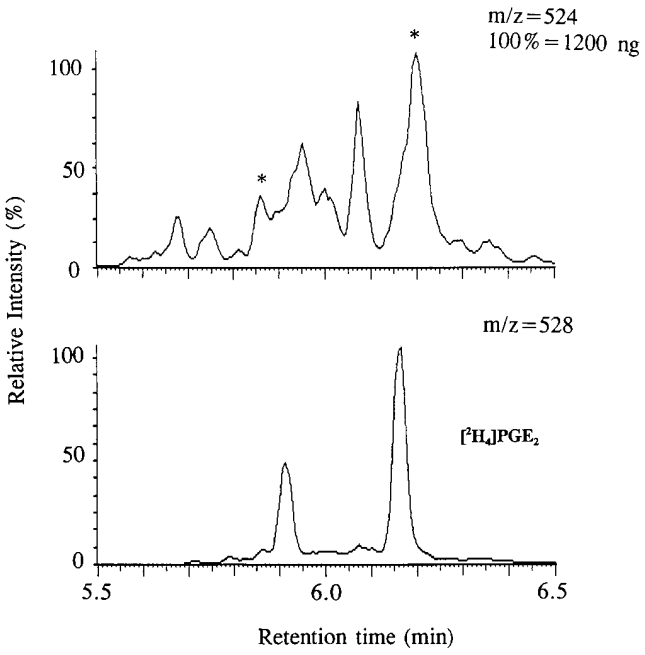

Fig. 1. Selected ion current chromatograms obtained from the analysis of crude hydrolyzed liver extract for D_2/E_2-IsoPs. The *m/z* 524 chromatogram shows a series of peaks representing endogenous D_2/E_2-IsoPs and the *m/z* 528 chromatogram represents the syn and anti O-methyloxime isomers of the [$^2H_4$]PGE_2 internal standard. The starred (*) peaks in the *m/z* 524 chromatogram represent material that co-elutes on GC with the syn and anti O-methyloxime isomers of chemically synthesized 15-E_{2t}-IsoP. The quantity of D_2/E_2-IsoPs represented by the two starred peaks was 1.4 μg and the amount represented by all of the peaks in the chromatogram was 4.3 μg.

D_2/E_2-IsoPs as represented by all of the peaks in the chromatogram was 4.3 μg.

HPLC separation of 15-E_{2t}-IsoP

The crude liver hydrolysate containing the mixture of D_2/E_2-IsoPs was initially subjected to normal phase HPLC using the solvent system hexane–isopropyl alcohol–acetic acid 87:13:0.1 (v/v/v). Aliquots of fractions eluted from the HPLC were then analyzed for D_2/E_2-IsoPs by GC/MS and for radioactivity (**Fig. 2A**). Radiolabeled 15-E_{2t}-IsoP eluted in this system between 15–17.5 min. Compounds representing endogenous D_2/E_2-IsoPs were present that had the same retention time on GC as 15-E_{2t}-IsoP, but which eluted with different retention volumes from that of 15-E_{2t}-IsoP on HPLC (11–13 ml and 17–20 ml). Radiolabeled 15-E_{2t}-IsoP elutes at a volume approximately 1.5 ml after unlabeled 15-E_{2t}-IsoP using this HPLC system. Significantly, as shown in Fig. 2A, an endogenous D_2/E_2-IsoP peak (+) was detected that co-eluted with unlabeled 15-E_{2t}-IsoP.

Material eluted from the HPLC between 13–17.5 ml in Fig. 2A was then subjected to reversed phase HPLC using an isocratic solvent system of acetonitrile–water–acetic acid 30:70:0.1 (v/v/v). Aliquots of fractions collected were again analyzed for endogenous D_2/E_2-IsoPs by GC/MS and radioactivity (Fig. 2B). Radiolabeled 15-E_{2t}-IsoP eluted from the HPLC with a retention volume of 30.5–34 ml. Analysis of aliquots of the eluted fractions by GC/MS showed that almost all of the unlabeled D_2/E_2-IsoP material detected in the chromatogram eluted at the retention time of unlabeled 15-E_{2t}-IsoP (31–33.5 ml) except for a small amount of additional material that eluted at the trailing end (note shoulder) of the large endogenous D_2/E_2-IsoP peak (33.5–35 ml).

