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Short Communication

Application of liquid radiochromatographic techniques to the metabolic profiling of xenobiotically-mediated arachidonate metabolism*

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Introduction

Numerous *in vitro* studies have established the capacity of macrophages to metabolize arachidonic acid (AA) to a variety of biologically active compounds [1]. These include products derived from lipoxygenase pathways such as 12- and 15-hydroxyeicosatetraenoic acid (12-HETE and 15-HETE) [2], and cyclooxygenase metabolites such as various prostaglandins (PGs) and thromboxane B_2 (TXB_2) [3].

This paper describes the suitability of on-line radiometric detection, after reversed-phase LC separation (LC–RD), for studying the action of different xenobiotics on the metabolism of arachidonic acid, and that of the xenobiotic itself. In previous studies on Toxic Oil Syndrome (TOS) [4–6], the effects of different xenobiotics such as phenol (P), *N*-phenyllinoleamide (NPLA) or pentachlorophenol (PCP) on the AA metabolism in mouse peritoneal macrophages (MPM) were investigated. Both NPLA and PCP have been detected in rapeseed cooking oils associated with TOS [7, 8]. LC–RD has been applied previously to the study of the metabolism of ring G tritium-labelled NPLA (*N*-[ring-*G*-^{3}H]PLA) and carbon-14-labelled NPLA (*N*-P[1-^{14}C]LA) by mouse peritoneal macrophages and human platelets.

Experimental

Reagents

Tritiated arachidonic acid (^{3}H-AA, specific activity 60 Ci mmol^{-1}) was supplied by New England (Boston, MA, USA). Tritiated standards: ^{3}H-12-HETE (143 Ci mmol^{-1}), ^{3}H-15-HETE (191 Ci mmol^{-1}), ^{3}H-5-HETE (149 Ci mmol^{-1}), leukotriene B_4 (^{3}H-LTB_4, 228 Ci mmol^{-1}) and ^{3}H-LTC_4 (51 Ci mmol^{-1}) were purchased from Amersham Co. (Bucks, UK). NPLA, *N*-[^{3}H]PLA and *N*-P[^{14}C]LA were synthesized as previously described [9, 10]. PCP was purchased from Kromxpeck Analítica (San Cugat, Barcelona, Spain) and P from Panreac (Barcelona, Spain). Unisolve-1 scintillation fluid was obtained from Koch-Light (Haverhill, Suffolk, UK). Reagents and solvents used for LC and liquid–liquid extractions were analytical or spectroscopic grade. Minimum essential medium Eagle (modified) with Earle's salts (EMEM) was supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids mixture (1%), penicillin and streptomycin (100 IU ml^{-1}) and foetal bovine serum (10%) (Flow Laboratories, McLean, VA, USA).

Instrumentation

The LC–RD equipment comprised the

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following components: a Rheodyne 7125 injector; two Kontron 414 series pumps; a 200 series gradient controller (Kontron Analytical, Zurich, Switzerland) and a Ramona Ray Test radioactivity detector (Isomess, Straubenhardt, Germany).

Isolation of cells

Swiss mice (25–30 g body weight) were injected intraperitoneally with 2 ml of a 2.5% thioglycollate saline solution. Three days later, the animals were killed and exanguined, and the peritoneal mouse cells were collected by washing the peritoneal cavity with Dulbecco's phosphate buffer saline (PBS). Cells were centrifuged at 1400**g** for 20 min at 16°C, resuspended in supplemented EMEM and 2 ml aliquots each containing 5×10^6 cells ml^{-1} were distributed into the 35 mm wells of plastic plates. Macrophage monolayers were obtained according to the method described by Dimitriu [11].

Human platelets were obtained from healthy volunteers who had not received any medication in the preceding 7 days. Washed platelets were prepared according to the method described by Rao *et al.* [12]. Briefly, the anticoagulated blood was centrifuged to prepare platelet rich plasma (PRP). The PRP was mixed with 0.5 parts by volume of a dextrose citric citrate mixture and centrifuged. This operation was repeated 2 or 3 times to remove erythrocytes. Finally, the platelet pellet was resuspended in Hank's buffer (pH 7.3), and the cellular density was adjusted to 5×10^8 cells ml^{-1}.

