Diabetes-induced changes in specific lipid molecular species in rat myocardium

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Intrinsic cardiac dysfunction during the diabetic state has been causally linked to changes in myocardial lipid metabolism. However, the precise alterations in the molecular species of myocardial polar and non-polar lipids during the diabetic state and their responses to insulin have not been investigated. Herein we demonstrate four specific alterations in rat myocardial lipid molecular species after induction of the diabetic state by streptozotocin treatment: (i) a massive remodelling of triacylglycerol molecular species including a >5-fold increase in tripalmitin mass and a 60% decrease in polyunsaturated triacylglycerol molecular species mass (i.e. triacylglycerols containing at least one acyl residue with more than two double bonds); (ii) a 46% increase in myocardial phosphatidylinositol mass; (iii) a 44% increase in myocardial plasmenylethanolamine mass and (iv) a 22% decrease in 1-stearoyl-2-arachidonoyl phosphatidylethanolamine content. Each of the changes in phospholipid classes, subclasses and individual molecular species were prevented by insulin treatment after induction of the diabetic state. In sharp contrast, the alterations in triacylglycerol molecular species were not preventable by peripheral insulin treatment after induction of the diabetic state. These results segregate diabetes-induced alterations in myocardial lipid metabolism into changes that can be remedied or not by routine peripheral insulin treatment and suggest that peripheral insulin therapy alone may not be sufficient to correct all of the metabolic alterations present in diabetic myocardium.

Key words: insulin, palmitate, phospholipid, plasmalogen, triacylglycerol.

INTRODUCTION

Diabetes mellitus is a complex polygenic disorder of intermediary metabolism that is accompanied by a vast array of lethal end-organ sequelae. By far, the cardiovascular sequelae of diabetes represent the most frequent cause of death in both Type-I and Type-II diabetes. Although Type-I diabetes is due to insulin deficiency and Type-II diabetes is due to insulin resistance, the myocardial sequelae are both related to an increased dependence on fatty acid (instead of nearly equal amounts of fatty acids and glucose) for myocardial energy metabolism. Moreover, this shift towards increased fatty acid utilization in both Type-I and Type-II diabetes results in a cardiomyopathy whose mechanisms are thought to be related to the kinetics of lipid uptake, utilization and disposal, but whose actual mechanisms are incompletely understood [1,2]. Many studies have demonstrated altered fatty acid metabolism in diabetic myocardium documented by increased rates of fatty acid uptake, enhanced oxidation of fatty acids and increases in non-esterified fatty acid levels [2,3]. Similarly, numerous investigations have examined alterations in total triacylglycerol (TAG) and phospholipid mass in diabetic myocardium, but differing conclusions have been reached [3–7]. For example, some investigators have demonstrated an increased mass of TAGs in diabetic myocardium, whereas others have not found any alteration in total TAG pools in control versus diabetic myocardium [3–5]. Moreover, no information is available about alterations in individual molecular species of either phospholipids or TAGs during the diabetic state or their response to insulin therapy.

Membrane-associated signalling processes are a critical part of the complex pathways that transduce insulin-mediated changes in cellular metabolism. For example, insulin induces the uptake of glucose by fusion of intracellular GLUT4-containing vesicles to the plasma membrane [8,9], facilitates the phosphorylation of membrane phosphoinositides by activation of phosphoinositide 3-kinase (PI 3-kinase) [10,11] and mobilizes critical signalling enzymes (e.g. Akt kinase) and proteins (e.g. insulin receptor substrates 1 and 2) to membrane surfaces [12,13]. Perhaps this is best illustrated by the bidirectional modulatory influences of PI 3-kinase and PTEN (phosphatase and tensin homologue deleted on chromosome 10) as crucial determinants of insulin-mediated alterations in intermediary metabolism [14,15]. Moreover, alterations in membrane physical properties modulate insulin-receptor autophosphorylation [16]. Accordingly, alterations in membrane-lipid physical properties and scaffolding interactions play a crucial role in insulin-mediated regulation of cellular metabolism.

Recently, substantial advances in the molecular-species analysis of phospholipids derived from biological sources have been achieved through exploiting the high-efficiency volatilization of zwitterionic phospholipids by electrospray ionization MS (ESI-MS) [17,18]. By appropriate manipulation of ionization conditions (e.g. sample pH and counter-ion content, voltage plate potential and polarity), the molecular-species analysis of phospholipids present in biological membrane systems can be obtained directly from chloroform extracts by ESI-MS [19,20]. Accordingly, we exploited this technique to perform the first detailed molecular-species analysis of polar and non-polar lipids in diabetic myocardium and investigated the ability of insulin to prevent these alterations. We report four specific alterations in lipids from rat myocardium rendered diabetic by streptozotocin treatment (a well-accepted model of Type-I diabetes) utilizing

Abbreviations used: ESI-MS, electrospray ionization MS; PhosCho, phosphatidylcholine; PhosEth, phosphatidylethanolamine; PhosGly, phosphatidylglycerol; TAG, triacylglycerol; PI 3-kinase, phosphoinositide 3-kinase.

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ESI-MS analyses. First, over a 5-fold increase in tripalmitin mass and a 60% decrease in TAG molecular species containing polyunsaturated fatty acids was present in diabetic rat myocardium. Moreover, there was also a 44% increase in plasmenylethanolamine mass and a 46% increase in phosphatidylinositol mass. Finally, a 22% reduction of 18:0/20:4 PhosEth (phosphatidylethanolamine) occurred in the absence of decreases in non-esterified arachidonic acid or total myocardial arachidonic acid content. Each of the alterations in phospholipid class, subclass and individual molecular-species profiles was completely prevented by insulin treatment. In sharp contrast, the dramatically altered TAG molecular-species profile was not prevented by peripheral insulin treatment after induction of the diabetic state. Collectively, these results segregate changes in myocardial lipid metabolism in the diabetic state into changes that are either modified easily by routine peripheral insulin treatment or difficult (or perhaps impossible) to prevent by peripheral insulin administration alone. Accordingly, they raise the intriguing possibility that factors other than insulin may contribute to altered TAG metabolism and cardiac dysfunction in the diabetic state.

EXPERIMENTAL PROCEDURES

Materials

Commercially available phospholipids including 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0/14:0 PhosCho), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine (15:0/15:0 PhosEth) and 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphoglycerol (14:0/16:0 PhosGly) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). TAGs including triheneicosanoin (21:0/21:0/21:0-TAG; used as the internal standard in TAG determinations by GC), tripalmitin (16:0/16:0/16:0-TAG), triolein (18:1/18:1/18:1-TAG) and trilinolein (18:2/18:2/18:2-TAG) were purchased from Nu Chek Prep (Elysian, MN, U.S.A.). Streptozotocin, insulin and most other reagents were obtained from Sigma (St. Louis, MO, U.S.A.).

Treatment and maintenance of animals

Male Sprague–Dawley rats (350–450 g body weight) were purchased from Charles River Labs (Wilmington, MA, U.S.A.). Rats were housed separately, kept on a 12-h:12-h light/dark cycle, and given free access to water and a standard rodent diet comprised of 20% protein, 5% fat (including 2% linoleic acid, 0.2% linolenic acid, < 0.01% arachidonic acid, 0.5% ω-3 fatty acids, 0.8% total saturated fatty acids and 1.0% total monosaturated fatty acids), 5% fibre, 32% starch, 6% ash and 3% added minerals (Pico-Vac diet, PMI Nutrition International, St. Louis, MO, U.S.A.).