Altering the polarity of a compound by derivatization and rechromatographing the compound can provide a powerful approach for purification and separation of biomolecules (11). Thus, the material eluted from the HPLC between 30.5–33 ml in Fig. 2B was converted to a PFB ester and rechromatographed on normal phase HPLC using a solvent system of hexane–isopropyl alcohol 90:10 (v/v). Pooling only the material that eluted between 30.5–33 ml eliminated most of the material eluting as a shoulder on the trailing edge of a large chromatographic peak representing D_2/E_2-IsoPs that co-eluted with unlabeled 15-E_{2t}-IsoP. Figure 2C shows the results of this HPLC step. The

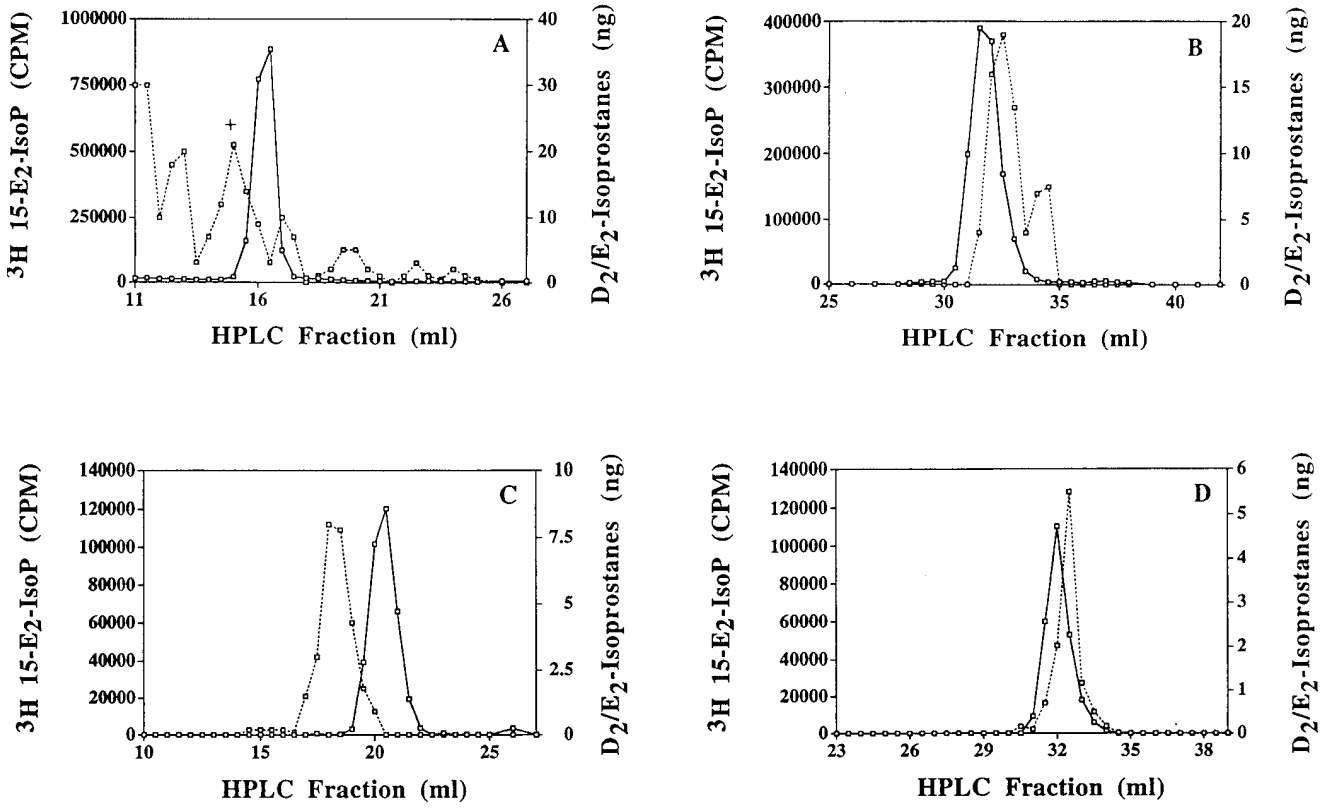

Fig. 2. HPLC purification of a mixture of D_2/E_2-IsoPs shown in Fig. 1. Details regarding solvent systems used are described in the text. Tritiated 15-E_{2t}-IsoP was added to the mixture at the beginning and aliquots of fractions eluted were assayed for radioactivity (open squares, solid lines). Aliquots were also assayed and quantified by GC/MS for the presence of D_2/E_2-isoP peaks with the same GC retention time as that for authentic 15-E_{2t}-IsoP (open squares, dotted lines). All HPLC purifications were carried out isocratically. (A) Normal phase HPLC as free acids of the initial mixture of D_2/E_2-IsoPs as shown in Fig. 1; (+) denotes fractions in which chemically synthesized unlabeled 15-E_{2t}-IsoP elutes using this solvent system. (B) Reversed phase HPLC as free acids of the material eluted at the retention volume between 13–17.5 min in Fig. 2A. (C) Normal phase HPLC as PFB esters of the material eluted at the retention volume between 30.5–33 ml in Fig. 2B. (D) Reversed phase HPLC as PFB esters of the material eluted at the retention volume between 17.5–21 ml in Fig. 2C.

radiolabeld 15-E_{2t}-IsoP eluted between 19–21.5 ml. As before, a large peak representing endogenous D_2/E_2-IsoPs that co-eluted with unlabeled 15-E_{2t}-IsoP was detected (17.5–20.5 ml).

