

# Fatty Acid Uptake and Incorporation into Phospholipids in the Rat Lens

Jessica R. Nealon,<sup>1</sup> Stephen J. Blanksby,<sup>1</sup> Paul J. Donaldson,<sup>2</sup> Roger J. W. Truscott,<sup>\*3</sup> and Todd W. Mitchell<sup>\*4</sup>

**PURPOSE.** Phospholipids are a major component of lens fiber cells and influence the activity of membrane proteins. Previous investigations of fatty acid uptake by the lens are limited. The purpose of the present study was thus to determine whether exogenous fatty acids could be taken up by the rat lens and incorporated into molecular phospholipids.

**METHODS.** Lenses were incubated with fluorescently labeled palmitic acid and then analyzed by confocal microscopy. Concurrently, lenses incubated with either fluorescently labeled palmitic acid or the more physiologically relevant <sup>13</sup>C<sub>18</sub>-oleic acid were sectioned into nuclear and cortical regions and analyzed by highly sensitive and structurally selective electrospray ionization tandem mass spectrometry techniques.

**RESULTS.** The detection of fluorescently labeled palmitic acid, even after 16 hours of incubation, was limited to approximately the outer 25% to 30% of the rat lens. Mass spectrometry also revealed the presence of free <sup>13</sup>C<sub>18</sub>-oleic acid in the cortex but not the nucleus. No evidence could be found for incorporation of fluorescently labeled palmitic acid into phospholipids; however, a low level of <sup>13</sup>C<sub>18</sub>-oleic acid incorporation into phosphatidylethanolamine (PE), specifically PE (PE 16:0/<sup>13</sup>C<sub>18</sub> 18:1) was detected in the lens cortex after 16 hours.

**CONCLUSIONS.** These data demonstrate that uptake of exogenous (e.g., dietary fatty acids) by the lens and their incorporation into phospholipids is minimal, most likely occurring only during de novo synthesis in the outermost region of the lens. This finding adds support to the hypothesis that once synthesized there is no active remodeling or turnover of fiber cell

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Phospholipids are a major component of lens fiber cells and also interact with integral membrane proteins such as Ca<sup>2+</sup>-ATPase,<sup>1,2</sup> caveolin 1 and 2,<sup>3,4</sup> and aquaporin-0 (AQPO),<sup>5,6</sup> as well as  $\alpha$ -crystallin.<sup>7,8</sup> This interaction is crucial for the structure and function of membrane proteins. For example, phospholipids are thought to play a vital role in the conversion of nonjunctional- to junctional-AQPO as fiber cells age.<sup>8</sup> Both the phospholipid<sup>9,10</sup> and protein<sup>11</sup> composition of the lens is specific to the particular animal species. It is therefore reasonable to surmise that membrane phospholipid composition is optimized to maintain cellular function.

We have recently demonstrated that altering dietary fatty acid composition is unable to influence the phospholipid composition of the rat lens.<sup>12</sup> This finding suggests that, once synthesized, lens phospholipid composition remains unchanged. An inability to replace oxidatively damaged phospholipids is likely to have significant consequences for membrane integrity (and thus function) over an animal's lifespan. Borchman et al.<sup>13</sup> have previously hypothesized that a lack of cellular machinery may result in a lack of active lipid turnover in the lens center. This, however, does not explain the lack of dietary influence on the outer, metabolically active fiber cells. One explanation for this observation is that phospholipid synthesis is highly regulated. Another possible explanation is that fatty acids obtained from the diet are not taken up by the lens; however, previous studies suggest that lipids enter the aqueous humor from the blood stream bound to albumin<sup>14</sup> or as part of high-density lipoproteins.<sup>15</sup> From there, it is believed that they are taken up by the lens epithelium as free fatty acids<sup>16</sup> or bound to albumin<sup>17</sup> by a mechanism that may involve caveolae and clathrin-coated vesicles.<sup>18</sup> It is also possible that fatty acid binding proteins play a role in the delivery of fatty acids to the lens, and these have been identified in mouse,<sup>19</sup> cow,<sup>20</sup> and rat lenses<sup>21</sup>; however, no studies have been conducted into its function in the lens.