Diabetes was induced by a single intravenous injection (in the tail vein) of streptozotocin (35 mg/kg body weight in 0.2 ml of 0.1 M citrate buffer, pH 4.5) as described previously [21]. This dose of streptozotocin has been demonstrated to produce moderate diabetes with impaired glucose-induced insulin secretion similar to subtotal pancreatectomy [22]. Diabetic animals did not gain as much weight over 6 weeks (375 ± 25 g at the baseline and 384 ± 35 g after 6 weeks, $n = 12$) as age-matched control animals (408 ± 26 g at the baseline and 494 ± 36 g after 6 weeks, $n = 5$) but otherwise remained in good health. Control rats received citrate buffer (0.2 ml) alone. Diabetes was confirmed within 48 h by blood glucose levels > 3 mg/ml as measured by chemstrips (bG, Boehringer-Mannheim). In animals for which diabetes was not confirmed, a second dose of streptozotocin was given. Once the presence of diabetes was confirmed, half of the diabetic animals (treated group) were given a combination of regular insulin (Humulin, 2 U) and NPH insulin (5 U) subcutaneously once per day (in the afternoon). Blood glucose levels were checked daily (in the morning) and insulin doses were adjusted to maintain levels between 1 and 2 mg/ml. Half of the control animals (treated groups) were also given insulin (Humulin, 1 U; NPH insulin, 1 U) as a control, and insulin doses were adjusted to maintain blood glucose levels between 0.6 and 1 mg/ml. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, 1996) and were approved by the Animal Studies Committee at Washington University.

Preparation of phospholipids and TAGs from rat heart

Rats were killed by inhaling carbon dioxide. The hearts were excised quickly and immersed in ice-cold buffer (250 mM sucrose/25 mM imidazole, pH 8.0, at 4 °C). After removing extraneous tissue and epicardial fat, each heart was quickly dried and immediately freeze-clamped at the temperature of liquid nitrogen. Myocardial wafers were pulverized into a fine powder with a stainless-steel mortar and pestle. A myocardial sample ($\approx$ 50 mg) was weighed from each rat heart and lipids were extracted by the method of Bligh and Dyer [23]. Appropriate amounts of internal standards [e.g. 14:0/14:0 PhosCho (1.7 nmol/mg protein), 15:0/15:0 PhosEth (1.6 nmol/mg protein) and 14:0/16:0 PhosGly (2.0 nmol/mg protein) for phospholipid analyses, 21:0/21:0/21:0-TAG (3 nmol/mg protein) for TAG quantification or arachidic acid (20:0) for free fatty acid determination] were added during lipid extraction. The lipid extracts were dried under a nitrogen stream, dissolved in chloroform, filtered with Millex-HV 0.45 μm filters (Millipore, Bedford, MA, U.S.A.), re-extracted and dried under a nitrogen stream. The final lipid residue was immediately resuspended in either 500 μl of chloroform/methanol (1:2, v/v) for ESI-MS analyses of phospholipids or in 500 μl of hexane/isopropanol (1:1, v/v) for TAG analyses.

ESI-MS of phospholipids

ESI-MS analyses were performed using a Finnigan TSQ-7000 Spectrometer (Finnigan MAT, San Jose, CA, U.S.A.) equipped with an ESI source and controlled by Finnigan ICIS software operated on a DEC alpha workstation as described previously [17,19]. Typically, a 5 min period of signal-averaging in the profile mode was employed for each spectrum of lipid extract from rat heart. All samples of heart-tissue extracts were appropriately diluted in chloroform/methanol (1:2) prior to direct infusion into the ESI source using a Harvard syringe pump at a flow rate of 2 μl/min. Anionic phospholipids in the diluted chloroform extracts of rat hearts were analysed by ESI-MS in the negative-ion mode and quantified by comparisons of the individual ion peak intensity with internal standard (i.e. 14:0/16:0 PhosGly) after correction for ^{13}C isotope effects.

Prior to the analyses of choline and ethanolamine glycerophospholipids in the diluted tissue extracts, LiOH in methanol (50 nmol/mg protein) was added. Choline glycerophospholipids and sphingomyelins in the diluted tissue extracts were directly quantified as their lithium adducts by comparisons with the internal standard (i.e. lithiated 14:0/14:0 PhosCho) after correction for ^{13}C isotope effects in the positive-ion mode. Similarly, analyses of commercially available TAGs or TAG fractions from HPLC purification of rat myocardial extracts were performed by positive-ion ESI-MS after addition of LiOH in methanol (10 nmol/mg protein). After rendering the

solution mildly basic by addition of LiOH (50 nmol/mg protein), ethanolamine glycerophospholipids were directly quantified by comparisons with internal standards (i.e. 15:0/15:0 PhosEth) after correction for ^{13}C isotope effects in the negative-ion mode. The selectivity of ESI-MS for direct analysis of distinct phospholipid classes was achieved by exploiting differential ionization propensities in the positive- and negative-ion modes of each phospholipid class [17]. The internal standards (i.e. 14:0/16:0 PhosGly, 14:0/14:0 PhosCho and 15:0/15:0 PhosEth) were selected based on their solubility and the lack of any demonstrable endogenous molecular ions in that region, which was verified by acquiring a mass spectrum without internal standards. Identification of ion peaks was achieved utilizing tandem-MS analyses in the molecular-ion scanning mode. Quantification of ion peaks corresponding to multiple individual molecular ions was substantiated using molecular-ion ESI-MS/MS analyses as described previously [24]. Plasmalogen molecular species were distinguished from alkyl-acyl phospholipid molecular species by treating tissue extracts with acidic vapours prior to mass-spectroscopic analyses as described previously [25]. The masses of all ions were rounded to the nearest integer.

Quantification of rat heart TAGs and free fatty acids using capillary GC

Bligh and Dyer extracts from control and diabetic rat hearts containing exogenously added internal standard (i.e. 21:0/21:0/21:0-TAG) were used for TAG analysis. The extracts were loaded on to an Ultrasphere-Si HPLC column (250×4.6 mm, 5 μm; Beckman) and TAGs were resolved using a mobile phase comprised of hexane/isopropanol (1:1, v/v) with a linear gradient of 0–6% H_2O for 45 min at a flow rate of 1 ml/min. Under these conditions, TAGs eluted at $\approx$ 10 min. The collected fractions were dried under a nitrogen stream and the masses of TAGs were quantified by capillary GC after acid methanolysis by comparisons with a 21:0/21:0/21:0-TAG standard as described previously [26].

Analysis of free fatty acids was performed from Bligh and Dyer extracts of control and diabetic rat myocardium containing exogenously added arachidic acid (20:0). Extracts were first loaded on to a Whatman prepacked Si column and were resolved using step gradients comprised of hexane and ethyl ether as described previously [27]. Free fatty acids eluted at 92:8 hexane/ethyl ether (v/v). The collected fractions were dried under a nitrogen stream prior to acid methanolysis and the mass of free fatty acid was quantified by capillary GC after acid methanolysis by comparison with arachidic acid as described previously [26].

Miscellaneous

Protein concentration was determined with a Bio-Rad protein assay using BSA as standard. Results in this study are presented as means $\pm$ S.E.M. Differences between mean values were determined by unpaired Student's t tests. $P < 0.05$ was considered significant.

RESULTS

Analysis of ethanolamine glycerophospholipid individual molecular species from myocardium of control and diabetic rats

ESI-MS analyses of chloroform extracts of control rat myocardium in the negative-ion mode (after addition of LiOH) demonstrated two predominant peaks corresponding to ethanolamine glycerophospholipids at m/z 767 (18:0/20:4 PhosEth)