Compounds eluted between 17.5–21 ml in the HPLC step shown in Fig. 2C were then pooled. This material was subjected to further purification by reversed phase HPLC using a solvent system of acetonitrile–water 48:52 (v/v). The results of the analyses for radioactivity and endogenous D_2/E_2-IsoPs in the eluted fractions are shown in Fig. 2D. Only a single D_2/E_2-IsoP peak eluted from the HPLC column which co-eluted exactly with the PFB ester of unlabeled 15-E_{2t}-IsoP (31.5–33.5 ml). Radiolabeled 15-E_{2t}-IsoP eluted slightly before the endogenous D_2/E_2-IsoP compound.

Analysis of endogenous 15-E_2-IsoP by GC/MS

Material eluting from the fourth HPLC step between 32–32.5 ml was then analyzed by GC/MS. As shown in **Fig. 3**, two D_2/E_2-IsoP peaks were present in the *m/z* 524 chromatogram representing the syn and anti O-methyloxime isomers of putative 15-E_{2t}-IsoP. When this material was mixed with an equivalent amount of derivatized synthetic 15-E_{2t}-IsoP and analyzed by GC/MS, the two compounds co-chromatographed perfectly on capillary GC without any suggestion of a shoulder on the GC peaks (**Fig. 4**).

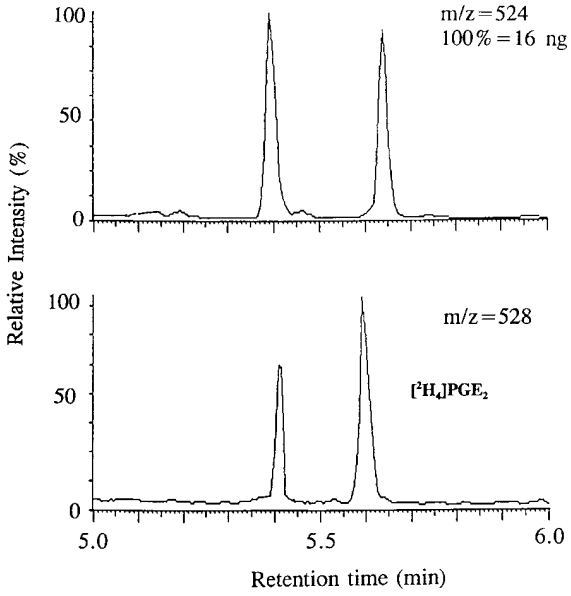

Fig. 3. Selected ion current chromatogram obtained from the GC/MS analysis of the material eluted at a retention volume between 32–32.5 in Fig. 2D. Only a single set of *m/z* 524 peaks representing the syn and anti O-methyloxime isomers of an endogenous D_2/E_2-IsoP, presumably 15-E_{2t}-IsoP, remains after the four HPLC purification procedures shown in Fig. 2. The peaks in the lower *m/z* 528 chromatogram represent the syn and anti O-methyloxime isomers of the deuteriated PGE_2 internal standard. The amount of 15-E_{2t}-IsoP in the fraction eluted between 32–32.5 ml is approximately 31 ng. The total amount of 15-E_{2t}-IsoP generated from 4 g of rat liver was approximately 65 ng which is approximately 1–2% of the total D_2/E_2-IsoPs generated.