The purpose of the present study was to determine whether exogenous fatty acids could be taken up by the rat lens and incorporated into molecular phospholipids. To investigate this, confocal microscopy was used to track the movement of BODIPY palmitate (BODIPY C<sub>16</sub>) into intact rat lenses. To rule out any influence on diffusion and enzyme specificity associated with the use of a relatively large fluorophore (palmitic acid has a mass of 256 Da vs. 474 Da for BODIPY palmitate), we performed complementary experiments where <sup>13</sup>C-labeled oleic acid (<sup>13</sup>C<sub>18</sub>-oleic acid) was added to intact lenses and its movement and incorporation into phospholipids were determined by analysis of sectioned lenses using electrospray ionization tandem mass spectrometry. Palmitate and oleate were chosen, as they are abundant fatty acids present in rat lens phospholipids.<sup>10</sup>

From the Schools of <sup>1</sup>Chemistry and <sup>4</sup>Health Sciences, University of Wollongong, Wollongong, New South Wales, Australia; the <sup>2</sup>Department of Optometry and Visual Science, New Zealand National Eye Centre, University of Auckland, Auckland, New Zealand; and the <sup>3</sup>Save Sight Institute, University of Sydney, Sydney, New South Wales, Australia.

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\* Each of the following is a corresponding author: Roger J. W. Truscott, Save Sight Institute, 8 Macquarie Street, Sydney, NSW, 2001, Australia; rjw@eye.usyd.edu.au.

Todd Mitchell, University of Wollongong, Northfields Avenue, Wollongong, NSW, 2500, Australia; toddm@uow.edu.au.

## METHODS

### Reagents

HPLC grade chloroform, methanol, and analytical grade ethanol were purchased from Crown Scientific (Moorebank, NSW, Australia). Analytical grade butylated hydroxytoluene (BHT), fatty acid free bovine serum albumin (BSA), and  $^{13}\text{C}_{18}$ -oleic acid (99%) were purchased from Sigma-Aldrich (Castle-Hill, NSW, Australia). 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid (BODIPY  $\text{C}_{16}$ ) and Medium 199 without phenol red were purchased from Invitrogen-Molecular Probes (Mount Waverley, NSW, Australia). OCT (Tissue-Tek) compound was purchased from ProSci Tech (Thuringowa, QLD, Australia). Unless stated, all other chemicals were purchased from Sigma-Aldrich.

### Lens Incubation

Fatty acid-free BSA was added to Medium 199 at a concentration of 1 mg/mL and filter-sterilized using a 0.22- $\mu\text{m}$  filter (Sartorius-Stedim Biotech, Dandenong South, VIC, Australia). BODIPY  $\text{C}_{16}$  or  $^{13}\text{C}_{18}$ -oleic acid were dissolved separately in ethanol and vortexed. Supplemented medium was pre-equilibrated at 37°C for 30 minutes before addition of BODIPY  $\text{C}_{16}$  to a final concentration of 110  $\mu\text{M}$  and incubated for a further 30 minutes before addition of the lens. For confocal microscopy, lenses were obtained from 18-week-old male Sprague-Dawley rats after euthanatization by a peritoneal injection of sodium pentobarbitone (400 mg/kg body weight) and immediately incubated for 1, 4, 6, and 16 hours at 37°C. For mass spectrometric analysis, lenses were incubated in supplemented medium 199 as described above with either  $^{13}\text{C}_{18}$ -oleic acid or BODIPY  $\text{C}_{16}$  added to a final concentration of 110  $\mu\text{M}$  for 16 hours. After incubation, the lenses were washed with PBS (0.15 M NaCl and 0.01 M  $\text{Na}_3\text{PO}_4$  [pH 7.4]) and frozen immediately at  $-80^\circ\text{C}$  for mass spectrometric analysis or fixed for histologic analysis.

All experiments were approved by the Animal Ethics Committee of the University of Wollongong and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Confocal Microscopy

Lenses used for confocal microscopy after incubation were prepared as reported previously.<sup>22,23</sup> In brief, the lenses were fixed in 0.75% aqueous paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 24 hours. They were washed three times in PBS, followed with cryoprotection by incubation in 10% sucrose in PBS for 1 hour at room temperature, 20% sucrose in PBS for 1 hour at room temperature, and 30% sucrose in PBS overnight at 4°C.

