Akt2, but not Akt1 or Akt3 Mediates Pressure-Stimulated Serum-Opsonized Latex Bead Phagocytosis Through Activating mTOR and p70 S6 Kinase

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Abstract   Monocytes and macrophages play critical roles in innate host defense and are sensitive to mechanical stimuli. Tissue pressure is often altered in association with inflammation or infection. Low pressure (20 mmHg), equivalent to normal tissue pressure, increases phagocytosis by primary monocytes and PMA-differentiated THP-1 macrophages, in part by FAK and ERK inhibition and p38 activation. PI-3K is required for macrophage phagocytosis, but whether PI-3K mediates pressure-stimulated phagocytosis is not known. Furthermore, little is known about the role played by the PI-3K downstream Kinases, Akt, and p70 S6 kinase (p70S6K) in modulating macrophage phagocytosis. Thus, we studied the contribution of PI-3K, Akt, and p70S6K to pressure-increased serum-opsonized bead phagocytosis. Pressure-induced p85 PI-3K translocation from cytosolic to membrane fractions and increased Akt activation by $36.1 \pm 12.0\%$ in THP-1 macrophages. LY294002 or Akt inhibitor IV abrogated pressure-stimulated but not basal phagocytosis. Basal Akt activation was inhibited 90% by LY294002 and 70% by Akt inhibitor IV. Each inhibitor prevented Akt activation by pressure. SiRNA targeted to Akt1, Akt2, or Akt3 reduced Akt1, Akt2, and Akt3 expression by 50%, 45%, and 40%, respectively. However, only Akt2SiRNA abrogated the pressure-stimulated phagocytosis without affecting basal. Pressure also activated mTOR and p70S6K. mTORSiRNA and p70S6K inhibition by rapamycin or p70S6KSiRNA blocked pressure-induced, but not basal, phagocytosis. Changes in tissue pressure during inflammation may regulate macrophage phagocytosis by activation of PI-3K, which activates Akt2, mTOR, and p70S6K. J. Cell. Biochem. 102: 353–367, 2007.   © 2007 Wiley-Liss, Inc.

Key words: mechanical stimuli; Akt; p70S6K; monocyte/macrophages; PI-3K; mTOR

Monocytes and macrophages play critical roles in innate host defense mechanisms and are recruited to sites of infection or inflammation. Mechanical stimuli such as pressure modulate cell morphology and function in other cell types [Basson et al., 2000; Li and Xu, 2000; Thamilselvan and Basson, 2004]. We have previously demonstrated that low extracellular pressure (20 mmHg), equivalent to normal

interstitial tissue pressure, increases phagocytosis by primary human monocytes and macrophages and by PMA-differentiated human monocytic THP-1 cells, in part by FAK and ERK inhibition and p38 activation [Shiratsuchi and Basson, 2004, 2005]. PI-3K regulates actin reorganization and Fc-receptor-mediated phagocytosis in macrophages [Defilippi et al., 1999; May and Machesky, 2001; Stephens et al., 2002; Aderem, 2003; Allen et al., 2005]. However, the contribution of PI-3K and its downstream kinases to pressure-induced macrophage phagocytosis is not known. We hypothesized that changes in extracellular pressure such as those that occur during inflammation or infection might modulate PI-3K and related downstream kinases such as Akt and ribosomal enzyme p70 S6 kinase (p70S6K) and that such changes might contribute to the regulation of macrophage phagocytosis by extracellular pressure.

Normal interstitial tissue pressure is 20–30 mmHg in humans [Guyton et al., 1971;

Aukland and Nicolaysen, 1981; Aukland and Reed, 1993; Mehik et al., 2003]. This interstitial pressure is often altered in association with inflammation or infection. Although interstitial tissue pressure has been reported to decrease by 1–150 mmHg in the setting of infection or inflammation [Guyton et al., 1971; Koller et al., 1993], tissue pressure in closed compartments during inflammation actually increases [Schnall et al., 1996; Bertram et al., 1997; Mehik et al., 2003; Schaser et al., 2003]. In addition, tissue pressures within solid tumors are elevated compared to normal tissue pressures [Zachos et al., 2001; Heldin et al., 2004; Diresta et al., 2005]. Thus, macrophages infiltrating into tumors might be exposed to such intratumoral-elevated pressure.