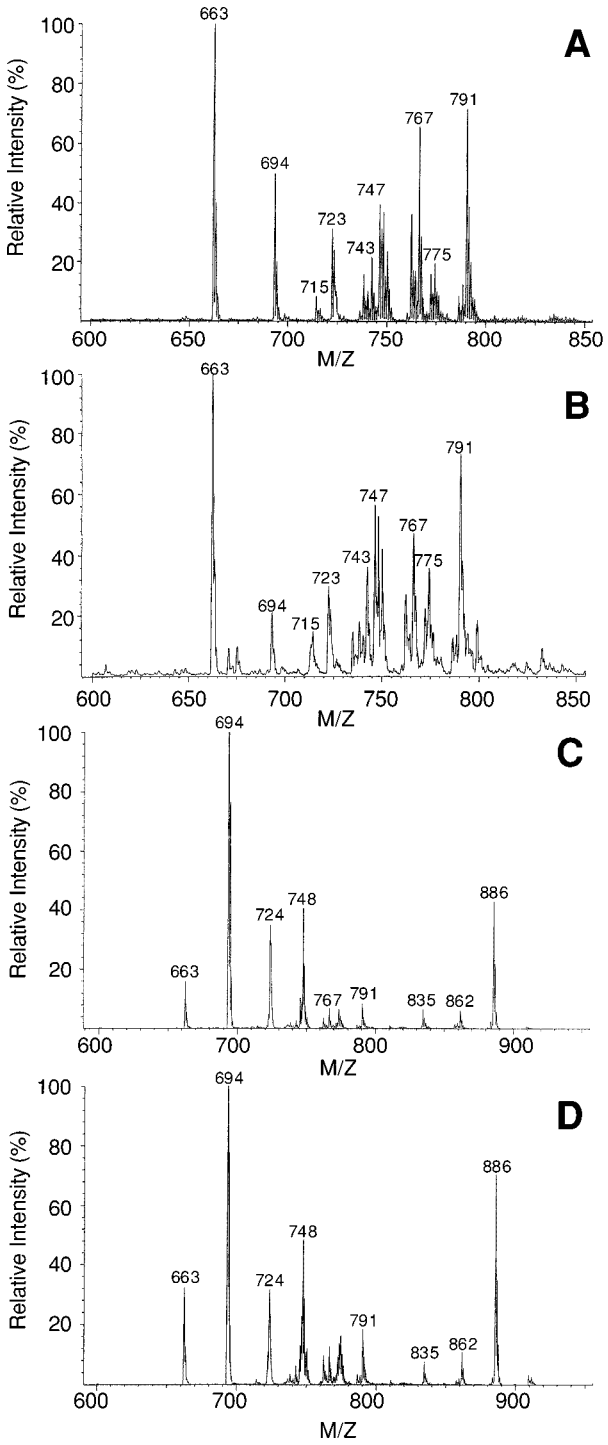

Figure 1 Negative-ion ESI mass spectra of ethanolamine glycerophospholipids and anionic phospholipids from myocardium of control and diabetic rats

Rat hearts were excised, rapidly frozen, pulverized, homogenized and phospholipids were extracted by the method of Bligh and Dyer [23] as described in the Experimental procedures section. Aliquots of the chloroform extracts were infused directly into the ESI source using a Harvard syringe pump at a flow rate of 2 μl/min after addition of LiOH (50 nmol/mg protein) (**A** and **B**) or without addition of LiOH (**C** and **D**). Negative-ion ESI-MS of the myocardial extracts from 6 week control (**A** and **C**) and 6 week diabetic (**B** and **D**) rats was performed as described in the Experimental procedures section. Individual ethanolamine glycerophospholipid or anionic phospholipid molecular species were identified using tandem MS and are listed in Tables 1 and 2, respectively. The internal standards were 15:0/15:0 PhosEth (m/z 663) or 14:0/16:0 PhosGly (m/z 694).

Table 1 Diabetes-induced alterations in the masses of ethanolamine glycerophospholipid molecular species in rat myocardium

Rat myocardial phospholipids were extracted by the Bligh and Dyer method [23] and analysed directly by negative-ion ESI-MS as described in the Experimental procedures section. Ethanolamine glycerophospholipids were quantified by comparisons of the individual ion peak intensities with an internal standard (i.e. 15:0/15:0 PhosEth), after correction for ^{13}C isotope effects. The results are expressed in nmol/mg of protein and represent means ± S.E.M. of a minimum of six separate animals. D (diacyl) and P (plasmenyl) indicate PhosEth and plasmenylethanolamine molecular species, respectively. Molecular species contributing < 0.5% of total ethanolamine glycerophospholipids are not listed. *$P < 0.05$ and †$P < 0.01$ compared with 6 week controls. The masses of all ions are rounded to the nearest integer. At the bottom of the Table are shown the numbers of molecular species containing four or more double bonds at the *sn*-2 fatty acyl chain and of arachidonic acid-containing ethanolamine glycerophospholipids.

Peak (*m/z*)	Assignment	6 Week control	6 Week diabetes	6 Week diabetes treated	6 Week control treated
715	D16:0/18:2	0.49 ± 0.05	0.60 ± 0.14	0.65 ± 0.06	0.46 ± 0.12
723	P16:0/20:4	1.50 ± 0.17	1.57 ± 0.18	1.81 ± 0.18	1.40 ± 0.07
727	P18:0/18:2	0.12 ± 0.01	0.30 ± 0.07†	0.23 ± 0.05*	0.14 ± 0.03
737	D16:1/20:4	0.21 ± 0.03	0.31 ± 0.06	0.24 ± 0.04	0.26 ± 0.02
739	D16:0/20:4	0.80 ± 0.08	0.76 ± 0.08	0.71 ± 0.04	0.71 ± 0.08
739	D18:2/18:2	0.09 ± 0.01	0.10 ± 0.02	0.20 ± 0.01	0.10 ± 0.01
741	D18:1/18:2	0.58 ± 0.08	0.66 ± 0.08	0.59 ± 0.10	0.52 ± 0.08
743	D18:0/18:2	0.80 ± 0.06	1.00 ± 0.20	0.81 ± 0.08	0.65 ± 0.07
743	D18:1/18:1	0.50 ± 0.03	0.80 ± 0.03	0.60 ± 0.05	0.51 ± 0.04
747	P16:0/22:6	1.07 ± 0.12	1.54 ± 0.18*	1.20 ± 0.09	1.06 ± 0.11
747	P18:2/20:4	0.70 ± 0.06	1.00 ± 0.10*	0.80 ± 0.06	0.71 ± 0.06
749	P18:1/20:4	1.18 ± 0.12	1.34 ± 0.16	1.10 ± 0.09	1.29 ± 0.11
749	P16:0/22:5	0.70 ± 0.07	0.90 ± 0.10	0.70 ± 0.06	0.80 ± 0.06
751	P18:0/20:4	0.65 ± 0.05	1.00 ± 0.10†	0.91 ± 0.07*	0.73 ± 0.05
751	P16:0/22:4	0.50 ± 0.04	0.70 ± 0.07*	0.60 ± 0.05	0.40 ± 0.03
761	D16:1/22:6	0.15 ± 0.03	0.26 ± 0.09	0.19 ± 0.04	0.17 ± 0.04
763	D16:0/22:6	1.76 ± 0.21	1.68 ± 0.18	1.50 ± 0.14	1.62 ± 0.15
765	D18:1/20:4	0.72 ± 0.04	0.55 ± 0.05*	0.68 ± 0.03	0.71 ± 0.03
765	D16:0/22:5	0.20 ± 0.01	0.20 ± 0.02	0.20 ± 0.01	0.20 ± 0.01
767	D18:0/20:4	3.31 ± 0.28	2.58 ± 0.28†	3.33 ± 0.27	3.48 ± 0.50
771	P18:2/22:6	0.22 ± 0.02	0.33 ± 0.05*	0.24 ± 0.04	0.24 ± 0.03
773	P18:1/22:6	0.80 ± 0.09	1.27 ± 0.16†	0.84 ± 0.12	0.86 ± 0.09
775	P18:0/22:6	0.72 ± 0.06	1.44 ± 0.15†	0.79 ± 0.07	0.78 ± 0.06
775	P18:1/22:5	0.22 ± 0.03	0.51 ± 0.06†	0.30 ± 0.02	0.20 ± 0.02
777	P18:0/22:5	0.47 ± 0.05	0.88 ± 0.09†	0.53 ± 0.07	0.26 ± 0.03
787	D18:2/22:6	0.44 ± 0.04	0.64 ± 0.07*	0.39 ± 0.04	0.46 ± 0.06
789	D18:1/22:6	0.60 ± 0.10	0.69 ± 0.07	0.54 ± 0.06	0.65 ± 0.10
791	D18:0/22:6	3.84 ± 0.40	3.77 ± 0.40	3.54 ± 0.38	3.76 ± 0.39
793	D18:0/22:5	0.57 ± 0.06	0.54 ± 0.07	0.55 ± 0.04	0.56 ± 0.07
795	D20:0/20:4	0.25 ± 0.03	0.20 ± 0.02	0.30 ± 0.01	0.20 ± 0.03
795	D18:0/22:4	0.20 ± 0.03	0.15 ± 0.01*	0.28 ± 0.01	0.12 ± 0.01
Total		24.36 ± 1.83	28.27 ± 2.13*	25.35 ± 1.31	24.01 ± 1.52
⩾4 Double bonds		21.78 ± 1.49	24.81 ± 1.85	22.27 ± 1.15	21.63 ± 1.32
Plasmenylethanolamines		8.85 ± 0.77	12.78 ± 1.01†	10.05 ± 0.52	8.87 ± 0.56
Arachidonic acid-containing		9.32 ± 0.92	9.31 ± 0.75	9.88 ± 0.51	9.48 ± 0.78