For sectioning, the lenses were positioned in an equatorial orientation so that the posterior surface of the lens was mounted onto prechilled chucks. The lens was then encased in OCT compound. Sections were cut at  $-20^\circ\text{C}$  on a cryotome with disposable blades (Thermo Shandon; ThermoFisher Scientific, Adelaide, SA, Australia). The lenses were sliced into 18- $\mu\text{m}$  sections and transferred to poly-L-lysine-coated microscope slides in PBS and then washed three times with PBS. Cell membranes were stained by incubation with wheat germ agglutinin (WGA) conjugated with Alexa Fluor 555 (Invitrogen-Molecular Probes) for 1.5 hours, in the dark at room temperature. Antifade reagent (10  $\mu\text{L}$ ) was added to each section and viewed with a Pascal confocal system (LSM5; Carl Zeiss Meditec, Inc., North Ryde, NSW, Australia). BODIPY  $\text{C}_{16}$  was imaged at 488 nm with an argon laser at 34%, with a BP505-530 filter and a 52- $\mu\text{m}$  pinhole. WGA was imaged at 543 nm using an HeNe laser at 100% with an LP 560 filter and a 54- $\mu\text{m}$  pinhole. Images were processed by Image J Software 1.41 (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). To create a plot of pixel intensity versus distance (see Fig. 2), we highlighted a section of the image from the epithelium to the central core (the same number of pixels were selected for each image for consistency of comparison), and a plot profile was generated within the Image J software, listing a

distance and the corresponding pixel intensity at that distance. These data were then normalized for both intensity (with the pixel intensity at the edge of the epithelium being set to 1) and distance (with the center of the lens set to 0 and the capsule set to 1).

### Lipid Extraction

After incubation, lenses were frozen at  $-80^\circ\text{C}$ , and sectioned into nucleus and cortex with a 3-mm trephine. Lenses were homogenized in chloroform-methanol (2:1 vol/vol) with 0.01% BHT at a ratio of 20:1 solvent to tissue. Total lipids were extracted as described elsewhere<sup>10</sup> and stored at  $-80^\circ\text{C}$  until analysis.

### Mass Spectrometry

All mass spectra were obtained with a triple quadrupole mass spectrometer (QuattroMicro; Waters, Manchester, UK) equipped with a z-spray electrospray ion source and controlled by allied software (Micromass Masslynx, ver. 4.0; Waters). Capillary voltage was set to 3000 V, source temperature to 80°C, and desolvation temperature to 120°C. Cone voltage was set to 50 V in negative ion mode and 35 V in positive ion mode. Nitrogen was used as the drying gas at a flow rate of 320 L/h. Samples were infused into the electrospray ion source at a flow rate of 10  $\mu\text{L}/\text{min}$  by the instrument's on-board syringe pump. Formation of ions was enhanced by the addition of aqueous ammonium acetate to a final concentration of approximately 50 mM.<sup>24</sup> Negative ion scans were performed to identify the presence of free  $[\text{M}-\text{FH}_2]^-$  BODIPY  $\text{C}_{16}$  and  $^{13}\text{C}_{18}$ -oleic acid in the mass range of  $m/z$  250 to 900.

Precursor ion scans for  $m/z$  453.5 (BODIPY  $\text{C}_{16}$ ), 299.3 ( $^{13}\text{C}_{18}$ -oleic acid), and 281.4 (unlabeled oleic acid) were performed in negative ion mode at collision energies of 20, 35, and 35 eV, respectively. Precursor ion scanning in positive ion mode for  $m/z$  184.1 (phosphocholine) using a collision energy of 35 eV was performed to determine incorporation of labeled fatty acids into phosphatidylcholines and sphingomyelins. Product ion scans were performed at a collision energy of 35 eV to further validate labeled fatty acid incorporation. In all precursor ion and product ion scans, argon was used as the collision gas at a pressure of 3 mTorr.

## RESULTS

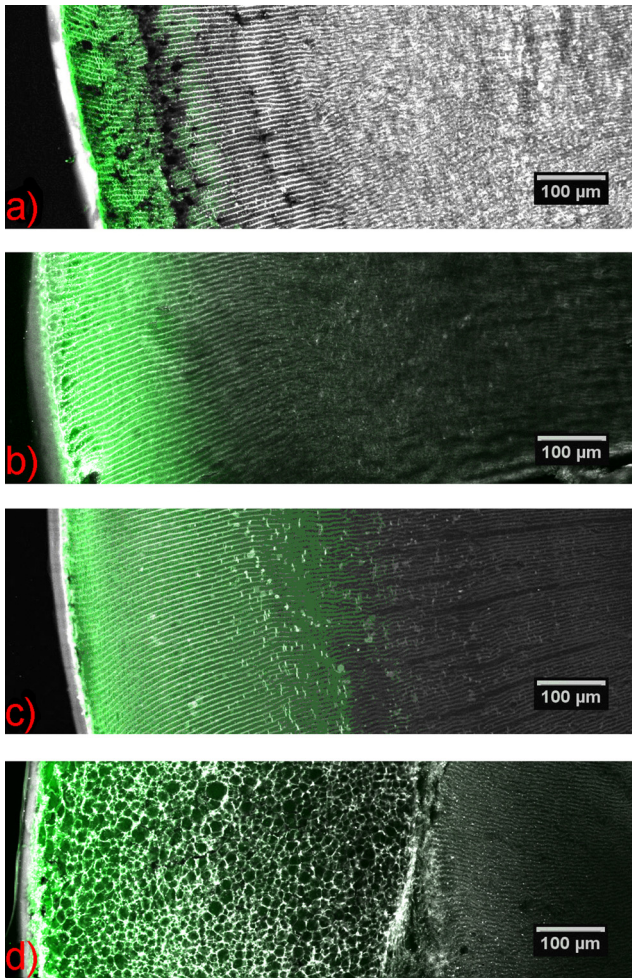
### Incubation of Intact Lenses with BODIPY Palmitate