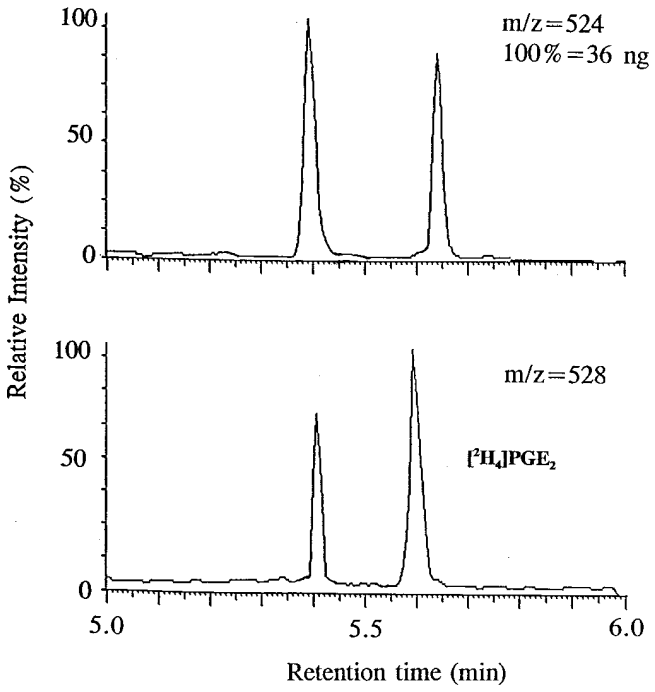

Fig. 4. Selected ion current chromatogram from the GC/MS analysis of the material eluted at a retention volume between 32–32.5 ml in Fig. 2D combined with a roughly equivalent amount of synthetic 15-E_{2t}-IsoP. Only a single set of *m/z* 524 peaks representing the syn and anti O-methyloxime isomers of 15-E_{2t}-IsoP is present. Note that these peaks are without any shoulders. The peaks in the lower *m/z* 528 chromatogram represent the syn and anti O-methyloxime isomers of the deuteriated PGE_2 internal standard.

DISCUSSION

These studies provide evidence that the E_2-IsoP, 15-E_{2t}-IsoP, is produced in vivo. This finding is of particular importance as a number of studies have shown that 15-E_{2t}-IsoP exerts potent biological activity, suggesting that this compound could participate as a mediator in the pathophysiology of oxidant injury (12–14). Identity of 15-E_{2t}-IsoP was made using HPLC and GC. While these methodologies strongly support the identification of this compound, the one analytical approach that would unequivocally prove that the D_2/E_2-IsoP that exactly eluted at the retention time of 15-E_{2t}-IsoP was endogenous 15-E_{2t}-IsoP would be NMR spectroscopy. However, it is not possible to obtain a sufficient quantity of this compound from a biological source for analysis by NMR. Given these limitations, the results of studies described herein provide evidence, as best as can be obtained, that 15-E_{2t}-IsoP is produced in vivo.

The abundance of 15-E_{2t}-IsoP formed relative to that of the other D_2/E_2-IsoPs can also be estimated from the results obtained. For these studies, a known amount of radiolabeled 15-E_2-IsoP was added to the crude liver homogenate containing a mixture of D_2/E_2-IsoPs and the intensity of peaks in this mixture (Fig. 1) was quantified. Material

co-eluting with 15-E_{2t}-IsoP was shown by the starred (*) peak in Fig. 1. As is evident in Fig. 2, a considerable portion of the material in the starred peak in Fig. 1 was separated in subsequent HPLC purification steps. As a result, after the fourth HPLC, the ratio of the amount of material present when quantified by GC/MS to the amount of radioactivity recovered showed that 15-E_{2t}-IsoP represented approximately 1–2% of the total D_2/E_2-IsoPs generated in vivo (approximately 70 ng/4 g liver tissue).

In light of the findings that 15-E_{2t}-IsoP is formed in vivo, future studies aimed at exploring the spectrum of biological activity of this IsoP and the mechanism by which it exerts these actions should be of relevance.

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