and *m/z* 791 (18:0/22:6 PhosEth) (Figure 1A; peaks at *m/z* 663 and 694 correspond to internal standards). Other easily demonstrable peaks at *m/z* 723, 747 and 775 (corresponding to 16:0/20:4, 16:0/22:6 and 18:0/22:6 plasmenylethanolamines containing highly unsaturated constituents) were also prominent (Figure 1A and Table 1). Molecular-ion scanning for fatty acids (e.g. palmitate, stearate, oleate, linoleate, arachidonate, docosahexenoate, etc.) was performed (spectra not shown) to confirm the identities of each of the moieties in Table 1. Tandem MS was also used to resolve ambiguities resulting from the presence of isobaric molecular species (e.g. the peak at *m/z* 751 was comprised of 18:0/20:4 and 16:0/22:4 plasmenylethanolamines in a 7:5 ratio), which are shown in Table 1. Overall, the results are remarkable for the fact that 90% of control rat myocardial ethanolamine glycerophospholipids were comprised of constituents containing four or more double bonds at the *sn*-2 position (Table 1).

Comparisons of ESI mass spectra of control and rat myocardium rendered diabetic by streptozotocin treatment (a model of Type-I diabetes) demonstrated a 24% decrease in a single molecular species of ethanolamine glycerophospholipid at *m/z* 767 (corresponding to 18:0/20:4 PhosEth) in diabetic rat myocardium (Figure 1B). Remarkably, plasmenylethanolamine content increased by 44% during the diabetic state (Figure 1B and Table 1). To determine the role of insulin in mediating these changes, a group of rats was made diabetic by streptozotocin injection and subsequently rendered euglycaemic by treatment with insulin for the 6 week experimental interval. Insulin treatment of diabetic rats restored the decreased levels of 18:0/20:4 PhosEth to normal and decreased the levels of plasmenylethanolamines to control values (Table 1). Insulin injection into control rats resulted in ethanolamine glycerophospholipid levels that were indistinguishable from controls (Table 1).

Analyses of individual molecular species of anionic phospholipids in normal and diabetic rat hearts

ESI mass spectra of chloroform extracts of rat hearts in negative-ion mode (without addition of LiOH) demonstrated the predominance of peaks at *m/z* 886 (18:0/20:4 phosphatidylinositol) and 748 (16:0/18:1 PhosGly) (Figure 1C). In addition, cardiolipins corresponding to singly charged moieties containing 18:2 fatty acyl chains (*m/z* 1448) and doubly charged moieties containing linoleate (*m/z* 724) were recognized and their struc-

Table 2 Diabetes-induced alterations in the masses of anionic phospholipid molecular species in rat myocardium

Rat myocardial phospholipids were extracted by the Bligh and Dyer method [23] and analysed directly by negative-ion ESI-MS as described in the Experimental procedures section. Anionic phospholipids were quantified by comparisons of the individual ion peak intensities with an internal standard (i.e. 14:0/16:0 PhosGly), after correction for ^{13}C isotope effects. The results are expressed in nmol/mg of protein and represent means ± S.E.M. of a minimum of six separate animals. $^{*}P < 0.05$ and †$P < 0.01$ compared with 6 week control. The masses of all ions were rounded to the nearest integer. PhosSer, phosphatidylserine; PhosIns, phosphatidylinositol.

Peak (*m/z*)	Assignment	6 Week control	6 Week diabetes	6 Week diabetes treated	6 Week control treated
724	Cardiolipin	6.39 ± 0.49	6.46 ± 0.61	5.83 ± 0.72	5.81 ± 0.71
746	16:0/18:2 PhosGly	0.43 ± 0.06	0.53 ± 0.06	0.48 ± 0.06	0.38 ± 0.05
748	16:0/18:1 PhosGly	1.70 ± 0.16	1.87 ± 0.08	1.79 ± 0.21	1.50 ± 0.09
770	16:0/20:4 PhosGly	0.05 ± 0.01	0.12 ± 0.01†	0.05 ± 0.01	0.08 ± 0.01
774	18:0/18:2 PhosGly	0.29 ± 0.03	0.53 ± 0.06†	0.27 ± 0.03	0.27 ± 0.05
776	18:0/18:1 PhosGly	0.07 ± 0.01	0.20 ± 0.01†	0.07 ± 0.01	0.07 ± 0.01
798	18:0/20:4 PhosGly	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.04 ± 0.01
Total		2.56 ± 0.23	3.27 ± 0.08†	2.68 ± 0.21	2.34 ± 0.18
761	16:0/18:1 PhosSer	0.02 ± 0.01	0.05 ± 0.01*	0.02 ± 0.01	0.03 ± 0.01
787	18:0/18:2 PhosSer	0.07 ± 0.01	0.17 ± 0.02†	0.06 ± 0.01	0.07 ± 0.01
789	18:0/18:1 PhosSer	0.08 ± 0.02	0.16 ± 0.03†	0.08 ± 0.02	0.10 ± 0.03
811	18:0/20:4 PhosSer	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.02	0.08 ± 0.02
813	18:0/20:3 PhosSer	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
835	18:0/22:6 PhosSer	0.33 ± 0.05	0.30 ± 0.04	0.36 ± 0.06	0.32 ± 0.09
837	18:0/22:5 PhosSer	0.10 ± 0.02	0.09 ± 0.01	0.12 ± 0.03	0.06 ± 0.02
839	20:0/20:4 PhosSer	0.04 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Total		0.70 ± 0.12	0.88 ± 0.06	0.77 ± 0.14	0.72 ± 0.14
862	18:0/18:2 PhosIns	0.35 ± 0.05	0.56 ± 0.14*	0.31 ± 0.05	0.32 ± 0.03
884	18:1/20:4 PhosIns	0.12 ± 0.02	0.13 ± 0.02	0.11 ± 0.01	0.17 ± 0.04
886	18:0/20:4 PhosIns	1.94 ± 0.19	2.76 ± 0.18†	1.89 ± 0.23	2.05 ± 0.12
910	18:0/22:6 PhosIns	0.06 ± 0.02	0.16 ± 0.04†	0.04 ± 0.01	0.09 ± 0.02
Total		2.46 ± 0.24	3.60 ± 0.19†	2.36 ± 0.18	2.63 ± 0.12
Grand total		12.11 ± 1.05	14.21 ± 0.88	11.64 ± 0.47	11.50 ± 0.48

tures confirmed by tandem MS (results not shown). Tandem-MS analyses were also employed to confirm the identities of inositol lipids by product ion analysis and of serine-containing classes by the neutral loss of polar head groups and/or product ion analyses of individual molecular ions. The results demonstrated that there were 12 nmol/mg of protein of anionic phospholipids (19 mol % of total phospholipids) in control rat myocardium, which was comprised of phosphatidylinositol (4 mol %), PhosGly (4 mol %), cardiolipin (10 mol %) and phosphatidylserine (1 mol %; Table 2, and see Table 5). Remarkably, the content of inositol glycerophospholipids increased by 44 % in rats subjected to streptozotocin-induced diabetes for 6 weeks (Figure 1D). Smaller increases in the amounts of PhosGlys and phosphatidylserine molecular species also occurred. No alterations in the single molecular species of cardiolipin present were identified (Figures 1D and Table 2). The dramatic increase in inositol glycerophospholipids, as well as the smaller increases in PhosGlys and phosphatidylserines, were completely abolished by the treatment of diabetic rats with insulin. Treatment of control rats with insulin resulted in spectra that were indistinguishable from untreated controls (Table 2).