Incubation of rat lenses with BODIPY  $\text{C}_{16}$  showed the presence of the fluorescently labeled fatty acid in the lens cortex after 1 hour (Fig. 1a). The diffusion distance was extended slightly with 4 and 6 hours of incubation by comparison to the 1-hour sample (Figs. 1b, 1c).

BODIPY  $\text{C}_{16}$  did not penetrate deeper into the lens nucleus with increased times of incubation, up to 16 hours. A plot distribution was obtained by averaging the pixel intensity across this distance with Image J software. As shown in Figure 2, even after a 16-hour incubation, BODIPY  $\text{C}_{16}$  (474 Da) was observed only in the outer 25% to 30% of the lens. The confocal image at 16 hours was notably irregular compared with the earlier time points, which may reflect a disruption of the fiber cell membranes by BODIPY  $\text{C}_{16}$  (Fig. 1d).

Further investigation of the distribution of BODIPY  $\text{C}_{16}$  into the lens was performed by separating the lens into nucleus and cortex and then analyzing lipid extracts from these sections by electrospray ionization tandem mass spectrometry. A negative ion mass spectrum of the rat cortex after 16 hours showed an abundant ion at  $m/z$  453.5, which corresponds to the  $[\text{M}-\text{FH}_2]^-$  free form of BODIPY  $\text{C}_{16}$  (data not shown). Further interrogation of the ion at  $m/z$  453.5 using collision-induced dissociation in a tandem mass spectrometry experiment revealed fragmentation that was identical with that obtained from an authentic sample of BODIPY  $\text{C}_{16}$ , confirming the





**FIGURE 1.** Diffusion of labeled fatty acid into rat lens. Equatorial cryosections showing the diffusion of BODIPY  $C_{16}$  into a rat lens after (a) 1, (b) 4, (c) 6, and (d) 16 hours. Green fluorescence corresponds to BODIPY  $C_{16}$  and the gray fluorescence corresponds to WGA Alexa Fluor 555.  $C_{16}$ , palmitic acid; WGA, wheat germ agglutinin.

presence of the fluorescent probe in the cortex. No such signal was detected in the nucleus.

No evidence was obtained for incorporation of BODIPY  $C_{16}$  into phospholipids in the range of  $m/z$  600 to 1000 as assessed by performing a negative ion precursor scan ( $m/z$  453.5) selective for any molecule containing BODIPY  $C_{16}$  and a positive ion precursor scan for phosphatidylcholines and sphingomyelins, which contain a phosphocholine head group ( $m/z$  184.1).

Analysis of the rat lens nucleus by mass spectrometry confirmed the data obtained by confocal microscopy in that no evidence of free or phospholipid-incorporated BODIPY  $C_{16}$  was found, even after 16 hours of incubation.

### $^{13}C_{18}$ -oleic Acid Incubation

Negative ion spectra of lipid extracts obtained from the cortex and nucleus of a lens incubated in  $^{13}C_{18}$ -oleic acid and also a control lens cortex and nucleus are shown in Figure 3. The spectra show  $^{13}C_{18}$ -oleic acid (Fig. 3A;  $m/z$  299.5,) present only in the incubated cortex at an ion abundance approximately one third that of the native oleic acid (Fig. 3A;  $m/z$  281.4). These data confirm the results shown in Figure 1, where free BODIPY  $C_{16}$  was detected in the cortex, but not in the nucleus.