Recent reports suggest that physical forces such as extracellular pressure and repetitive strain may alter other aspects of macrophage functions. Pressure (40–130 mmHg) dose-dependently increases monocyte migration [Singhal et al., 1997] and enhances scavenger receptor expression in macrophages [Sakamoto et al., 2001]. Constant low extracellular pressure (20 mmHg) stimulates spontaneous TNF-α production in macrophages, but inhibits LPS-induced IL-1β production [Shiratsuch and Basson, 2005]. Macrophage pro-inflammatory cytokine production is also promoted in response to very high cyclic pressure ($\approx 1,000$ mmHg), and the combination of cyclic strain and 75 mmHg pressure, or high pressure (over 70 mmHg) and endotoxin [Yang et al., 2000; McEvoy et al., 2002]. Increased pressure (40–90 mmHg), but not cyclic strain, has also been shown to increase the uptake of aggregated IgG by the mouse macrophage J774.16 cell line [Mattana et al., 1996]. The mechanisms or intracellular signals responsible for this effect have not been described.

PI-3Ks are activated in response to stress stimuli including stimulation with growth factors or neurotransmitters, osmotic conditions, and hydrostatic pressure in various cell types [Selvaraj et al., 2003; Kopakkala-Tani et al., 2004; Salvador-Silva et al., 2004; Wilkes et al., 2005], as well as by the crosslinking of integrins, also important cell-surface receptors for macrophage phagocytosis [May and Machesky, 2001; Aderem, 2003]. Phagocytosis of foreign bodies in macrophages requires PI-3K activation [Garcia-Garcia et al., 2002; Stephens et al., 2002; Aderem, 2003; Fenteany and

Glogauer, 2004] and PI-3K inhibitors blocks bacterial phagocytosis and engulfment [Allen et al., 2005]. PI-3K activation is required for macrophage-stimulating protein activated, but not basal, complement receptor (CR)-mediated phagocytosis and oridonin-enhanced phagocytosis in human macrophage-like U937 cells [Lutz and Correll, 2003; Liu et al., 2005]. Although PI-3K is well documented to play a critical role in macrophage phagocytosis through receptor activation [May and Machesky, 2001; Stephens et al., 2002; Aderem, 2003; Fenteany and Glogauer, 2004], its role in the macrophage phagocytosis response to extracellular pressure changes has not been explored.

Several signal proteins are activated downstream of PI-3K, but two important ones are Akt and p70S6K. Akt plays an essential role in cell survival and mobility [Datta et al., 1999; Pastukh et al., 2005; Song et al., 2005]. Three isoforms of Akt (Akt1, Akt2, and Akt3) have been identified with distinct functions and substrates [Datta et al., 1999; Testa and Tsichlis, 2005]. p70S6K is a downstream kinase of Akt which plays a prominent role in cell growth and G1 cell cycle progression and regulates the translation of mRNA containing a polypyrimidine tract [Chou and Blenis, 1995; Pullen and Thomas, 1997; Kawasome et al., 1998]. The roles of Akt and p70S6K in regulating macrophage phagocytosis in response to extracellular pressure have not been explored. In this study, we examined the contribution of PI-3K and its downstream kinases Akt and p70S6K to the stimulation of macrophage phagocytosis by extracellular pressure.

MATERIALS AND METHODS

Antibodies and Chemicals

Antibodies against total-Akt, phospho-Akt, total-Akt1, total-Akt2, phospho-p70S6K, and phospho-mTOR were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Antibody against total-p70S6K was obtained from Upstate USA, Inc. (Charlottesville, VA). Fluorescent-labeled latex beads (2.0 μ) were obtained from Polysciences, Inc. (Warrington, PA). LY294002, a specific PI-3K inhibitor, Akt inhibitor IV (cell-permeable benzimidazole compound) and rapamycin were obtained from Calbiochem (Santa Cruz, CA).

Cells and Cell Cultures

The human monocytic cell line THP-1 was obtained from the American Type Culture Collection (Rockville, MD), and maintained in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO), L-glutamine (200 mM), and 2-mercapto-ethanol (5×10^{-5} M) (Sigma). THP-1 cells (5×10^5 cells per 35 mm dish) were differentiated by stimulation with PMA (50 ng/ml, final concentration) for 3 days to obtain a macrophage-like phenotype which closely resembles human monocyte-derived macrophages as previously reported [Auwerx, 1991].

