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On the presence of phosphorylated sphingoid bases in rat tissues

A mass-spectrometric approach

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Abstract

A simple and straightforward procedure to analyze phosphorylated sphingoid bases has been developed. After phase separation of lipid extracts under alkaline conditions, the compounds were quantitatively recovered in the aqueous upper phase. Following a clean-up of the aqueous phase on C18-solid phase extraction columns, the amino-group of the bases was derivatized by means of phenylisothiocyanate addition. FAB-MS of the phenylthiocarbamate derivatives of sphingenine- and sphinganine-phosphate in the negative mode revealed the expected pseudo-molecular ions (M-1) at 513 m/z and 515 m/z, respectively. Moreover, a typical fragmentation pattern, characterized by the loss of the phenylthiocarbamate moiety (m/z = 135), was observed. When applied to rat tissues, the presence of sphingenine-phosphate in brain, kidney and liver could easily be demonstrated. Highest levels, amounting to 5 nmol/g of wet weight, were present in brain.

Key words: Aliphatic amine; Signal transduction; Sphingosine kinase; Sphingenine-phosphate; Sphingosine-phosphatase; Sphingosine-phosphate lyase

1. Introduction

The involvement of sphingolipids in signal transduction has been intensively studied during the last years. In addition to ceramide [1], sphingoid bases [2], sphingosylphosphocholine [3], lysoglycosphingolipids [4], and gangliosides [5], sphingosine-phosphate has also been added to the list of bioactive sphingolipids. Sphingenine-phosphate has been shown to influence the intracellular mobilization of calcium [6], to be mitogenic, probably via an activation of a phospholipase D activity [7], and to inhibit cell motility [8]. The lipid can be formed by phosphorylation of sphingenine [9] (or deacylation of ceramide-phosphate), while attenuation of the signal relies on the action of sphingosine-phosphate lyase [10] and/or sphingosine-phosphatase [11]. The presence of phosphorylated sphingoid bases in cells or tissues has seldom been investigated. Small amounts of 4*D*-hydroxy-sphinganine-phosphate have been found in the intestinal mucosa of rats given orally 4*D*-hydroxysphinganine and in the kidney of rats after intravenous administration of this sphingoid base [12]. Phosphorylation of the supplemented sphingoid bases has been documented only in platelets, which lack sphingosine-phosphate lyase [13], and in tissues of rats, given optical isomers of sphinganine that can be phosphorylated but not cleaved [14]. Very recently, the transient formation of labeled sphingenine-phosphate in Swiss 3T3 cells, grown in the presence of labeled serine, in response to PDGF and serum has been reported [15]. To our knowledge, no reports on the quantification of phosphorylated sphingoid bases in biological systems have appeared. Using tritiated sphinganine-phosphate, we were able to investigate the behavior of these zwitterionic lipids during phase extraction and to develop an analytical method based on mass spectrometry of the phenylthiocarbamate derivatives.

2. Materials and methods

2.1. Materials

Sphingolipids were obtained from Sigma, St. Louis, MO, USA. Sphingenine-phosphate [16], sphinganine-phosphate [16], [4,5-^{3}H]sphinganine-phosphate [17], 1-alk(en)ylphosphoethanolamine [11] were prepared as described. PITC and 4-biphenylcarbonylchloride were purchased from Aldrich, Bornem, Belgium and triethylamine and pyridine, both sequencing grade, from Pierce Europe, Oud Beijerland, the Netherlands. Solid phase extraction columns (C18 Bond Elut – 100 mg) were from Analytichem International, Harbor City, California, USA.

2.2. Lipid extraction and derivatisation

Fresh tissues (1 g of wet weight), obtained from male Wistar rats, were rinsed in cold physiological salt solution, patted dry and cut into smaller pieces and homogenized by means of a Polytron homogenizer in 3 ml of methanol/chloroform (2:1 v/v). In order to check the recoveries of phosphorylated sphingoid bases during the work-up, a duplicate was spiked with 2 nmol of [4,5-^{3}H]sphinganine-phosphate (94550 dpm). After extraction of the lipids for 30 min, denatured proteins were

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Abbreviations: FAB-MS, fast atom bombardment-mass spectrometry; PDGF, platelet derived growth factor; PITC, phenylisothiocyanate; PTC-, phenylthiocarbamate-; SPE, solid phase extraction.