Figure 4A shows mass spectra of the naturally occurring phospholipids found in the cortex of a rat lens. No major differences in anionic phospholipid profile were observed with the incubated cortex (Fig. 4B) in comparison to the control cortex. Furthermore, scans were performed to determine whether  $^{13}C_{18}$ -oleic acid had been incorporated into phospholipids. An initial precursor ion scan ( $m/z$  281.4) shown in Figure 5A, was performed to identify phospholipids containing unlabeled 18:1. It would be expected that any incorporation of  $^{13}C_{18}$ -oleic acid would occur in these highly abundant phospholipids. The corresponding precursor ion scan for  $^{13}C_{18}$ -oleic acid ( $m/z$  299.3) is shown in Figure 5B. The signal obtained from the  $^{13}C_{18}$ -oleic acid precursor ion scan is approximately 500 times lower than that obtained for precursors of native oleic acid.  $^{13}C_{18}$ -oleic acid was detected in one phospholipid present at  $m/z$  734.6. This ion was tentatively identified as phosphatidylethanolamine (PE) (16:0/ $^{13}C_{18}$ 18:1), as it was 18 Da higher than PE (16:0/18:1;  $m/z$  716.6), which is observed as a low-abundance ion in Figure 5A and has been identified in the rat lens.<sup>12</sup> The identification of PE (16:0/ $^{13}C_{18}$ 18:1) was confirmed using tandem mass spectrometry by the presence of an ion at  $m/z$  255.4 corresponding to the 16:0 fatty acid, and an ion at  $m/z$  299.5 corresponding to the labeled 18:1 fatty acid (data not shown). Phosphatidylcholines and sphingomyelins were analyzed using the precursor ion scan  $m/z$  184.1, and no evidence of  $^{13}C_{18}$ -oleic acid incorporation was observed.

## DISCUSSION

The present study suggests that movement of fatty acids into the lens over a 16-hour period is restricted to the outer 25% to 30% of the rat lens radius. Moreover, a minimal amount of exogenous fatty acid incorporation into molecular phospholipids was detected.

To the authors' knowledge only two previous studies have examined the uptake of fatty acids into the lens and their incorporation into fiber cell phospholipids. After incubating rabbit lenses in radio-labeled fatty acids for 3.5 hours, Broekhuysen and Bögemann<sup>16</sup> found most of the radioactivity in the epithelium and the equator, with small amounts in the peripheral, anterior, and posterior cortex. No radioactivity was observed in the lens center. A more recent study indicates that fluorescently labeled lauric acid (BODIPY  $C_{12}$ ) can enter the nucleus of cultured rat lenses when the incubation period is extended to 12 hours.<sup>17</sup>

The current finding that BODIPY or  $^{13}C$ -labeled fatty acids did not enter the rat lens nucleus after 16 hours is in contrast to that reported by Sabah et al.<sup>17</sup> An explanation for the discrepancy may be the significantly increased binding efficiency to BSA of both palmitic and oleic acid compared with lauric acid.<sup>25</sup> After incubating lenses in BODIPY  $C_{16}$  for 16 hours, we observed a disruption of fiber cell structure (Fig. 1d). Nevertheless, it did not result in further diffusion of fluorescently labeled palmitic acid into the lens. Similar vesiculation of fiber cells has been observed in rat lenses after an 18-hour incubation with the chloride channel inhibitor tamoxifen, probably owing to an impairment of cell volume regulation.<sup>26</sup> Disruption of fiber cell membrane structure by free BODIPY  $C_{16}$  could also impair cell volume regulation and may explain fiber cell swelling in the present study. From the low-magnification images it cannot be determined whether a similar phenomenon occurred in the study of Sabah et al.<sup>17</sup> and whether this had an impact of fatty acid diffusion. The plot shown in Figure 2 suggests that the fatty acids entered the rat lens via diffusion, and therefore a longer incubation period may have resulted in the labeled fatty acids penetrating farther into the

