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Revealing the Role of Phosphatidylserine in Shear Stress – Mediated Protection in Endothelial Cells

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Abstract

Previous studies have demonstrated that endothelial cells exposed to laminar shear stress are protected from apoptotic stimuli such as tumor necrosis factor (TNF)- α . The authors investigated the role of phosphatidylserine (PS) in this phenomenon. Western blot analysis of cleaved caspase 3 was used as an indicator of apoptosis and revealed that in the absence of serine, cells exposed to laminar shear stress were unable to protect against TNF- α -induced apoptosis, in contrast to sheared cells grown in regular medium. It was also found that shear-induced activation of the Akt pathway was significantly decreased in cells grown without serine. In addition, quantitation of PS using a novel isotopic labeling technique involving the use of formalin revealed that stearoyl-oleic PS (18:0/18:1) did not increase during shear treatment. These findings suggest that basal levels of PS are required to activate survival pathways in endothelial cells and thereby contribute to the overall protective mechanism initiated by shear stress.

Keywords

Endothelial Cells; ESI-ToF; Mass Spectrometry; TNF- α

The vascular endothelium is constantly exposed to shear stress due to blood flow. Studies on cultured vascular endothelial cells (VECs) have revealed that critical intracellular survival pathways are activated when cells are subjected to shear stress *in vitro*. These include but are not limited to the mitogen-activated protein (MAP) kinase, Janus kinase/extracellular signal-regulated kinase (JNK/ERK), and phosphoinositide 3-kinase (PI3K)/Akt pathway (Boo et al. 2002; Yan et al. 1999).

An intriguing phenomenon exhibited by sheared endothelial cells is the ability to resist apoptosis in the presence of apoptotic stimuli. For instance, VECs that have been exposed to shear exhibit protection against tumor necrosis factor- α (TNF- α) as well as serum deprivation (Dimmeler et al. 1999; Surapisitchat et al. 2001). A number of scenarios exist for shear stress-induced protection of endothelial cells, including nitric oxide inactivation of downstream caspases (Dimmeler et al. 1999). Recently it has been reported that increases and/or accumulation of phosphatidylserine (PS) within the inner leaflet of the plasma membrane can trigger activation of the Akt pathway and counteract the effects of apoptotic stimuli (Kim et al. 2000). Traditionally, phospholipids within the plasma membrane have been thought to act as a matrix for the support and organization of different membrane proteins. However, in addition to this structural function, phospholipids have been shown to be involved in specific signaling cascades necessary for response to external stimuli (Tyurina et al. 2000). Because shear stress is known to increase membrane fluidity and possibly phospholipid composition (Barbee et al. 1994; Butler et al. 2001; Haidekker et al. 2000), we hypothesized that shear increases the amount of PS within the cell membrane and is responsible for initiation of the Akt survival pathway, thus providing protection from apoptotic stimuli. In order to test this hypothesis, we developed a method for the sensitive and accurate relative quantification of PS.

Electrospray ionization mass spectrometry has proven to be extremely useful in studying phospholipids as compared to previous ionization methods, such as fast atom bombardment (Kim et al. 1994; Pulfer et al. 2003). Several approaches have been reported that attempted to relate phospholipid signal intensity to phospholipid concentration, with the most commonly used method being incorporation of a known amount of internal standard (Han 2002; Lehmann et al. 1997; Liebisch et al. 2002). However, issues such as obtaining an appropriate stable-isotope that is well outside the molecular envelope of the phospholipid of interest can hinder the use of this method (Pulfer et al. 2003). Proteomics-based mass spectrometry experiments frequently take advantage of isotopic labeling in order to quantify relative differences between samples; however, this approach, has not been widely used for lipid analysis. Of all the phospholipids, PS and phosphoethanolamine (PE) are the only lipids that contain a primary amine group, making this functional group a natural target for labeling. Chemicals such as methyl acetimidate have been shown to selectively react with primary amines, and isotopically labeled variants of the compound have been used for relative quantification of specific metabolites (Shortreed et al. 2006). Here we report the use of ^{12}C - and ^{13}C -formalin to isotopically label both PS from extracted lipid samples as well as commercially available PS standard in order to quantify levels of PS in sheared versus nonsheared cells. The neutral nature and small size of the added moiety is such that it has only a minor effect upon the chromatographic behavior of the lipids.