Analysis of choline glycerophospholipid individual molecular species in control and diabetic rat myocardium

Examination of chloroform extracts of control rat myocardium by ESI-MS in the positive-ion mode (after addition of LiOH) demonstrated the predominance of two PhosCho molecular species containing arachidonic acid (e.g. *m/z* 789 and 817, corresponding to lithiated 16:0/20:4 PhosCho and 18:0/20:4 PhosCho; Figure 2A and Table 3). In addition, the presence of di-unsaturated PhosChos (e.g. *m/z* 765 and 793, corresponding to 16:0/18:2 and 18:0/18:2-18:1/18:1 PhosCho, respectively) was also easily demonstrated. The ion clusters at *m/z* 864 and 888 probably were not choline molecular species since they did not contain odd numbers of N atoms (i.e. they possessed even-number masses).

To confirm that the peaks from *m/z* 741 to 841 resulted from volatilization of moieties containing phosphocholine, tandem MS was performed with ion scanning for the neutral loss of either 59 amu (i.e. trimethylamine) or the neutral loss of species of 183 or 189 amu (representing the loss of phosphocholine and lithiated phosphocholine, respectively; spectra not shown). However, neither choline nor phosphocholine loss could be identified in multiple tandem-MS analyses of the ion clusters at *m/z* 864 and 890, confirming that these peaks were not generated from choline glycerophospholipids (see below).

ESI mass spectra of extracts of diabetic rat myocardium in the positive-ion mode did not show substantial differences in choline glycerophospholipid molecular species in comparison with controls (Figure 2). Moreover, tandem MS did not demonstrate differences in the ratio of 18:0, 18:1 and 18:2 fatty acid chains in isobaric molecular species, indicating that this distribution was also unchanged. As anticipated, insulin treatment of diabetic or control rats did not alter total choline glycerophospholipid mass or the mole fraction of individual molecular species (Table 3).

Identification of the moieties giving rise to the ion clusters centred at *m/z* 864 and 888 in the positive-ion mode during ESI-MS

To determine the chemical identity of moieties giving rise to the ion clusters centred at *m/z* 864 and 888, constituents present in the chloroform extracts were first purified by gradient-elution HPLC and each of the peaks eluting from the silica HPLC

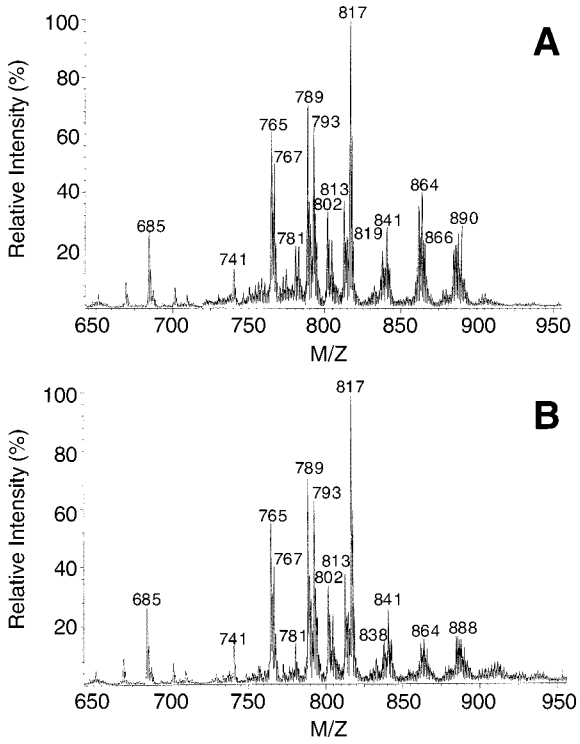

Figure 2 Positive-ion ESI mass spectra of choline glycerophospholipids in myocardium from control and diabetic rats

Rat hearts were excised, rapidly frozen in liquid N_2, pulverized and their phospholipids extracted by the method of Bligh and Dyer [23] as described in the Experimental procedures section. After addition of LiOH (50 nmol/mg protein) the chloroform extracts were infused directly into the ESI source using a Harvard syringe pump at a flow rate of 2 μl/min. Positive-ion ESI-MS spectra of myocardial extracts from 6 week control (**A**) and 6 week diabetic (**B**) rats were acquired as described in the Experimental procedures section. The internal standard was 14:0/14:0 PhosCho (*m/z* 685).

column was analysed by ESI-MS in the positive-ion mode. The peaks centred at *m/z* 864 and 888 eluted just after the solvent front, and prior to the application of the water gradient (results not shown). Tandem-MS analysis showed product ions consistent with the loss of aliphatic chains (Figure 3). For example, selection and collisional activation of the ion at *m/z* 864 after ESI in the positive-ion mode yielded several informative product ions (Figure 3A, inset) corresponding to the neutral loss of palmitic acid, linoleic acid and oleic acid (*m/z* 608, 584 and 582), respectively, in an approx. 1:1:1 ratio. In addition, the neutral loss of their lithium salts at *m/z* 602, 578 and 576 (also in a 1:1:1 ratio) was also present. Similarly, selection and collisional activation of the ion at *m/z* 888 after ESI in positive-ion mode yielded product-ion patterns corresponding to the loss of oleic and linoleic acids (Figure 3B). Collectively, the chromatographic elution profile and the tandem-MS demonstration of multiple aliphatic chains suggested that the clusters centred at *m/z* 864 and 888 resulted from TAG molecular species.

Positive-ion ESI mass spectra of an equimolar mixture of 16:0/16:0/16:0-TAG, 18:1/18:1/18:1-TAG and 18:2/18:2/18:2-TAG (5 pmol/μl each) in chloroform/methanol (1:1) after addition of LiOH (20 pmol/μl) yielded three major ion peaks with relative intensities of 2:3:5, respectively (spectra not shown). This analysis and other studies (results not shown) demonstrated that the sensitivity of individual TAG molecular species is different and is largely dependent on the degree of unsaturation present. However, the total ion current was directly proportional to the mass of each individual molecular species of TAG present, thereby allowing direct comparisons of the relative abundance of each specific individual molecular species with standards under different physiological perturbations. Collectively these results demonstrate that the relative contribution of individual molecular species of TAGs can be analysed by ESI-MS, but that absolute quantification of the total mass of each species is not feasible until the response factor of each molecular species is known.

Table 3 Diabetes-induced alterations in the masses of PhosCho molecular species in rat myocardium

Rat myocardial phospholipids were extracted by the Bligh and Dyer method [23] and analysed directly by positive-ion ESI-MS as described in the Experimental procedures section. PhosChos and sphingomyelins were quantified by comparisons of the individual ion peak intensities with an internal standard (i.e. 14:0/14:0 PhosCho) after correction for ^{13}C isotope effects. The results are expressed in nmol/mg of protein and represent means ± S.E.M. of a minimum of six separate animals. Values for diabetic or insulin-treated groups were not significantly different from those in the control group. The masses of all lithiated ions were rounded to the nearest integer.