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removed by centrifugation. The pellet was reextracted by adding 0.8 ml of water and 3 ml of methanol/chloroform (2:1 v/v). The latter step was repeated twice. The combined extracts, containing approximately 99% of the added radioactivity, were phase separated by adding 4 ml of chloroform and 4 ml of 0.1 M NH_4OH. After centrifugation, the upper phase was removed and the lower phase was washed once with 8 ml of 0.1 M NH_4OH/methanol (6:2 v/v). The combined yellowish alkaline upper phases, which contained 90% to 95% of the extracted radioactivity, were reduced in volume to approx. 5 ml by a gentle stream of dry nitrogen at 45°C, diluted two-fold with 0.1 M NH_4OH (in order to reduce their content of remaining methanol) and then applied to activated C18-SPE cartridges. Tubes were rinsed with 2 × 3 ml of 0.1 M NH_4OH and rinses were also applied to the SPE-cartridges which were subsequently washed with 10 ml of 0.1 M NH_4OH. Depending on the tissue, 5% to 10% of the radioactivity present in the alkaline aqueous upper phases did not bind to the reversed phase. Following an intermediate wash with 4 ml of 0.1 M NH_4OH in 35% (v/v) methanol in which almost no radioactivity was present, phosphorylated sphingoid bases were eluted with 8 ml of 0.1 M NH_4OH in 80% (v/v) methanol. The eluate was dried under nitrogen, dissolved in 0.1 ml of ethanol/pyridine/triethylamine/water (6:1:1:2 v/v) and derivatized by adding 10 μl of PITC. For the derivatization of standards, volumes were reduced two-fold and lipids were first redried from 50 μl of ethanol/pyridine/triethylamine/water (6:1:1:2). Reactions were allowed to proceed at room temperature for 40 min and the mixtures were dried under nitrogen and redried once from methanol. In case of tissue extracts, the derivatized extracts were subjected to an additional phase separation. After dissolving the yellow extracts in 3.8 ml of methanol/chloroform/water (2:1:0.8), phase separation was induced with 1 ml of chloroform and 1 ml of 0.25 M acetic acid. After an additional wash with 2 ml of theoretical upper phase, the lower phase containing 90% of the derivative was dried under nitrogen and stored at −20°C until analysis.

2.3. Mass-spectrometry

Derivatized standards and tissue extracts were dissolved in a 20 μl of methanol and approx. 0.5 μl was mixed with a drop of glycerol, placed at the tip of the probe. FAB-MS was performed with a Finnigan MAT TSQ 70 triple quadrupole mass spectrometer equipped with an Ion Tech FAB gun operated at 6 kV. For the tandem mass spectrometry experiments, Xe at 0.8 mTorr was used as the collision gas. The collision energy was 10 eV.

3. Results and discussion

The analysis of phosphorylated sphingoid bases in biological samples as described in this paper relies on three steps: (1) a selective, non-destructive extraction; (2) a selective, mild derivatisation; (3) mass characterization of the derivatized compound. Phosphorylated sphingoid bases are known to be poorly soluble in organic and aqueous solvents (see [10]). The distribution of μmolar solutions between organic and aqueous phases of the classical Folch [18] or Bligh-Dyer [19] extractions appeared to be governed mainly by the phosphate moiety. For tritiated sphinganine-phosphate, recoveries in the aqueous upper phase during acidic (0.1 M HCl), neutral and high ionic strength (1 M NaCl), and alkaline (0.1 M NaOH) conditions were 22%, 45% and 100%, respectively (in the absence of exogenous lipids). Recoveries of [4,5-^{3}H]sphinganine on the other hand were almost not influenced by the pH (being 90%, 95% and 100%, respectively, in the lower phases of the above mentioned extraction systems). Hence, an alkaline phase separation was chosen as starting step. In order to minimize the amount of sodium-ions which interfere with the mass spectrometry of the final preparations, 0.1 M NH_4OH was used. Extraction conditions are sufficiently mild to avoid hydrolysis of *N*-acylated sphingolipids and to limit the hydrolysis of 1-alk(en)yl-2-acylglycerophosphoethanolamine. The latter compounds would give rise to 1-alk(en)yl-glycerophosphoethanolamine, the behavior of which during phase separation and thin layer chromatography resembles that of phosphorylated sphingoid bases ([11]; Van Veldhoven, P.P. and Mannaerts, G.P., unpublished data), and which is also derivatized by our procedures (see below).