MATERIALS AND METHODS

PS and Other Materials

The synthetic PS standard was obtained from Avanti Polar Lipids (Birmingham, AL). Formalin labeling reagents were purchased from Sigma (Saint Louis, MO). All solvents were of high-performance liquid chromatography (HPLC) or analytical grade and were purchased from Sigma or Honeywell Burdick and Jackson (Morristown, NJ).

Rat Endothelial Cell Culture

All animal protocols were approved by the Medical College of Wisconsin (MCW) Institutional Animal Care and Use Committee. Unless otherwise noted, all chemicals were provided by Sigma (St. Louis, MO).

Seven-week-old Sprague-Dawley rats were anesthetized with an intraperitoneal injection of pentobarbital (0.1 mL/100 g), followed by removal of skin from each hindlimb. Femoral arteries and veins were isolated, ligated, and removed. The dissected vessels were placed in warm Krebs solution (NaCl 120 mM, KCl 4.7 mM, CaCl₂ 3.0 mM, MgCl₂ 1.43 mM, NaHCO₃ 25 mM, KH₂PO₄ 1.17 mM, glucose 11 mM, and EDTA 0.03 mM) and digested in 2% collagenase type 1 in a 37°C water bath for 1 h. The digests were centrifuged at 500 × g for 10 min. The resulting pellet was resuspended in complete medium (RPMI 1640; Cellgro, Herndon, VA), 20% fetal bovine serum, 1% 100× antibiotic, .4% gentamicin (Invitrogen, Grand Island, NY) and plated. Four days following isolation, the medium was changed to modified Eagle's medium (D-valine powder with L-glutamine) (US Biological, Swampscott, MA), 20% fetal bovine serum, 1% antibiotic, .4% gentamicin to prevent possible fibroblast contamination. Cells were maintained on this medium for 1 week until returning to complete medium and grown to confluence. All cells were passaged three times prior to treatment. Cells grown in serine-free medium (modified Eagle's medium) were passaged in complete medium and after 1 day in complete medium were switched to the serine-free medium until confluent.

Shearing Protocol

Rai VECs grown in standard 100 × 20-mm cell culture plates were 85% to 90% confluent at the time of shearing. All cells were sheared in unrecirculated culture medium using a modified cone plate viscometer, as described by Sdougos et al., that has been adapted for 100 × 20-mm cell culture dishes (Rieder et al. 1997). The cone's fixed 0.5-degree angle was rotated at a constant speed to provide equal levels of shear across the plate. VECs were sheared at 10 dynes/cm² for 18 h. Shearing was performed in a standard incubator with the environment controlled at 5% CO₂, 20% O₂, and at 37°C.

Western Blotting

For detection of Akt, phosphorylated Akt, and caspase 3 cleavage, cells were lysed in RIPA buffer (200 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 M HEPES, 1 mM EDTA) plus protease inhibitors. Both adhered as well as unbound cells were collected following treatments. Cell lysates were gently sonicated on ice and centrifuged for 1 h (100,000 × g) and the supernatant containing the cytosolic fraction was recovered. Total protein was determined using a detergent-compatible assay kit provided by Bio-Rad (Hercules, CA). Proteins were loaded at 30 µg per well and were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto nitrocellulose membrane (Bio-Rad) and were blocked for at least 2 h in Tris-buffered saline (TBS), 0.3% bovine serum albumin (BSA), and 0.1 % Tween 20. Blots were then incubated with a monoclonal antibody against cleaved caspase 3 (Asp175-5A1) (no. 9664), a polyclonal antibody against Akt (no. 9272), or with a polyclonal antibody against phospho-(Ser/Thr) Akt (no. 4060), all at a 1:1000 dilution (Cell Signaling, Danvers, MA). Washed blots were then incubated with a secondary antibody conjugated to horseradish peroxidase and visualized with the use of SuperSignal West Dura chemiluminescence substrate detection system (Pierce, Rockford, IL).