Peak (*m/z*)	Assignment	6 Week control	6 Week diabetes	6 Week diabetes treated	6 Week control treated
PhosCho					
741	16:0/16:0	0.87 ± 0.08	0.96 ± 0.06	0.90 ± 0.06	0.87 ± 0.05
765	16:0/18:2	3.29 ± 0.18	3.84 ± 0.25	3.31 ± 0.14	3.17 ± 0.16
767	16:0/18:1	1.51 ± 0.12	1.55 ± 0.08	1.49 ± 0.06	1.50 ± 0.08
789	16:0/20:4	4.28 ± 0.21	4.71 ± 0.28	4.43 ± 0.26	4.38 ± 0.27
791	18:1/18:2	0.84 ± 0.07	0.96 ± 0.08	0.89 ± 0.06	0.80 ± 0.05
793	18:0/18:2	2.11 ± 0.16	2.31 ± 0.18	2.21 ± 0.15	2.02 ± 0.14
793	18:1/18:1	1.07 ± 0.08	1.27 ± 0.09	1.22 ± 0.10	1.09 ± 0.06
795	18:0/18:1	0.53 ± 0.04	0.67 ± 0.05	0.61 ± 0.03	0.54 ± 0.04
813	16:0/22:6	2.48 ± 0.21	2.55 ± 0.16	2.58 ± 0.15	2.58 ± 0.16
815	16:0/22:5	0.90 ± 0.07	0.87 ± 0.06	0.88 ± 0.05	0.94 ± 0.04
817	18:0/20:4	4.51 ± 0.28	4.81 ± 0.35	4.66 ± 0.35	4.61 ± 0.34
817	16:0/22:4	1.28 ± 0.07	1.47 ± 0.07	1.41 ± 0.06	1.45 ± 0.07
837	18:0/22:6	0.89 ± 0.07	0.85 ± 0.04	0.87 ± 0.06	0.89 ± 0.08
839	18:0/22:5	0.97 ± 0.05	0.95 ± 0.08	0.96 ± 0.05	1.01 ± 0.10
841	18:0/22:4	1.98 ± 0.11	1.84 ± 0.09	2.03 ± 0.15	2.11 ± 0.18
Total		27.51 ± 1.68	29.61 ± 2.01	28.45 ± 1.91	27.96 ± 1.75
Sphingomyelin					
710	16:0/18:0	0.38 ± 0.06	0.41 ± 0.03	0.42 ± 0.04	0.39 ± 0.02
738	18:0/18:0	0.32 ± 0.04	0.34 ± 0.03	0.33 ± 0.03	0.31 ± 0.03
Total		0.70 ± 0.09	0.75 ± 0.06	0.75 ± 0.06	0.70 ± 0.04

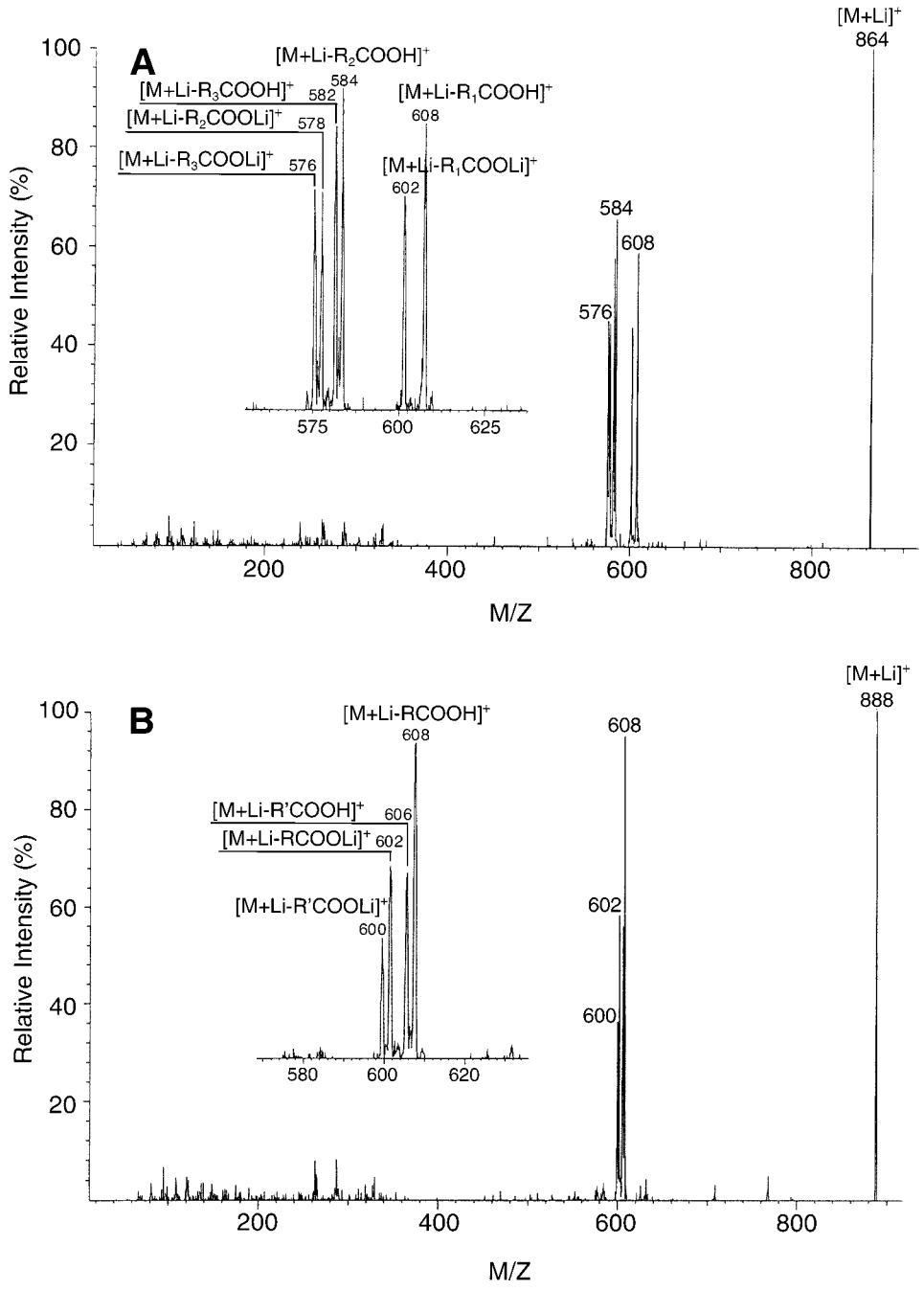

Figure 3 Identification of molecular ions between 820 and 900 in positive-ion ESI spectra (Figure 1) of rat myocardial extracts as TAGs by using positive-ion ESI tandem MS

ESI-MS/MS demonstrates that (**A**) molecular ion *m/z* 864 corresponds to lithiated 16:0/18:1/18:2-TAG and that (**B**) molecular ion *m/z* 888 corresponds to lithiated 18:1/18:2/18:2-TAG. The stereospecificity of acyl constituents cannot be assigned by this method. Insets: show enlarged sections of the main panels.

To further analyse alterations in the TAG content of control and diabetic rat hearts, HPLC purification of TAGs followed by acid methanolysis and capillary GC analyses was performed. The total TAG content in diabetic hearts was similar to controls (Table 4), which is in agreement with previous studies on total TAG mass in myocardium from streptozotocin-treated rats [6]. However, three new and intriguing results from molecular-species analysis were found. First, the acyl-chain constituents of TAGs in diabetic rat hearts were substantially different from controls, with a 2-fold increase in palmitic acid content of TAGs from 0.41 nmol/mg protein in controls to 0.81 nmol/mg protein in diabetics. Second, the peak corresponding to tripalmitin (*m/z* 814) in ESI-MS analysis of HPLC-purified TAGs increased by over 5-fold, and over a 2-fold rise in TAG species containing two molecules of palmitic acid and one molecule of palmitoleic acid (*m/z* 812) was present (Figure 4). Third, insulin

Table 4 Diabetes-induced alterations in the fatty acyl content of TAGs and in non-esterified fatty acids in rat myocardium

Rat myocardial TAGs and free fatty acids were extracted by the Bligh and Dyer method [23] and resolved by HPLC or prepared silica column as described in the Experimental procedures section. The masses of TAGs and non-esterified fatty acids were quantified by capillary GC after acid methanolysis by comparisons with internal standards. The results are expressed in nmol/mg of protein and represent means ± S.E.M. of a minimum of six separate animals. NEFA, non-esterified fatty acid; nd, not detected. *$P < 0.05$ and †$P < 0.01$ compared with 6 week control.