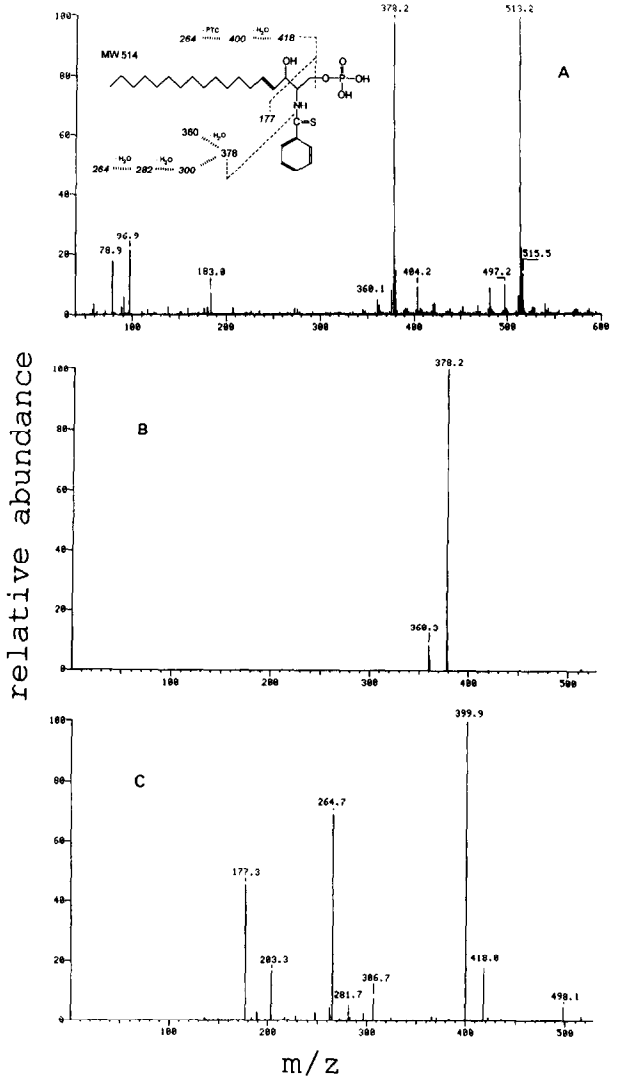

Fig. 1. Mass spectra of PTC-derivatives of sphingenine-phosphate. In panel A, the FAB-spectrum of sphingenine-phosphate (150 nmol of starting product), after derivatisation with PITC, is shown. The corresponding daughter spectra in the negative and positive mode are shown in panel B and C, respectively. In the fragmentation pattern inserted in panel A, the size of the molecular pseudo-ions, expected to be seen in the positive mode, are written in italic numbers.

Binding of the phosphorylated sphingoid bases to the C18-phase appeared not to be controlled by pH. Almost

