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HPTLC analysis of sphingomylein, ceramide and sphingosine in ischemic/reperfused rat heart[1]

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Abstract

Since the sphingomylein–ceramide–sphingosine pathway, especially ceramide, has been shown to induce programmed cell death (apoptosis), and since apoptosis may be involved with ischemic/reperfused injury in the heart, it became desirable to quantitate the three components in ischemic/reperfused rat heart. One group of rat hearts ($n = 6$) was isolated and perfused with Krebs–Henseleit buffer using the Langendorff non-recirculating mode. The hearts were perfused for 10 min, made ischemic for 30 min and reperfused for 120 min. Hearts were collected and stored at −70°C before ischemia, after ischemia and after 30, 60 and 120 min of reperfusion. The hearts were homogenized, and lipids were extracted using the Folch method. The lipids were then chromatographed on Whatman silica gel 60 Å high-performance thin-layered chromatography (HPTLC) plates. The plates were developed with iodine, photographed using Photoshop software and quantitated using NIH Imaging software. The results show a 50% decrease of sphingomylein during reperfusion with a corresponding increase in ceramide with sphingosine showing a smaller decrease as compared with the ceramide increase.

Keywords: Apoptosis; Ceramide; Heart; HPTLC; Ischemia/reperfusion; Sphingomylein; Sphingosine

1. Introduction

Sphingomylein is a major phospholipid located in the cellular membranes of myocytes. Certain signals can trigger sphingomyleinases to breakdown sphingomylein to phosphocholine and ceramide. Ceramide can act as a second messenger setting off cell proliferation, differentiation, growth and, most importantly for the heart, programmed cell death (apoptosis) [1–6]. Ceramidases can then break down ceramide to sphingosine and fatty acid [5,7]. Sphingosine can also act as a second messenger stimulating phospholipase D leading to an increase in phosphatidic acid and diacylglycerides [8]. Since our previous studies demonstrated apoptosis and phospholipase D stimulation in the ischemic/reperfused myocardium [9–12], and since the signal transduction studies for apoptosis involves sphingomylein breakdown [13], it became necessary to exam the trends in sphingomylein, ce-

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ramide and sphingosine in ischemic/reperfused heart.

Since the late 1960's, sphingomylein has been measured using silica thin-layered chromatography (TLC), elution of the phospholipid and quantitation by phosphorus determination [14–18]. Incorporation of radioactivity, TLC and scintillation counting has also been used for both sphingomylein and ceramide [19,20]. Gas chromatography [21], high-performance liquid chromatography [22–24] and mass spectrophotometry [25] are also likely candidates for quantification of all three compounds, but like the other methods, they are laborious, expensive and not easily available. In this report we describe a HPTLC method using the new computer technology offered by such software as Photoshop and NIH Imaging for quantification of sphingomylein, ceramide and sphingosine.

2. Experimental

2.1. Preparation of heart biopsies

Sprague Dawley male rats weighing about 230–250 g were anesthetized with intraperitoneal pentobarbital (80 mg kg^{-1}). Hearts were excised and rapidly mounted on a non-circulating Langendorff apparatus. Retrograde perfusion was established at a pressure of 100 cm H_2O with oxygenated normothermic Krebs–Henseleit bicarbonate (KHB) buffer of the following composition: (in mM) NaCl 118; KCl 4.7; $CaCl_2$ 1.7; $NaHCO_3$ 24; KH_2PO_4 1.2; $MgSO_4$ 1.2; glucose 10 [26]. After equilibration for 10 min at 37°C with non-circulating KHB buffer, the hearts were made ischemic for 30 min followed by 120 min of reperfusion. Hearts ($n = 6$) were collected and stored at -70°C before ischemia, after ischemia and after 30, 60 and 90 min of reperfusion.