	6 Week control		6 Week diabetes		6 Week diabetes treated	
Fatty acid	TAG	NEFA	TAG	NEFA	TAG	NEFA
$C_{16:0}$	0.41 ± 0.07	0.52 ± 0.09	0.81 ± 0.12†	0.80 ± 0.15†	0.75 ± 0.11†	0.65 ± 0.09
$C_{16:1}$	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.01
$C_{18:1}$	0.35 ± 0.06	0.13 ± 0.05	0.79 ± 0.11†	0.28 ± 0.09*	0.71 ± 0.11†	0.22 ± 0.06
$C_{18:2}$ (*n*-6)	0.34 ± 0.08	0.33 ± 0.08	0.32 ± 0.07	0.46 ± 0.12	0.35 ± 0.08	0.42 ± 0.12
$C_{18:2}$ (*n*-4)	0.64 ± 0.11	0.20 ± 0.08	0.32 ± 0.09†	0.31 ± 0.09	0.45 ± 0.07	0.27 ± 0.09
$C_{18:3}$	0.04 ± 0.02	0.04 ± 0.02	nd	0.06 ± 0.02	nd	0.06 ± 0.02
$C_{20:3}$	0.08 ± 0.02	0.02 ± 0.01	nd	nd	nd	nd
$C_{20:4}$	0.19 ± 0.05	0.03 ± 0.01	0.13 ± 0.05	0.07 ± 0.03	0.14 ± 0.04	0.05 ± 0.02
$C_{22:6}$	0.06 ± 0.02	0.02 ± 0.01	nd	nd	0.02 ± 0.01	0.01 ± 0.01
Total NEFA	2.14 ± 0.32	1.31 ± 0.28	2.40 ± 0.38	2.02 ± 0.44	2.45 ± 0.36	1.71 ± 0.35
Total TAG	0.71 ± 0.11	—	0.80 ± 0.13	—	0.82 ± 0.12	—

treatment of diabetic rats did not substantially decrease the content of tripalmitin or the content of palmitic acid in TAGs (Figure 4 and Table 3). Although the response factor for individual TAG molecular species is markedly different (e.g. tripalmitin gives only half of the signal of triolein), direct comparisons of ratios between identical molecular species within a sample are valid. Collectively, three independent methods (i.e. capillary GC, ESI-MS of chloroform extracts and ESI-MS of HPLC-purified TAG) each demonstrate the dramatic increase in saturated TAG molecular species in diabetic myocardium that is not readily reversible by peripheral insulin treatment.

Measurement of non-esterified fatty acids in control and diabetic rat myocardium

To compare and contrast the alterations present in molecular-species composition of diabetic rat myocardium observed by ESI-MS (Table 5), it was essential to determine if the observed changes resulted from the depletion of specific fatty acids in the fatty acid pool. The total mass of non-esterified fatty acids was ≈ 20–30 % higher in diabetic myocardium and similar increases in each individual molecular species examined were present (Table 4). Accordingly, these results show that the specific differences in the phospholipid molecular species were not due to differences in the content of individual molecular species of non-esterified fatty acid precursors present in myocardium that are utilized in the synthesis of phospholipids.

DISCUSSION

The present results identify three specific and readily reversible changes in myocardial phospholipid composition due to insulin deficiency, including: (i) an increase in phosphatidylinositol mass; (ii) an increase in plasmenylethanolamine mass and (iii) a decrease in 18:0/20:4 PhosEth. Additionally, we demonstrated a dramatic (> 5-fold) increase in the amount of tripalmitin present in the TAG fraction of myocardium from streptozotocin-treated rats to the point where it represented the major TAG molecular species present in diabetic myocardium. Remarkably, this increase in tripalmitin mass was not readily reversible by peripheral insulin treatment although all other lipid alterations were entirely prevented by insulin treatment.

Alterations in phospholipid classes, subclasses and individual molecular species have previously been shown to modulate the physical properties of cell membranes [28,29], the enzymic activity of transmembrane proteins [30,31], the recruitment of critical intracellular signalling proteins to membrane surfaces [7,32], the propensity for membrane fusion [33,34] and the production of lipid second messengers of signal transduction [31,35]. The essential role of membrane constituents in insulin-mediated signalling has been underscored by the demonstration of the importance of: (i) membrane physical properties in modulating the magnitude of insulin-receptor autophosphorylation [36]; (ii) plasmenylethanolamines in facilitating membrane fusion [33,34,37]; (iii) PI 3-kinase in mediating insulin-induced increases in glucose transport, amino acid transport and glycogen synthesis [38–40] and (iv) lipid second messengers and activation of their downstream targets in modulating cellular responses to insulin [41]. Accordingly, the alterations identified herein provide new insights into factors that contribute to the altered insulin responses present in diabetic myocardium.

A proximal effect of insulin in cellular signalling is the association of PI 3-kinase activity with IRS 1 and 2, which is an obligatory event in the translocation of the GLUT4 transporter from an intracellular membrane compartment to the plasma membrane [38,39]. This study demonstrates that the major substrate (by mass) for Class-I and Class-III PI 3-kinases undergoes a 46 % increase in myocardium from diabetic rats and that this increase is readily preventable by insulin administration. Furthermore, inositol glycerophospholipids and their downstream hydrolysis products (e.g. diacylglycerol and 1,4,5-inositol trisphosphate) play critical roles in many cellular responses through their effects on protein recruitment (e.g. FYVE and pleckstrin homology domains), protein kinase C activation and cellular calcium-ion homoeostasis [42]. Moreover, recent studies have identified the importance of the interaction of the serine/threonine protein kinase Akt with polyphosphoinositides in the plasma membrane, which is essential in mediating insulin's effects. The importance of membrane interactions in facilitating the activation of Akt is underscored by the fact that activation of Akt may either occur through the interaction of its pleckstrin homology domain with membrane constituents, or, alternatively, be genetically engineered by addition of a myristoylation site which renders the Akt kinase membrane-bound and constitutively active [43]. The importance of Akt kinase and protein–

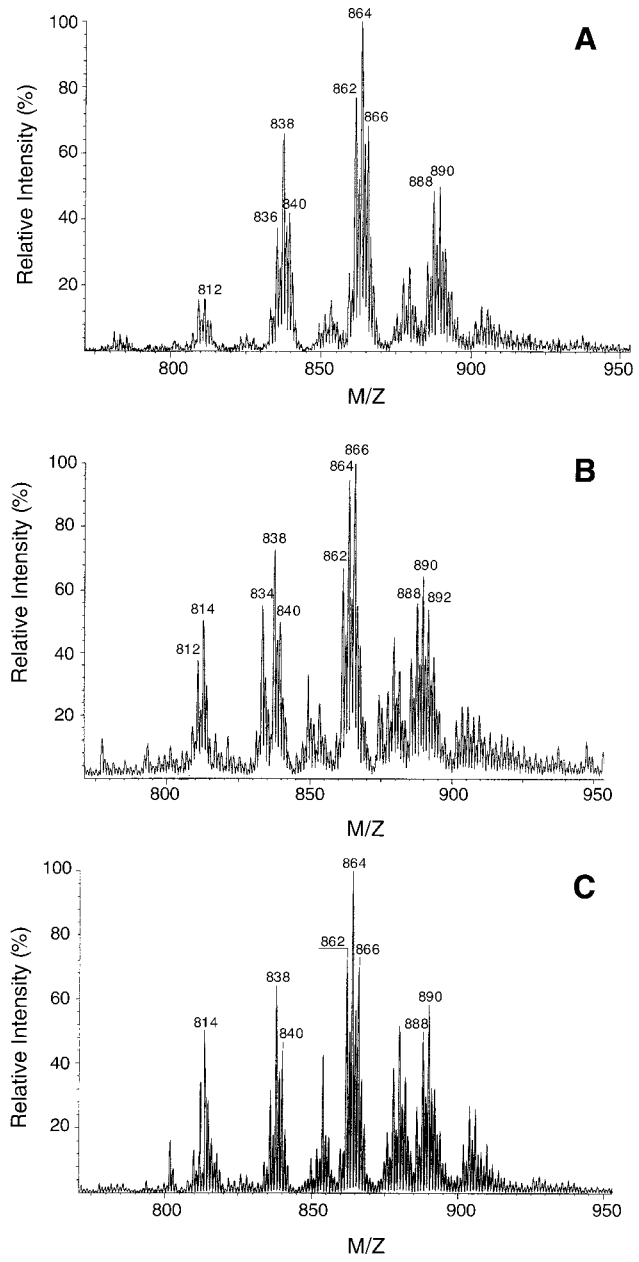

Figure 4 Positive-ion ESI mass spectra of HPLC-purified TAGs from control and diabetic rat myocardium