Incubation procedures

Mouse peritoneal macrophages were incubated for 2 h both in the absence and presence of three different xenobiotics, namely NPLA, P and PCP, at 1 mM concentration in supplemented EMEM. After exposure to the xenobiotic, cells were washed four times to remove any traces of the xenobiotic and then incubated for 8 min with ^{3}H-AA (0.5 μCi) in 2 ml PBS. Subsequently, the incubation medium was collected and eicosanoids were extracted four times with 1 ml of ethyl acetate.

Finally, NPLA metabolism by MPM and human platelets was studied. For this purpose, monolayers of MPM were incubated, respectively, with 2 ml of *N*-[ring ^{3}H]PLA and *N*-P[1-^{14}C]LA solutions in serum-free EMEM (2 h, 37°C). Washed human platelets were also incubated with 1 ml of labelled NPLA solutions in Hank's solution (2 h, 37°C). Thereafter, the incubation medium was collected and the labelled NPLA metabolites were extracted twice by addition of two volumes of ethyl acetate. For LC–RD analysis, the dried residues were also resuspended in chromatographic buffer.

Liquid radiochromatographic techniques

LC–RD profiles of AA metabolites were obtained by using a 5 μm (25 × 0.46 cm) reversed-phase Ultrasphere IP column (Beckman, San Ramón, CA, USA). The eluent was methanol–water–trifluoroacetic acid–triethylamine (80:20:0.1:0.05, v/v/v/v), running isocratically at a flow rate of 1.5 ml min^{-1} for 14 min and then under a linear gradient to 100% methanol from 14 to 17 min [13].

LC–RD profiles of *N*-[ring ^{3}H]PLA and *N*-P[1-^{14}C]LA metabolites were obtained using a 10 μm (30 × 0.4 cm) reversed-phase Spherisorb ODS-2 column (Tracer Analítica, San Cugat, Barcelona, Spain). The eluent was water (adjusted to pH 3.4 with acetic acid)–acetonitrile at 1.5 ml min^{-1} flow rate. A stepped gradient programme consisting of 25-90% acetonitrile from 3 to 23 min and 90-100% in 5 min was used.

Results and Discussion

The ethyl acetate extraction recoveries for ^{3}H-15-HETE, ^{3}H-12-HETE, ^{3}H-5-HETE, ^{3}H-LTB_4 and ^{3}H-LTC_4 were (mean ± standard deviation): 98.8 ± 0.1; 93.6 ± 0.47; 92.9 ± 0.16; 98.1 ± 0.32 and 62.7 ± 4.33, respectively. Although it has been reported that the ethyl acetate extraction is non-selective for several arachidonate metabolites, such as peptidoleukotrienes [14], recoveries corresponding to the major metabolites synthesized by mouse peritoneal macrophages (12-HETE and 15-HETE) were higher than 90%.

Figure 1 shows the LC–RD profiles obtained from unexposed and NPLA-, P-, and PCP-exposed cells (1 mM, 2h, 37°C) before ^{3}H-AA incubation for 8 min. In all cases, 12-HETE was the major metabolite, in agreement with previous reported data [15]. 15-HETE in low concentrations was produced but there was no evidence of conversion to leukotrienes [16].

Figure 2 shows the effects of NPLA, PCP and P on the biosynthesis of the major lipoxygenase AA metabolites. NPLA slightly in-

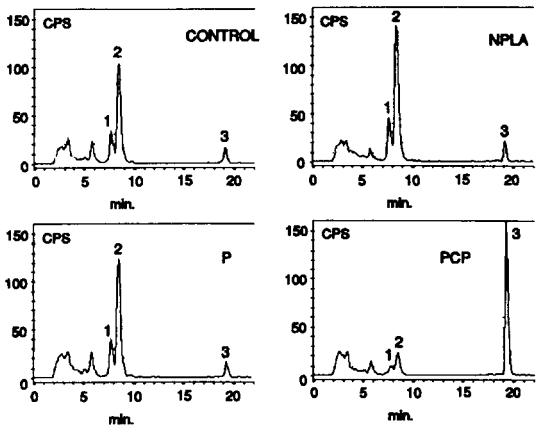

Figure 1
LC–RD profiles of the products generated from exogenous ^{3}H-AA (8 min incubation time) by MPM previously unexposed (control) and NPLA-, P-, and PCP-exposed cells. 1, ^{3}H-15-HETE; 2, ^{3}H-12-HETE; 3, ^{3}H-AA substrate.