Lipid Extraction

Approximately 4.0 × 10⁶ rat vascular endothelial cells were pelleted by centrifugation (800 × g, 10 min) and washed using phosphate-buffered saline. Cell pellets were then homogenized by sonication in 400 µL of mammalian protein extraction reagent (M-PER) (Pierce, Rockford, IL). Membranes were pelleted via centrifugation (100,000 × g, 1 h) and subjected to lipid extraction according to the method of Bligh and Dyer (Bligh and Dyer 1959). Briefly, the pellet was resuspended in 2 mL water 2.5 mL chloroform, and 5 mL methanol. Another 2.5 mL chloroform was added and the mixture was vortexed for 30 s. Subsequently another 2.5 mL

water was added and the mixture was vortexed for another 30 s. The chloroform layer was recovered and reduced to dryness using a vacuum centrifuge prior to formalin labeling.

Formalin Labeling of PS

Lipid extracts were resuspended in 1 mL of 80:20 methanol:water. ^{12}C -Formalin was added to a final concentration of 30 mM. Pyridine borane was also added to a final concentration of 50 mM. In addition, 2 mg of synthetic stearyl-oleic phosphatidylserine (18:0/18:1) (840039P) (Avanti Polar Lipids, Birmingham, AL) was dissolved in 1 mL of labeling solvent and was labeled with ^{13}C -formalin. The reactions went overnight at room temperature. The following day the ^{12}C mixture and the ^{13}C mixture were mixed, reduced to dryness, then resuspended in running buffer (30% aqueous ammonium formate (pH 5):55% isopropyl alcohol: 15% tetrahydrofuran).

Mass Spectrometry

Liquid chromatography was performed using an HPLC system consisting of an LC Packings Famos autosampler and UltiMate solvent pump (Dionex, Sunnyvale, CA). A C30 column (YMC Carotenoid $3\mu\text{m}$ 2.0×150 mm) (Waters, Milford, MA) was used for separation of the analytes. An isocratic separation was performed with a running buffer of 30% aqueous ammonium formate (pH5):55% isopropyl alcohol: 15% tetrahydrofuran. All solvents were HPLC grade and used as received. Column temperature was maintained at 40°C . The flow rate was $4\mu\text{L}/\text{min}$, and the LC effluent was directed to the capillary electrospray ionization source of a MicroTOF time-of-flight mass spectrometer (Bruker Daltonics, Billerica, MA). Positive-ion mode was performed using 0.4 bar N_2 as a nebulizer gas and 4.0 L/min N_2 drying gas at 200°C .

Data Analysis

Extracted ion chromatograms were created for the light and heavy isotope peaks, 818 and 820 m/z respectively. Peak detection was then performed by visual inspection to define front and back boundaries of the two isotopologues. Peak profiles were constructed from the selected ion chromatograms in which the ion intensities for both the isotopologues at each second within the chromatographic peak were plotted against each other (Pan et al. 2006). The slope of the fitted line was then used as an estimator of the abundance ratio between the sample and standard. The final abundance ratio was then normalized by the number of cells per sample. (A representative peak profile is shown in Figure 1.)

RESULTS

To determine if shear stress protection from $\text{TNF-}\alpha$ was altered in cells that could not produce phosphatidylserine de novo, cells were grown to confluency in regular complete medium or modified Eagle's medium that did not contain the amino acid serine. The cells were then subjected to following treatments: nontreated control, $\text{TNF-}\alpha$ treatment. (10 ng/mL, 12 h), shear stress exposure (10 dynes/cm², 18 h), and preexposure to shear stress (10 dynes/cm², 18 h) followed by $\text{TNF-}\alpha$ treatment (10 ng/mL, 12 h).