2.2. Extraction of lipids

Lipids were extracted according to the modified method of Folch et al. [27] as described by Das et al. [28]. Approximately 500 mg of myocardial tissue was homogenized in a 10-ml solution of ice-cold chloroform–methanol mixture (2:1, v:v) containing 0.005% butylated hydroxytoluene (BHT) [Sol. A] using a Polytron homogenizer (Brinkman, NY). Sol. A (5 ml) was added and mixed, followed by 4 ml minus 0.7 times the gram molecular weight of the sample in millilitres of 0.9% NaCl and vortexing. The extracts were centrifuged for 10 min at 1000 *g*, and the lower (organic) layer was collected. Two additional extracts were attained by repeating the process with 10.8 ml of Sol. B (chloroform:methanol:saline, 86:14:1; v:v:v). The pooled organic extracts were dried under N_2 at 40°C, and the pellets were dissolved in Sol. A at 0.5 ml g^{-1} wet wt.

2.3. Lipid estimation

The lipid extracts were fractionated by one-dimensional HPTLC techniques on (Whatman, Clifton, NJ) silica gel 60 Å HPTLC plates using chloroform:methanol:petroleum ether:acetate:borate, 42:18:32:8:1.8, v:v:v:v:wt. for sphingomylein as shown by Gilfillan et al. [29] with slight modifications. Toluene:methanol, 7:3, v:v was used for ceramide separation, and chloroform:methanol:ammonium hydroxide, 65:25:4, v:v:v for sphingosine separation. The plates were developed with iodine overnight, immediately photographed with a Kodak 200 camera and a Mac powermate computer using Photoshop Ver. 4.0 software (Adobe Systems, Mountain View, CA). Quantification was obtained using density measurements with NIH Imaging Ver. 1.59 software (National Institutes of Health, Bethesda, MD). Standard curves were obtained by chromatographing authentic standards.

3. Results

3.1. HPTLC detection of sphingomylein, ceramide and sphingosine

As shown in Fig. 1, our excellent separation of the phospholipids is obtained with sphingomylein showing a decrease in intensity with the onset of

reperfusion. Since sphingomylein is present in large amounts, only a small amount of the lipid extract (6 μl of a 0.5 ml g^{-1} wet wt. solution) needed to be plated. Fig. 2 shows a 50% decrease in sphingomylein with reperfusion. In Fig. 3, ceramide is seen just below diacylglyceride. Although there is much less ceramide than sphingomylein, it only led to plating 2 μl more of the lipid extract (5 μl of a 0.5 ml g^{-1} wet wt. solution) since a different mobile phase was used. There is still a good separation of the other lipids. Fig. 4 shows a significant increase in ceramide with ischemia and reperfusion. Since sphingosine is present at the lowest quantity, a great deal of lipid extract (15 μl of a 0.5 ml g^{-1} wet wt. solution) must be plated causing a poor separation of the other lipids (Fig. 5). However, if the right amount of extract is plated (not too much), sphingosine can be separated and quantitated. As shown in Fig. 4, there is a small decrease in sphingosine with ischemia and reperfusion. A detection limit of 50 pmol was achieved for sphingomylein, ceramide and sphingosine with r values for the standard curves of 0.9968, 0.9937, and

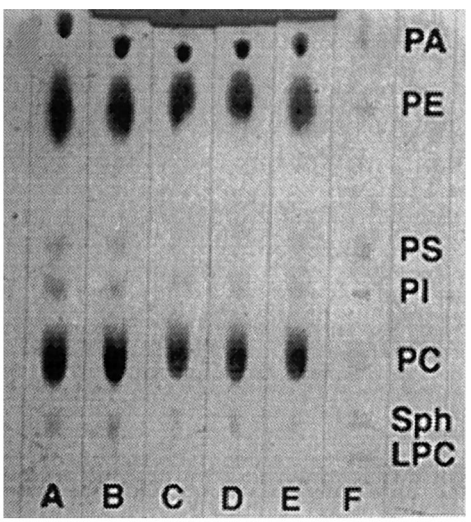

Fig. 1. HPTLC of rat heart sphingomylein. Lipid extracts (6 μl of a 0.5 ml g^{-1} wet wt. solution) were chromatographed on silica gel 60Å HPTLC plate using chloroform:methanol:petroleum ether:acetate:borate, 42:18:32:8:1.8, v:v:v:v:wt as the mobile phase. The plates were developed with iodine and photographed with a Kodak 200 camera and a Mac powermate computer using Photoshop software: (A) before ischemia; (B) after ischemia; (C) after 30 min reperfusion; (D) after 60 min reperfusion; (E) after 120 min reperfusion; (F) 51.3 nmol of sphingomylein standard (Avanti Polar Lipids, Albaster, AL). PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; Sph, sphingomylein; LPC, lysophosphatidylcholine.