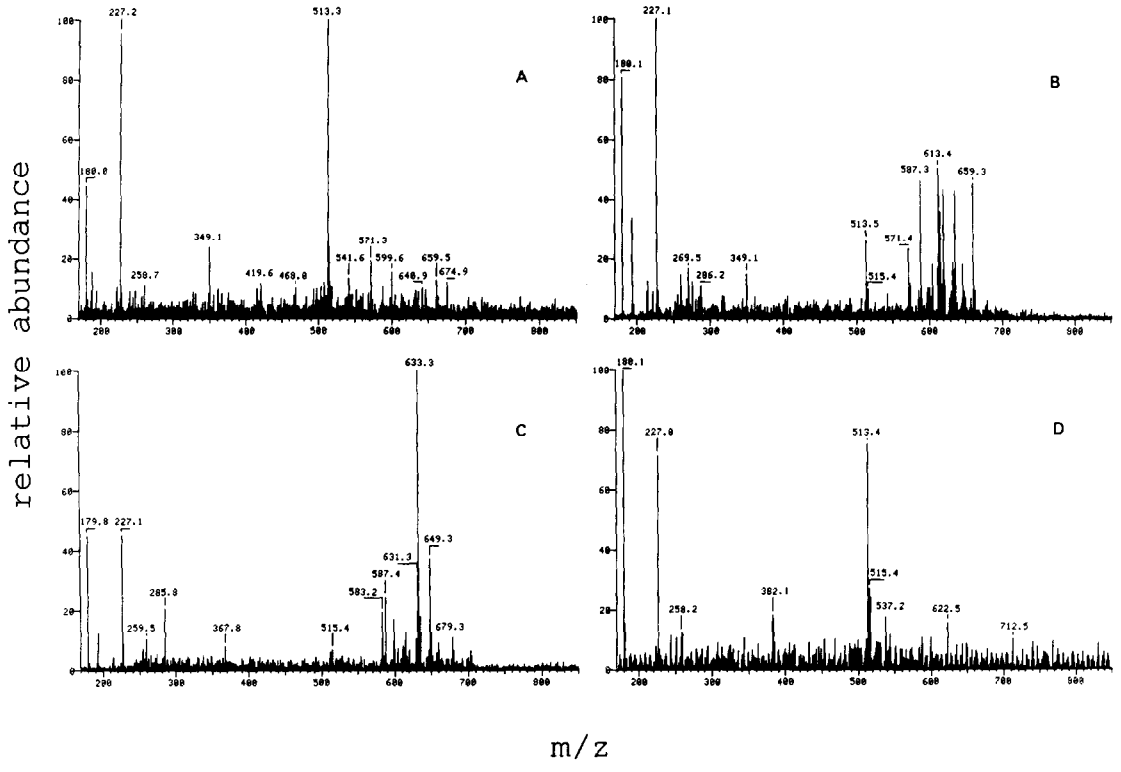

Fig. 2. Mass spectra of PTC-derivatives of rat tissue extracts. Mass spectra of extracts of rat brain (A), rat kidney (B), and rat liver (C), prepared and derivatized with PTC as described in the method section, were analysed for the loss of the PTC-moiety in the negative mode (–NLOSS = 135). In panel D, the corresponding spectrum for 1.6 nmol of sphingenine-phosphate + 0.4 nmol of sphinganine-phosphate standard, carried throughout the extraction procedure, is shown.

complete binding was observed whether the bases were applied in 0.1 M HCl, 0.1 M ammonium acetate, pH 7.0, or 0.1 M NH_4OH (although solubility is lower in neutral and acidic solvents). Hence, the alkaline upper phases, after reducing their methanol content, could be directly applied to the C18 columns. In addition, we did not have to fear precipitation of the phosphorylated sphingoid bases at neutral or acidic pH. Elution from the reversed phase occurred between 50 and 70% (v/v) methanol, either under acidic or alkaline conditions. Although the alkaline nature of the eluent might cause some damage to the matrix during long-term use, we adhered to the described protocol, since in this way the aminogroup of the sphingoid bases was in the appropriate unprotonated form for the derivatization.

For the derivatization an agent compatible with the following criteria was chosen: (1) selectivity for the primary amino group and no reactivity towards amides and, preferentially, secondary amines; (2) mild derivatization conditions to avoid formation of any sphingosine-phosphate by hydrolysis of *N*-acylated sphingolipids such as ceramide-phosphate or sphingomyelin (although their presence in the extracts is unlikely) or from sphingosylphosphocholine (a compound more likely to be present in the extracts); (3) stability of the derivative. Therefore, the formation of biphenylcarbonyl derivatives and of phenylthiocarbamates was investigated. Biphenylcarbonylchloride has already been used to derivatize sphingosylphosphocholine and psychosine [20], followed by HPLC separation and detection at 280 nm. In our hands, however, the biphasic nature of the reaction caused problems, and during the work-up of the samples, a considerable amount of the derivative was lost. With PITC, a complete conversion was obtained as monitored by TLC (data not shown). The volatility of the reaction mixture and reagent is an additional advantage. Moreover, a specific fragmentation pattern could be obtained with these derivatives, not only for sphingenine-phosphate (see below), but also for other lysosphingolipids (unpublished data).