Figure 2 shows the results from Western blot analysis against cleaved caspase 3 as an indicator of apoptosis. There was a significant increase of cleaved caspase 3 in cells treated with $\text{TNF-}\alpha$ from cells grown in both the regular medium and serine-free medium, confirming that $\text{TNF-}\alpha$ treatment results in apoptosis regardless of cellular medium. Shear stress treatment alone did not result in increased amounts of cleaved caspase 3 regardless of medium. However, when cells were exposed to shear stress prior to $\text{TNF-}\alpha$ treatment, levels of cleaved caspase 3 remained low in regular medium, unlike the significant increase in cleaved caspase 3 observed in cells grown in serine-free medium.

To test whether shear stress activation of Akt was attenuated in endothelial cells grown in serine-free medium, Western blot analysis was performed against total Akt and phosphorylated Akt in the following groups: nontreated control cells, cells exposed to shear stress (10 dynes/cm², 1 h), cells grown in serine-free medium, and cells grown in serine-free medium and exposed to shear stress (10 dynes/cm², 1 h).

As shown in Figure 3, endothelial cells grown in regular medium exposed to 1 h of shear stress had a significantly higher P-Akt/Akt ratio compared to nonsheared cells grown in regular medium. However, when cells grown in serine-free medium were exposed to shear for 1 h, an increase in the P-Akt/Akt ratio was not observed, suggesting that serine is required for shear stress-mediated protection in endothelial cells.

In order to test the hypothesis that PS was increased in sheared endothelial cell membranes compared to nonsheared cells, an isotopic labeling strategy was implemented for the analysis and quantification of PS using electrospray ionization time-of-flight (ESI-ToF) mass spectrometry.

To validate the technique and to show that the loss of protection in cells grown in serine-free medium is due to PS depletion, lipids were extracted from endothelial cells grown in serine-free medium as well as from cells grown in regular medium containing serine. Figure 4 shows that the amount of labeled PS from endothelial cells grown in serine-free medium was significantly lower than labeled PS from cells grown in regular medium; however, an increase in PS extracted from cells exposed to shear stress (10 dynes/cm², 18 h), compared to the nontreated control group, was not observed.

DISCUSSION

The overall findings of this study indicate that in the absence of extracellular serine, shear stress-mediated protection from the apoptotic effects of TNF- α is lost. Furthermore, cells grown in serine-free medium have a reduction in the amount of activated Akt when exposed to shear stress compared to sheared cells grown in regular medium. Together these results suggest that PS in the membrane of endothelial cells may contribute to shear stress protection. Further it appears that basal levels of PS may be sufficient for shear stress-mediated protection from TNF- α because no increase in PS was observed in sheared cells compared to nonsheared cells.

It is widely accepted that exogenous serine incorporates into phospholipids by base exchange with phosphatidylethanolamine in order to convert to PS (Xu et al. 1991). Kennedy and colleagues demonstrated that de novo synthesis of PS via the Kennedy pathway (Kennedy 1956) takes place in a subfraction of the endoplasmic reticulum termed the mitochondria-associated membrane (MAM), where two enzymes are responsible for performing base-exchange reactions with other phospholipids (Kuge 1989). More recently, Xu and colleagues were able to show that radiolabeled exogenous serine translocates into the cell and to the mitochondria where this reaction takes place. In addition they demonstrated total incorporation of the labeled serine into the plasma membrane within 48 h (Xu et al. 1991).