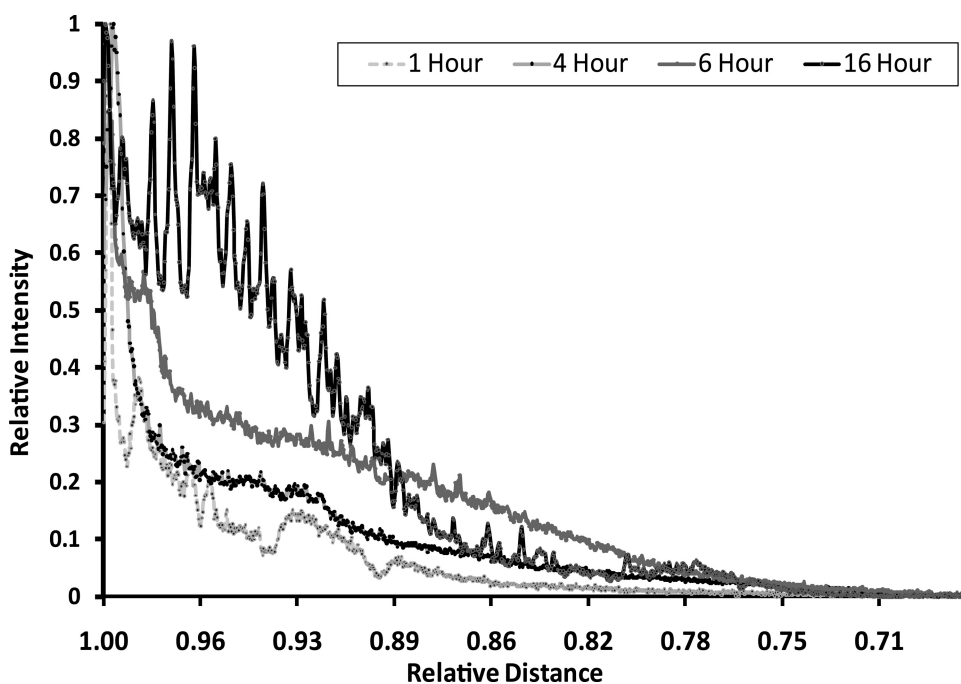


FIGURE 2. Diffusion of BODIPY C<sub>16</sub> into the rat lens as a function of time. The intensity of fluorescence versus distance from the lens epithelium was plotted after 1, 4, 6, and 16 hours. C<sub>16</sub>, palmitic acid.

lens. Given the cellular damage observed after 16 hours, we were reluctant to incubate the lenses for longer periods.

This study provides, for the first time, structural evidence, obtained with confocal microscopy and electrospray ionization tandem mass spectrometry, of both the extent and specificity of fatty acid diffusion and incorporation into lens phospholipids. We could find no evidence for incorporation of BODIPY C<sub>16</sub> into lens phospholipids using mass spectrometry. This suggests that this modified fatty acid is either not inserted into phospholipids in the lens or that the degree of incorporation is

too low to detect. Sabah et al.<sup>17</sup> observed co-migration of fluorescence with that of phospholipids by thin-layer chromatography (TLC), which they interpreted as incorporation of BODIPY C<sub>12</sub> into sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine. Only nonlabeled phospholipid standards were run on these TLC plates and the effect on migration of adding the large fluorophore was not shown.

In the present study, a very low level of <sup>13</sup>C<sub>18</sub>-oleic acid incorporation into PE (16:0/18:1) was detected in the rat lens cortex. This low level was near the limit of detection of the