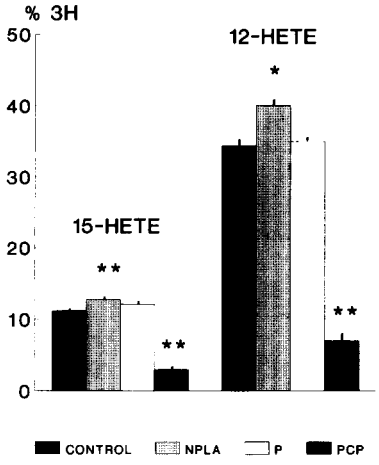

Figure 2
Bar plot depicting the percentage of radioactivity (mean ± standard error of the mean) as ^{3}H-15-HETE and ^{3}H-12-HETE recovered in the supernatant fluid of MPM incubated with ^{3}H-AA for 8 min and previously unexposed (n = 14) and exposed to NPLA (n = 8), P (n = 6) and PCP (n = 6);. * $P < 0.005$ vs control (one way ANOVA and subsequent multiple comparisons by Scheffe test). ** $P < 0.001$ vs control.

creased 12- and 15-HETE levels compared to the controls, whereas P had no effect on these lipoxygenase pathways in MPM. A significantly suppressed metabolism of ^{3}H-AA was observed in PCP-exposed cells, probably due to the toxic nature of this xenobiotic. This has been demonstrated by light microscopy studies in which cellular viability was found to be lower than 10% when measured by the trypan blue exclusion method. In contrast, cell viability after exposure to NPLA and P was higher than 95%.

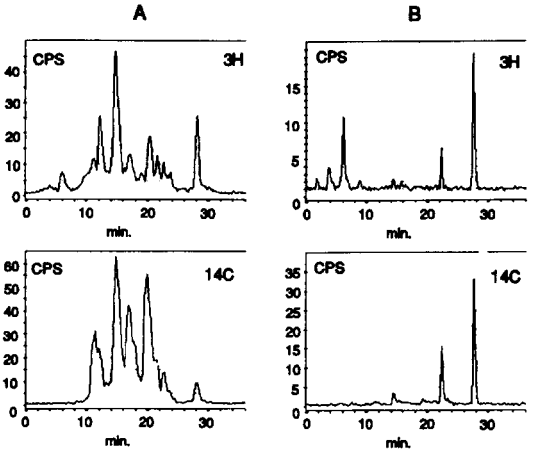

Figure 3
LC–RD profiles of N-[ring-^{3}H]PLA (upper traces) and N-P[1-^{14}C]LA (lower traces) metabolites recovered in (A) the supernatant fluid of MPM after 2 h incubation time, (B) the supernatant fluid of human platelets after 2 h incubation time.

Finally, by using LC–RD techniques, the N-[ring ^{3}H]PLA and N-P[1-^{14}C]LA metabolism by mouse peritoneal macrophages and human platelets was studied. The LC–RD profiles of NPLA metabolites are shown in Fig. 3. Results indicated that MPM and human platelets can metabolize NPLA to several products, and by comparing the differential ^{3}H and ^{14}C traces, metabolites labelled only in the ring and/or in the fatty acid chain were detected. Although an unequivocal identification of these metabolites awaits the results of on-going studies by mass spectrometric techniques, it is known that MPM and human platelets are a rich source of 12-lipoxygenase [15, 17] which is responsible for free fatty acid metabolism, not only of AA as shown herein (Fig. 1), but also of other acids such as linoleic acid [18, 19]. Thus, by taking into account the linoleyl moiety of NPLA, the overall qualitative similarity of the ^{3}H and ^{14}C profiles by MPM (Fig. 3A), and the lack of mixed function oxidase activity of macrophages [20] acting on the N-phenyl moiety, it would seem that most of the components of the LC–RD profile are metabolites arising from lipoxygenase activity on the fatty acid moiety of NPLA.

In contrast, human platelets seem to induce more clearly defined differential patterns (Fig. 3B) and reduced metabolic transformation. N-phenyl ring metabolism seems to be relatively prominent in this case as shown in the ^{3}H trace (Fig. 3B).

In conclusion, on-line LC–RD appears to be a rapid and suitable technique for carrying out studies of the effects of xenobiotics on AA metabolism, and on xenobiotic metabolism in a complex biological matrix. By using LC–RD, it is not possible to identify the structure of the metabolites, but the fate of the labelled moieties can be readily monitored. Therefore, LC–RD is a useful screening technique prior to final identification by GC–MS.

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