Myocardial TAGs from control (**A**), diabetic (**B**) or insulin-treated diabetic (**C**) rat myocardium were purified by straight-phase HPLC with an initial mobile phase comprised of hexane/isopropanol (1:1, v/v) and employing a linear gradient of 0–6% H_2O as described in the Experimental procedures section. Positive ion ESI-MS was performed after the addition of LiOH.

membrane interactions is further emphasized by the ablation of insulin-stimulated Akt kinase activity by inhibition of PI 3-kinase after wortmanin treatment [44].

Insulin-mediated stimulation of glucose uptake in myocardium and skeletal muscle is mediated by the directed translocation of vesicles enriched in the glucose transporter (GLUT4) to the plasma membrane. Directed translocation is a multistep process that includes priming by both Akt and PI 3-kinases, translocation of GLUT4-containing vesicles from intracellular compartments to the plasma membrane and, finally, fusion of GLUT4-containing vesicles with the plasma membrane [45]. With regard to the first two parts of this process, both pharmacological (e.g. wortmanin) and molecular-biological (e.g. membrane attachment) methods have demonstrated the importance of both PI 3-kinase and the protein kinase Akt in priming the system [43,46]. With regard to the third part of this process, plasmenylethanolamine molecular species increase by 44% in myocardium from diabetic rats and plasmenylethanolamines facilitate membrane fusion [33,34]. Accordingly, the increased plasmenylethanolamine content in diabetic rat myocardium is anticipated to facilitate the fusion of membrane bilayers (and hence the delivery of GLUT4 protein to the plasma membrane) after insulin challenge. We speculate that these alterations reflect either an adaptive process (i.e. an attempt to facilitate insulin-induced GLUT4 translocation after insulin deprivation) or perhaps the diabetes-induced proliferation of peroxisomes, since the rate determining step of plasmalogen biosynthesis occurs in the peroxisomal compartment.

The selective decrease in 18:0/20:4 PhosEth in myocardium from streptozotocin-treated diabetic rats occurred in the absence of depletion of either overall arachidonic acid content in cellular phospholipids (i.e. the arachidonic acid content of each of the phospholipid classes was either normal or increased) or in cellular non-esterified arachidonic acid content. Since ethanolamine glycerophospholipids are the major precursor pool of the arachidonic acid released after cellular stimulation in most cell types, the results suggest that diabetic myocardium may possess a decreased ability to respond to stimuli by releasing suboptimal amounts of arachidonic acid. The observed decrease in 18:0/20:4 PhosEth could be due either to a decreased synthetic capacity for the production of this molecular species or, alternatively, may result from the persistent stimulation of one or more phospholipases. Indeed, alterations in β-adrenergic receptor function have been reported in diabetes. Recently the β_2-adrenergic receptor in cardiac myocytes has been found to be linked to the release of arachidonic acid and alterations in cellular contractility [47]. Accordingly, we speculate that increased deacylation–reacylation cycling is present in the diabetic heart mediated by the β_2-receptor pathway and that arachidonic acid depletion (from chronic β_2-adrenergic overstimulation) may be one mechanism that contributes to the decreased contractility manifest in diabetic myocardium.

Many previous studies have examined alterations in total myocardial TAG mass in the streptozotocin-treated rat model, although no prior studies have examined the molecular-species distribution of TAGs from any tissue in the diabetic state [7,10–16]. The most dramatic finding of this study is that a single molecular species of TAG, tripalmitin, increases over 5-fold in the diabetic state. Moreover, this increase was specific since it occurred in the absence of any major alterations in total TAG mass or major alterations of other TAG molecular species. Remarkably, the increase in tripalmitin mass was not ameliorated by peripheral insulin treatment. This is not the first alteration in muscle metabolism of the streptozotocin-treated rat that could not be prevented by peripheral insulin treatment. Previously, tyrosine phosphorylation of a 66 kDa protein was shown to increase in streptozotocin-diabetic rats and this observed increase was not ameliorated by insulin treatment [46]. Two possibilities seem likely. Either the peripheral delivery of insulin does not recapitulate the physiological responses of complex secretion, the glucose regulatory events (i.e. impedance mismatching of glucose increases and insulin delivery into the portal circulation), or alternatively other products may be present in the β-cell that contribute to alterations in TAG metabolism. In this regard, pancreatic β-cell secretory granules contain numerous poly-

Table 5 Diabetes-induced alterations in the different phospholipid classes quantified by ESI-MS

The cumulative amounts of major phospholipid pools from Tables 1, 2 and 4 as quantified by ESI-MS are expressed in nmol/mg of protein and represent means ± S.E.M. of a minimum of six separate animals. *$P < 0.05$ and †$P < 0.01$ compared with 6 week controls.

Phospholipid	6 Week control	6 Week diabetes	6 Week diabetes treated	6 Week control treated
Choline glycerophospholipid	27.51 ± 1.68	29.61 ± 2.01	28.45 ± 1.91	27.96 ± 1.75
Ethanolamine glycerophospholipid	24.36 ± 1.83	28.27 ± 2.13*	25.35 ± 1.31	24.01 ± 1.52
Phosphatidylserine	0.70 ± 0.12	0.88 ± 0.06	0.77 ± 0.14	0.72 ± 0.14
Phosphatidylinositol	2.46 ± 0.24	3.60 ± 0.09†	2.36 ± 0.18	2.63 ± 0.12
PhosGly	2.56 ± 0.23	3.27 ± 0.08†	2.68 ± 0.21	2.34 ± 0.18
Cardiolipin	6.39 ± 0.49	6.46 ± 0.61	5.83 ± 0.72	5.81 ± 0.71
Sphingomyelin	0.70 ± 0.09	0.75 ± 0.06	0.75 ± 0.06	0.70 ± 0.04
Total	64.68 ± 3.56	72.84 ± 3.37	66.19 ± 3.24	64.17 ± 4.15

peptides including lipoprotein lipase and secretory phospholipase A_2. Moreover, the glucose-induced β-cell secreted lipoprotein lipase has important effects on β-cell metabolism, as assessed in lipoprotein lipase-knockout mice [48].

Although palmitic acid has traditionally been regarded either as a primary fuel for heart cells or an aliphatic scaffold utilized in cellular membranes, recent work by independent groups has identified novel roles for palmitic acid in insulin sensitivity and calcium sequestration in muscle cells. For example, Schmitz-Peiffer et al. [49] have demonstrated an important role for palmitate, but not other non-esterified fatty acids, in decreasing the sensitivity of muscle cells to insulin. Similarly, Rys-Sikora and Gill [50] demonstrated that translocation of calcium between distinct subcellular compartments is mediated by a GTP-activated process specifically requiring palmitate (i.e. other non-esterified fatty acids were ineffective). Accordingly, we speculate that some of the abnormalities in muscle-cell responses to insulin (e.g. insulin resistance) in the diabetic state may be related to this dramatic increase in a mobilizable intracellular storage depot of palmitic acid (i.e. tripalmitin) that can be released by intracellular lipases. If this hypothesis is correct, then alterations in myocardial function in streptozotocin-induced diabetic rats should be ameliorated by islet transplantation (since they may provide other bioactive substances that modulate TAG metabolism) but not by insulin treatment. Experiments towards this clinically relevant experimental goal are currently in progress in our laboratory.

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