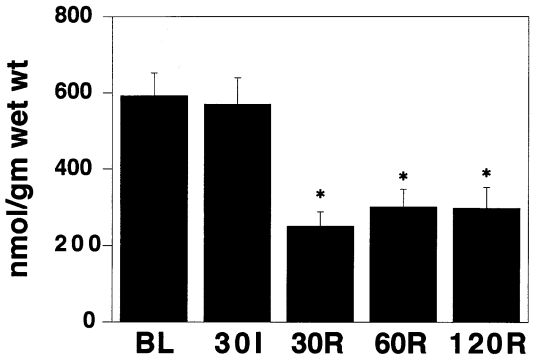

Fig. 2. Quantification of rat heart sphingomylein during ischemia/reperfusion. Quantification was obtained using density measurements with a Mac powermate computer and NIH Imaging software. BL, baseline before ischemia; 30I, 30 min ischemia; 30R, 30 min reperfusion; 60R, 60 min reperfusion; 120R, 120 min reperfusion. Results are means ± SE for six different animals per group. $*P < 0.05$ compared with (BL) baseline.

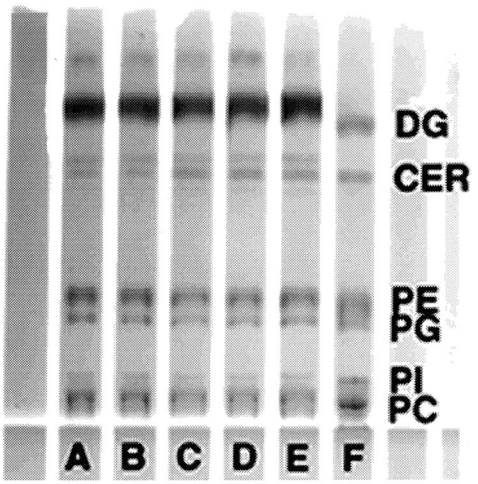

Fig. 3. HPTLC of rat heart ceramides during ischemia/reperfusion. Chromatography is the same as Fig. 1 except toluene:methanol, 7:3, v:v was used as a mobile phase. A–E, 5 μl of lipid extract; F, 113 pmol of ceramide standard (Avanti). DG, diacylglyceride; CER, ceramide; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine.

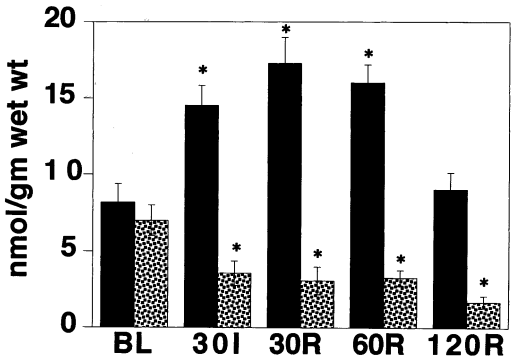

Fig. 4. Quantification of ceramide and sphingosine in rat hearts during ichemia/reperfusing. Measurements were made as Fig. 2. BL, baseline before ischemia; 30I, 30 min ischemia; 30R, 30 min reperfusion; 60R, 60 min reperfusion; 120R, 120 min reperfusion. Results are means ± SE for six different animals per group. $^*P < 0.05$ compared with BL (baseline). ■, ceramide; ▩, sphinosine.

0.9897 respectively. Standard errors ranged from ± 20 pmol for low, ± 340 pmol for middle and ± 895 pmol for high values. Control samples were spiked with 1 nmol of sphingomylein and 200 pmol of ceramide and sphingosine. Quantitation of these samples showed a 5% variation of the increased lipid.