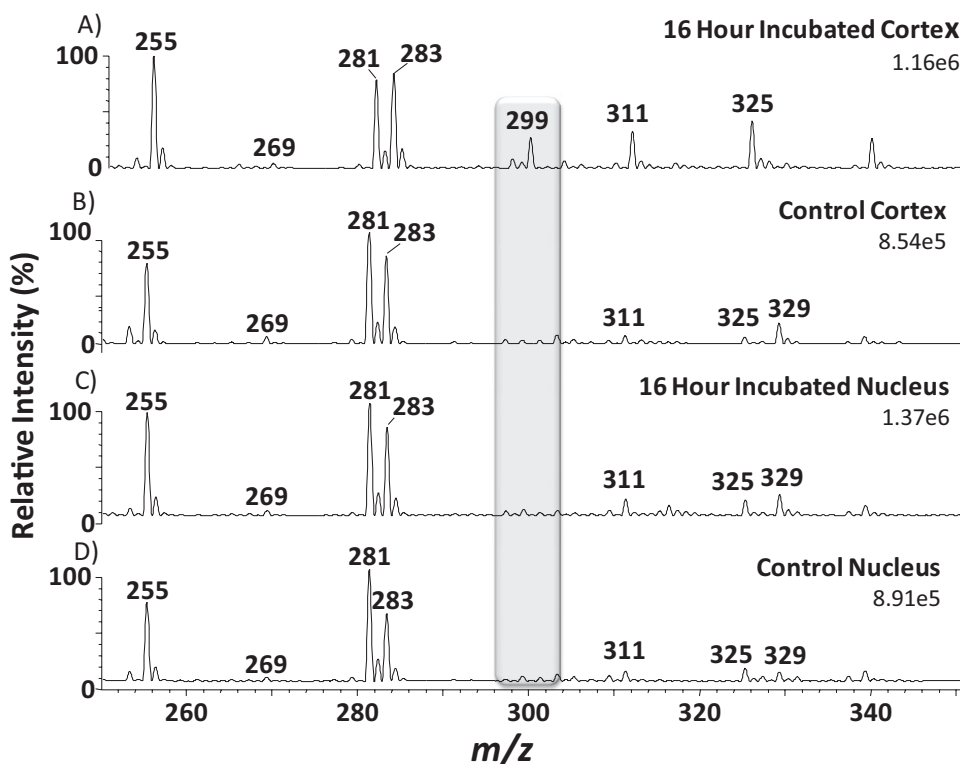
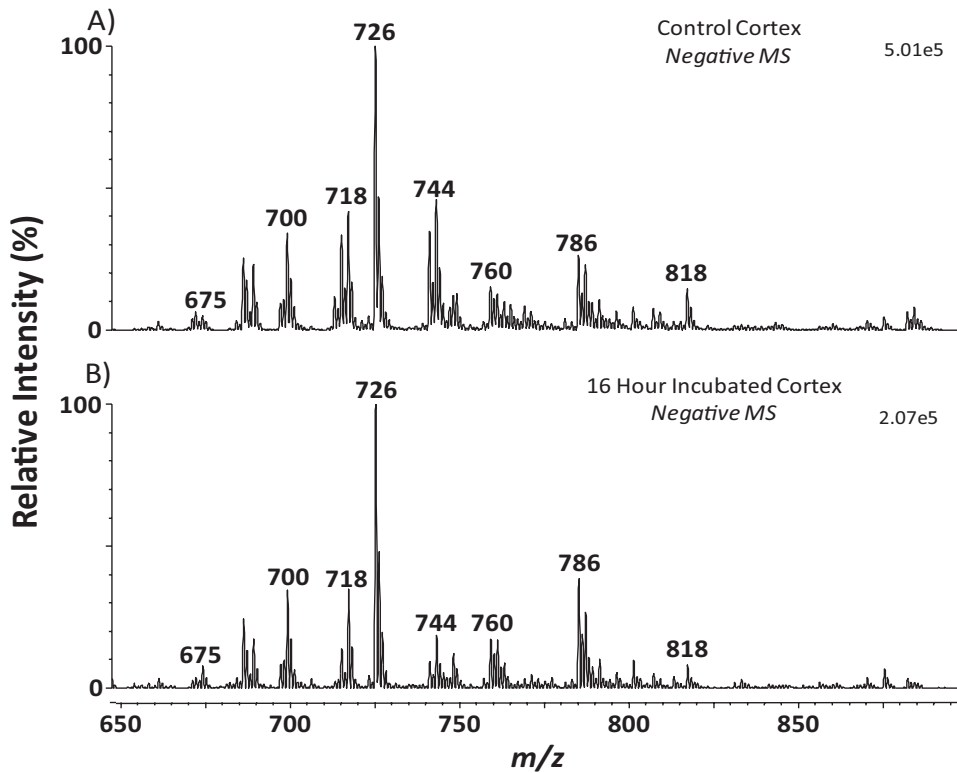