Although PS is an essential phospholipid for the growth of mammalian cells, it comprises only ~10% of the plasma membrane (Kuge et al. 1998). It is unique in that it is exclusively sequestered in the inner leaflet of the plasma membrane. Reno and colleagues demonstrated that this asymmetry is disturbed during onset of apoptosis as PS translocates to the outer leaflet of the plasma membrane (Reno et al. 1998). Furthermore, Kim and colleagues suggested a mechanism in which accumulation of PS activates the Akt survival pathway and protects against serum starvation-induced apoptosis in neuronal cells (Akbar et al. 2005). Together these data imply that the translocation of PS from the inner to the outer membrane may not be

a result of apoptosis, but may contribute to this process by decreasing the activation of survival pathways in the cell.

PS is only one of many components that comprise the lipid membrane in endothelial cells. Furthermore, there exist 31 different subspecies of PS with stearoyl-oleoyl-phosphatidylserine (SOPS), a PS species that contains a fatty acid chain containing an 18-saturated carbon chain and a fatty acid containing 18 carbons with 1 double bond, 18:0/18:1 being the most abundant (Takamura et al. 1990). Because SOPS is the most abundant subspecies of PS in endothelial cells, it was chosen for measurement. In the current study, lipid extractions from endothelial cells were treated with ^{12}C -formalin and commercially available SOPS (2 mg/mL) was treated with ^{13}C -formalin prior to LC/MS analysis. The major advantage of using formalin isotopes for PS quantification is that formalin reacts with amino groups, a functional group only found on PS and phosphatidylethanolamine (PE), thus adding specificity to the labeling scheme. The molecular weight of SOPS is 790 daltons. Thus a doublet of peaks was identified at 818 and 820 daltons for the light-labeled and heavy-labeled PS, respectively.

There are several possible explanations for the observation that an increase in PS was not observed in sheared cells. Although SOPS is the most abundant species of PS within endothelial cells, it still only accounts for 18% of the total amount of PS (Takamura et al. 1990). Therefore, increases in total PS could be due to an increase in a different subspecies and not necessarily SOPS. Alternatively, it is reasonable to hypothesize that a threshold amount of PS is sufficient to activate survival pathways in the presence of shear stress and that when PS levels fall below this critical amount, no activation can occur. The observation that serine depletion inhibited the protective effect while reducing PS levels is compatible with this hypothesis.

Despite not observing an increase in SOPS, cells grown in the absence of serine showed SOPS depletion and a loss of shear stress-induced protection from $\text{TNF-}\alpha$ stimulation. One possible explanation for this could be that the loss of protection, measured as an increase in cleaved caspase 3 during $\text{TNF-}\alpha$ stimulation, is due to an overall reduction in cell integrity when serine is not available. However, increases in cleaved caspase 3 (apoptosis) were not observed in nontreated cells grown in serine-free medium, suggesting loss of protection is a direct result of disruption of the PS signaling pathway.

Phospholipids provide not only structure to the cell, but engage in cell signaling activities. These lipids are commonly translocated within the plasma membrane due to numerous stimuli such as shear stress (Dimmeler et al. 1998). In fact, studies measuring membrane fluidity using fluorescent probes have indicated that an increase in fluidity due to shear stress occurs within 5 s of the onset of flow (Haidekker et al. 2000). This remarkably quick alteration in the plasma membrane coincides with the hypothesis that in response to shear stress, the membrane activates the Akt pathway, which has been shown to activate endothelial nitric oxide synthase (eNOS) (Fleming et al. 2005), thus increasing amounts of nitric oxide within the cell, and ultimately protecting the cell by nitrosylating caspases, key mediators of apoptosis.