With glycerol as the matrix, excellent and clean FAB-mass spectra were obtained in the negative mode clearly showing the expected pseudo-molecular ion (M-1) of 513 m/z for sphingenine-phosphate (Fig. 1A). Analysis of sphinganine-phosphate-PTC showed a pseudo-molecular ion of 515 m/z (data not shown). For both compounds, the most abundant fragment (378 m/z and 380 m/z, respectively) corresponded to the loss of the PTC-moiety (m/z = 135). Also the fragmentation of the parent pseudo-molecular ion was consistent with their proposed

structure (Fig. 1B and C). As expected, in the positive mode, fragments containing the phosphate-group were not recorded, but the typical W′ (m/z 282) and W′ (m/z 264) ions [21] were seen.

When biological extracts were examined via FAB-MS for those compounds which had lost the PTC-moiety (–NLOSS = 135), the profiles for liver, kidney and brain were quite different (Fig. 2). In brain, the most intense molecular ion (513 m/z) corresponded to that of sphingenine-phosphate-PTC (Fig. 2A). Also in kidney, the presence of sphingenine-phosphate was evident (Fig. 2B). In liver, the 513 m/z peak was just above noise level (Fig. 2C). By daughter analysis the pseudo-molecular ion at 513 m/z in liver extracts was verified to be due to sphingenine-phosphate-PTC. Most of the other molecular ions, seen in liver and kidney extracts, could be identified as monoesterified glycerophosphoethanolamine-PTC derivatives containing palmitoyl (M-1 = 587) or oleoyl (M-1 = 613) as acyl chain. In brain almost no ester-compounds were observed, but the presence of alkenylglycerophosphoethanolamine-PTC derivatives was very prominent (palmityl: M-1 = 571; stearyl: M-1 = 599).

With the exception of liver in which the 515 and 513 pseudo-molecular ion peaks were of similar intensity, the presence of sphinganine-phosphate could not be demonstrated in kidney and brain. In kidney and even more pronounced in brain, a pseudo-molecular ion at 541 m/z was observed which corresponded to the C_{20}-sphingenine-phosphate-PTC derivative.

Presently, we are considering the synthesis of some analogue which can be used as an internal standard for the quantitation of phosphorylated sphingoid bases. Due to the rather high molecular mass of the investigated compounds resulting in additional molecular ion peaks, [4,5-^{2}D]sphinganine-phosphate (M-1 of PTC-derivative of 517 m/z) does not seem to be a good choice, but sphinganine-phosphonate (M-1 of PTC-derivative of 499 m/z), first synthesized by Stoffel and Grol [22], might be a good candidate. Nevertheless, a rough estimation was obtained by spiking a duplicate biological extract with 2 nmoles of phosphorylated bases (1.6 nmol of sphingenine-phosphate plus 0.4 nmol of sphinganine-phosphate) and normalising the intensities of the m/z 513 peaks to that of other prominent peaks, representing one of the acyl- or alk(en)ylglycerophosphoethanolamines-derivatives. Concentration of sphingenine-phosphate in liver, kidney and brain are estimated at 0.2–0.3 nmol, 0.5–0.8 nmol and 3.5–5 nmol/g of wet tissue, respectively. Similar values were obtained by comparing the relative intensities of the reaction side products, diphenylthiourea (m/z 228) and ethylamine-PTC (m/z 180), seen in the biological samples and in a standard containing a 1.6 nmol of sphingenine-phosphate plus 0.4 nmoles of sphinganine-phosphate and carried through the whole procedure (Fig. 2D), with the intensity of the m/z 513 ion. Although these values are approximate, it is remarkable that they are in the same range as the sphingenine concentration reported in rat and mouse tissues [23,24].

Combined with our previous work on the turnover of exogenously added sphinganine-phosphate to cultured fibroblasts [11] and that of other groups on the bioactivity of sphingenine-phosphate [6–8] and its generation in response to growth factors [15], these new data certainly favor a role of sphingenine-phosphate as a biomodulator. In this respect, the high sphingenine-phosphate content of brain might deserve special attention. In a previous paper [11] we proposed that sphingosine-phosphatase and sphingosine-phosphate lyase are involved in the attenuation of bioactive sphingenine-phosphate and the catabolic removal of this lipid, respectively. Preliminary data indicate that the sphingenine-phosphate levels, as reported above, correlate better with the phosphatase activities (expressed on a weight basis), being 5- to 6-fold higher in brain (and other neuronal tissues) than in liver (De Ceuster, P., Mannaerts, G.P. and Van Veldhoven, P.P., unpublished data), than with the lyase activities which are approx. 4-fold lower in brain than in liver [10].

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