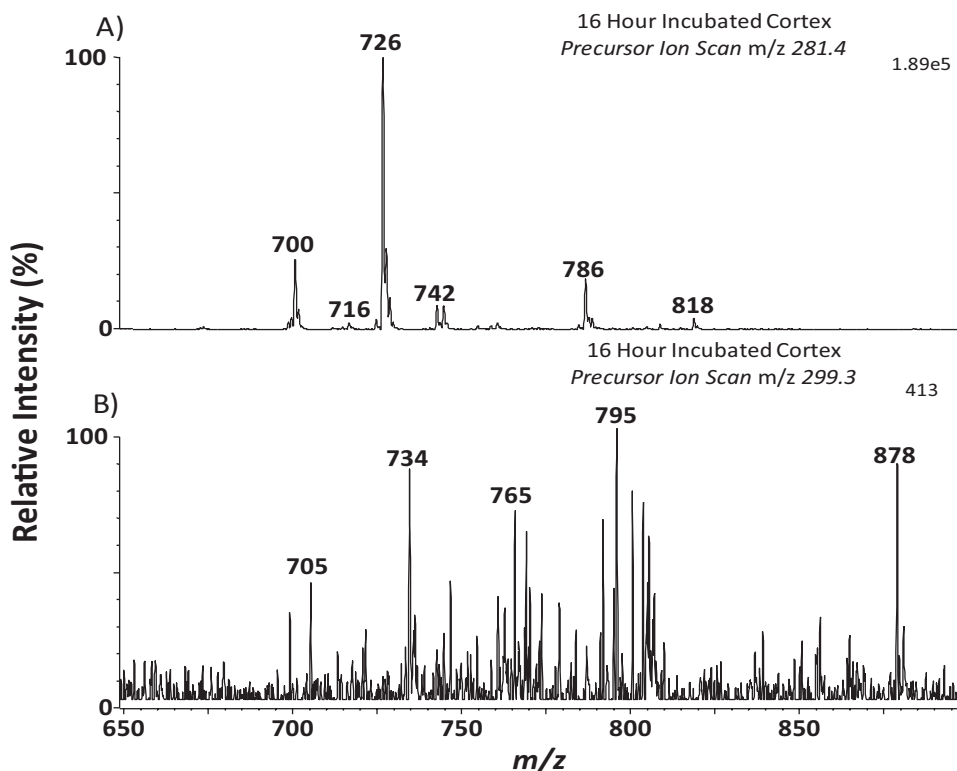
FIGURE 3. Negative ion mass spectra of control lenses and lenses incubated with <sup>13</sup>C<sub>18</sub>-oleic acid. (A) Cortex incubated with <sup>13</sup>C<sub>18</sub>-oleic acid for 16 hours, (B) control cortex, (C) nucleus incubated with <sup>13</sup>C<sub>18</sub>-oleic acid for 16 hours, (D) control nucleus. Shaded region: illustrates the position of <sup>13</sup>C<sub>18</sub>-oleic acid at *m/z* 299.5.



**FIGURE 4.** Negative ion mass spectra of lipid extracts from a control rat lens cortex (A) and a  $^{13}\text{C}_{18}$ -oleic acid-incubated rat lens cortex (B), showing the profile of acidic phospholipids present.

mass spectrometer ( $\sim 550$  picomoles/g tissue) and indicates that over the period of the experiment (16 hours) the extent of incorporation into phospholipids was low. This low level is not due to the availability of free fatty acids, because the ion abundance of free oleic acid was approximately 3 times that of free  $^{13}\text{C}_{18}$ -oleic acid (see Fig. 3), compared with approximately 500 times for phospholipids containing native versus  $^{13}\text{C}_{18}$ -

oleic acid (see Fig. 5). Broekhuysse and Bögemann<sup>16</sup> also reported incorporation of radio-labeled fatty acids into phospholipids in the outer cortex regions of the rabbit lens after 3.5 hours; however, no molecular characterization was conducted. Previous work suggests that the de novo synthesis of fiber cell membrane components (fatty acids, cholesterol, and AQP0) is restricted to the outer 3% to 6% of the lens radius.<sup>27</sup> This



**FIGURE 5.** Profile of oleic acid-containing phospholipids in a lipid extract from a rat lens cortex after incubation with  $^{13}\text{C}_{18}$ -oleic acid for 16 hours. (A) A negative precursor ion scan for  $m/z$  281.4 showing the profile of acidic phospholipids containing native oleic acid. (B) A negative precursor ion scan for  $m/z$  299.3 showing the profile of acidic phospholipids containing  $^{13}\text{C}_{18}$ -oleic acid.



localization is also likely to be the case for phospholipids and would explain the low levels of fatty acid incorporation that we observed when analyzing the whole cortex, if incorporation occurred exclusively during de novo synthesis in the very outer cortical region. This result implies that, at least over a 16-hour period, fatty acids from external sources (for example, diet), do not replace fatty acids that have already been incorporated into the membrane. These findings do not rule out replacement of membrane fatty acids occurring over longer periods, such as months or years. If this were true it would make the lens very different from other tissues as intravenously administered fatty acids are incorporated into phospholipids within 2 to 10 minutes in brain,<sup>28</sup> heart, kidney, and liver.<sup>29</sup> In vitro studies of cultured neuroblastoma cells have also demonstrated incorporation of fatty acids within 2 to 10 minutes.<sup>30</sup>

In summary, the present study adds support to the hypothesis that, once synthesized, there is no active remodeling or turnover of fiber cell phospholipids.

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