The elevation in P-Akt:Akt was significantly attenuated in sheared cells grown in serine-free medium in the apparent absence of an increase in SOPS. Dimmeler and colleagues concluded that shear-induced Akt activation occurs in a time-dependent manner with maximum response at 1 hr of exposure to laminar shear stress. In addition, significant suppression of this response was seen through the use of the PI3K inhibitor wortmannin, suggesting that shear stress-mediated activation of Akt is through PI3K signaling (Hu and Chien 1997). Although it is well accepted that shear stress initiates activation of the survival Akt pathway, the mechanism by which this occurs is poorly understood. Recently, Fleming et al. have suggested that phosphorylation of platelet endothelial cell adhesion molecule-1 (PECAM-1), found on the surface of endothelial cells, may be responsible for Akt activation during exposure to shear

stress (Idris and Donnelly 2001). Our data support the hypothesis that suppression of Akt activation in sheared cells may be due to decreased levels of serine and that these low levels inhibit an unknown protective mechanism. Although this mechanism is yet to be defined, previous evidence supports a role for protein kinase C (PKC) in phosphatidylserine-dependent shear stress-induced activation of Akt, Hu and Chien demonstrated that PKC, more specifically the PKC β isoform, was not only significantly increased in sheared endothelial cells, but this increase was predominantly seen in the cortical region of the cell near the plasma membrane (McIntire et al. 1987). In addition, Idris et al. have revealed that all known isoforms of PKC contain a PS binding site in their conserved regulatory domains and conventional forms of PKC (PKC α , PKC β , and PKC γ) require calcium as well as phosphatidylserine for proper activation (Dimmeler et al. 1999). Together these data suggest that PS is required for shear stress-mediated Akt activation through a mechanism involving PKC.

In conclusion, the present study demonstrated that PS plays a role in shear stress-mediated protection from TNF- α in endothelial cells. By implementing a novel isotopic labeling strategy, which allowed for relative quantification using ESI-ToF, it was demonstrated that the presence of PS is critical for shear stress-mediated protection as well as activation of the Akt survival pathway.

References

- Akbar M, Calderon F, Wen Z, Kim HY. Proceedings of the National Academy of Sciences of the United States of America 2005;102:10858–10863. [PubMed: 16040805]
- Barbee KA, Davics PF, Lal R. Circulation Research 1994;74(1):163–171. [PubMed: 8261591]
- Bligh EG, Dyer WJ. Canadian Journal of Biochemistry and Physiology 1959;37:911–917. [PubMed: 13671378]
- Boo YC, Sorescu G, Boyd N, Shiojima I, Walsh K, Du J, Jo H. Journal of Biological Chemistry 2002;277:3388–3396. [PubMed: 11729190]
- Butler PJ, Norwich G, Weinbaum S, Chien S. American Journal of Physiology: Cellular Physiology 2001;280:C962–C969.
- Dimmeler S, Assmus B, Hermann C, Haendeler J, Zeiher AM. Circulation Research 1998;83(3):334–341. [PubMed: 9710127]
- Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Nature 1999;399:601–605. [PubMed: 10376603]
- Dimmeler S, Hermann C, Galle J, Zeiher AM. Arteriosclerosis, Thrombosis, and Vascular Biology 1999;19:656–664.
- Fleming I, Mohamed A, Galle J, Turchanowa L, Brandes RP, Fisslthaler B, Busse R. Cardiovascular Research 2005;65(4):897–906. [PubMed: 15721870]
- Haidekker MA, L'Heureux N, Frangos JA. American Journal of Physiology: Heart and Circulatory Physiology 2000;278:H1401–H1406. [PubMed: 10749738]
- Han X. Analytical Biochemistry 2002;302:199–212. [PubMed: 11878798]
- Hu YL, Chien S. Journal of Histochemistry and Cytochemistry 1997;45:237–249. [PubMed: 9016313]
- Idris I, Gray S, Donnelly R. Diabetologia 2001;44(6):659–673. [PubMed: 11440359]
- Kennedy EP. Canadian Journal of Biochemistry and Physiology 1956;34:334–348. [PubMed: 13304754]
- Kim HY, Hamilton J. Lipids 2000;35(2):187–195. [PubMed: 10757550]
- Kim HY, Wang TC, Ma YC. Analytical Chemistry 1994;66:3977–3982. [PubMed: 7810900]
- Kuge O. Seikagaku 1989;61:1259–1264. [PubMed: 2614158]
- Kuge O, Hasegawa K, Saito K, Nishijima M. Proceedings of the National Academy of Sciences of the United States of America 1998;95:4199–4203. [PubMed: 9539713]
- Lehmann WD, Koester M, Erben G, Keppler D. Analytical Biochemistry 1997;246:102–110. [PubMed: 9056189]