4. Discussion

In the present study, we describe a rapid, inexpensive and reliable method for the estimation of sphingomylein, ceramide and sphingosine. Recent studies have identified a sphingomylein cycle whereby the breakdown product, ceramide, is implicated as the intracellular signalling agent for apoptosis [5]. Sphingomylein is a major phospholipid located primarily in the outer leaflet of the myocardial membrane and rapidly undergoes hydrolysis by the action of sphingomyelinase producing ceramide and phosphocholine [30]. Phosphocholine is released into the aqueous solution, while ceramide functions as an intracellular messenger in mediating suicidal cell death.

A recent study from this laboratory indicated translocation of phosphatidylserine and phosphatidylethanolamine associated with myocardial apoptotic cell death [31]. We have also demonstrated a role for phospholipase D signalling in the ischemic/reperfused myocardium [13]. Since phospholipase D attacks phosphatidylcholine to produce phosphatidic acid [11], and since sphingosine has been shown to stimulate phospholipase D activity in different cell types [32], it seems logical to speculate that ischemia/reperfusion would cause the breakdown of sphingomylein and activate the ceramide signalling cascade.

Our search for a reliable method for the simultaneous estimation of sphingomylein, ceramide and sphingosine was quite unsuccessful. Several methods combining TLC, GC and HPLC are available to quantitate these lipids [14–25], but no method is available to estimate them simultaneously. We felt the need to develop a rapid, reproducible and reliable method which can be routinely used for this purpose. To this end, we

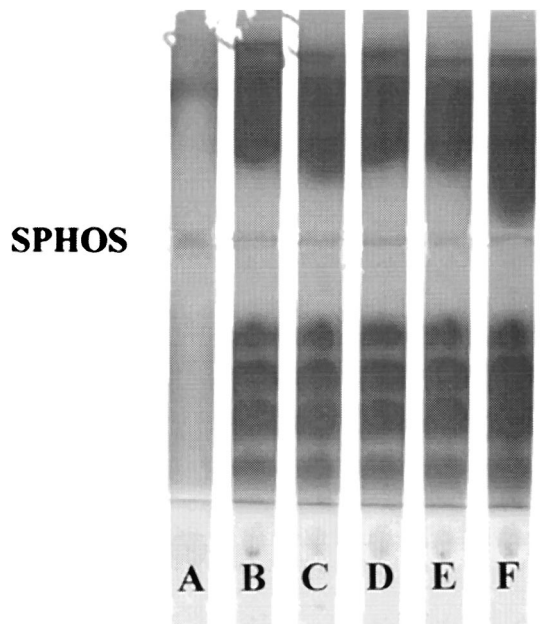

Fig. 5. HPTLC of rat heart sphingosine during ischemia/reperfusion. Chromatography is the same as Fig. 1 except chloroform:methanol:ammonium hydroxide, 65:25:4, v:v:v was used as a mobile phase: (A) 400 pmol of sphingosine standard (Avanti); B–F, 15 μl of a 0.5 ml g^{-1} wet wt. solution (lipid Extract); (B) before ischemia; (C) after ischemia; (D) 30 min reperfusion; (E) 60 min reperfusion; (F) after 120 min reperfusion. Sphos, sphingosine.

have developed this HPTLC technique to estimate sphingomylein, ceramide and sphingosine in three simultaneous runs of 20 min. The smaller silica particle size (4.5 μm) of the HPTLC plates is similar to packed HPLC columns and shortens the development time from 2 h to 20 min. This HPTLC densitometric method represents a significant improvement over previously published TLC methods. HPTLC provides better resolutions, accuracy and reproducibility for the identification and determination of lipid extracts. Previous studies using HPTLC plates provided accurate estimations of phosphatidylethanolamine and phosphatidylserine [33]. In this study, the authors clearly demonstrate better resolutions and accuracy compared with results from TLC plates. In this present study, we were able to detect changes as low as 50 pmol of ceramide or sphingosine.

In summary, we have developed a rapid, reliable and reproducible method for the estimation of sphingomylein, ceramide and sphingosine in biological tissue such as the heart. The simplicity of the technique should make it the method of choice for routine use.

Acknowledgements

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