- Liebisch G, Drobnik W, Lieser B, Schmitz G. *Clinical Chemistry* 2002;48:2217–2224. [PubMed: 12446479]
- Melntire LV, Frangos JA, Rhec BC, Eskin SG, Hall ER. *Annals of the New York Academy of Sciences* 1987;516:513–524. [PubMed: 3439746]
- Pan C, Kora G, Tabb DL, Pelletier DA, McDonald WH, Hurst GB, Hettich RL, Samatova NF. *Analytical Chemistry* 2006;78:7110–7120. [PubMed: 17037910]
- Pulfer M, Murphy RC. *Mass Spectrometry Reviews* 2003;22:332–364. [PubMed: 12949918]
- Reno F, Burattini S, Rossi S, Luchetti F, Columbaro M, Santi S, Papa S, Falcieri E. *Histochemistry and Cell Biology* 1998;110(5):467–476. [PubMed: 9826126]
- Rieder MJ, Carmona R, Krieger JE, Pritchard KA Jr, Greene AS. *Circulation Research* 1997;80:312–319. [PubMed: 9048650]
- Shortreed MR, Lamos SM, Frey BL, Phillips MF, Patel M, Belshaw PJ, Smith LM. *Analytical Chemistry* 2006;78:6398–6403. [PubMed: 16970314]
- Surapisitchat J, Hoefen RJ, Pi X, Yoshizumi M, Yan C, Berk BC. *Proceedings of the National Academy of Sciences of the United States of America* 2001;98:6476–6481. [PubMed: 11353829]
- Takamura H, Kasai H, Arita H, Kito M. *Journal of Lipid Research* 1990;31:709–717. [PubMed: 2351875]
- Tyurina YY, Shvedova AA, Kawai K, Tyurin VA, Kommineni C, Quinn PJ, Schor NF, Fabisiak JP, Kagan VE. *Toxicology* 2000;148:93–101. [PubMed: 10962127]
- Xu ZL, Byers DM, Palmer FB, Spence MW, Cook HW. *Journal of Biological Chemistry* 1991;266(4):2143–2150. [PubMed: 1899236]
- Yan C, Takahashi M, Okuda M, Lee JD, Berk BC. *Journal of Biological Chemistry* 1999;274:143–150. [PubMed: 9867822]

FIG. 1.

Representative peak profile plot of the two isotopologues. Ion intensities taken at time intervals of 1 s within the chromatographic peaks of both the heavy and light isotope are plotted against each other. The slope of the best-fit line represents the abundance ratio.

FIG. 2.

Quantitative densitometry of five separate experiments comparing cleaved caspase 3 in nontreated control cells, cells treated with 10 ng/mL of TNF- α for 12 h, cells exposed to shear for 18 h (10 dynes/cm²), and cells first exposed to shear (10 dynes/cm² for 18 h) then treated with 10 ng/mL of TNF- α for 12 h, grown in either regular or serine-free medium ($n = 5$).

*Significant difference versus no treatment group, $p < .05$.

FIG. 3.

Quantitative densitometry of six separate experiments comparing the ratio of phosphorylated Akt to nonphosphorylated Akt in nontreated control cells, cells exposed to shear stress (10 dynes/cm², 1 h), cells grown in serine-free medium, and cells exposed to shear stress that were grown in serine-free medium.

*Significant difference versus no treatment group, $p < .05$.

FIG. 4.

Comparison of PS levels (abundance ratio) in nontreated control cells, cells grown in serine-free medium, and sheared cells ($n=3$). *Significant difference versus no treatment group, $p < .05$.