Peripheral venous blood was obtained from healthy volunteers with consent approved by Wayne State University School of Medicine and John D Dingell VA Medical center. Peripheral monocyte monolayers were prepared as described previously [Shiratsuchi and Basson, 2005].

Assay for Phagocytosis

Fluorescent labeled latex beads (2.0 μ) were opsonized with 10% unheated FBS for 60 min at 37°C prior to the experiments. PMA-differentiated THP-1 (1×10^6) in 1 ml of tissue culture medium supplemented with 10% FBS in a 35 mm Petri dish were mixed with opsonized latex beads at a multiplicity ratio of 1:5, and incubated for 2 h at 37°C. One set of dishes was placed in a calibrated pressure box described previously [Shiratsuchi and Basson, 2004, 2005]. The pressure inside the pressure box was set at 20 mmHg, and the pressure box was placed inside an incubator at 37°C and monitored every 15 min. The second set of dishes was placed inside the same incubator but under ambient pressure conditions. After 2 h, macrophage monolayers were washed vigorously with phosphate buffered saline (PBS) to remove extracellular beads, fixed with methanol for 10 min, and then counterstained with Methylene Blue. The number of intracellular latex particles was determined by counting fluorescent beads within cells under a fluorescence microscope. Data were expressed as percent phagocytosis, calculated by the total number of cells with at least one bead as a percentage of the total number of cells counted.

PI-3K translocation. PI-3K translocation assay was performed using a slightly modifica-tion of the method described by Li et al. [1999]. Briefly, THP-1 macrophages exposed to ambient or increased pressure for 30 min were rinsed with ice-cold PBS and lysed with lysis buffer A (50 mM HEPES, 50 mM NaCl, 1 mM MgCl$_2$, 5 mM NaF, 2 mM EDTA (pH 7.4), 1 mM PMSF, 1 mM DTT, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Lysates were centrifuged at 42,000 rpm for 60 min at 4°C to separate cytosol and membrane enriched fractions. The pellet (membrane) was re-suspended in 1% Triton-X-100 in lysis buffer A and centrifuged at 12,000 rpm for 10 min to remove nuclei. Each sample was subjected to Western immunoblotting using anti-PI-3K p85 antibody to visualize the protein.

Western Blotting

THP-1 macrophages were incubated under ambient or increased pressure (20 mmHg) conditions for 30 min, rinsed once with cold PBS and lysed with lysis buffer B (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM Na pyrophosphate, 50 mM NaF, 10% glycerol, 1% Triton-X-100, 1% deoxycholic acid (pH 7.6) supplemented with 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin). Protein concentrations in cell lysates were measured using the bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein were loaded in each lane. Cell lysates were resolved under reducing conditions by 10% SDS—PAGE and then transferred to nitrocellulose membranes. After being blocked with 5% bovine serum albumin in tris-buffered saline (TBS) with 0.1% Tween-20, the membranes were incubated with antibodies against activated or total forms of protein overnight at 4°C, washed three times with 0.1% Tween-20-TBS and then incubated for 60 min with 2,000:1 peroxidase-conjugated anti-rabbit IgG. The membrane-bound peroxidase activity was detected using ECL Plus Western Blotting Detection kits (Amersham, Arlington Heights, IL). Chemiluminescent images were captured and analyzed using a Kodak Digital Science Image Station 440CF. All blots were studied within the linear range of exposure.

Transfection of siRNA

To inhibit Akt1, Akt2, or p70S6K protein expression, THP-1 cells were transfected with small interfering RNA (SiRNA). The SiRNA

targeted to Akt1, Akt2, and Akt3 were purchased from Dharmacon (Lafayette, CO). The SiRNA duplex targeted to human p70S6K, 5′-GCCGTGTTTGATTTGGATTTGdTdT/dTd-TCGGCACAAACTAAACCTAAAC-5′, directed toward the mRNA target, 5′-GCCGTGTTT-GATTTGGATTTG-3′ was designed using Deqor, a web-based tool for the design and quality control of SiRNAs and synthesized by Dharmacon. Non-targeted SiRNA duplex (NT1) used as a control was purchased from Dharmacon. THP-1 cells (4×10^5 cells per 60 mm Petri dish) were stimulated with PMA (50 ng/ml) for 2 days before transfection. Transfection of duplex SiRNAs were performed using Oligo-fectamine (Gibco, Gaitherburg, MD) according to the manufacturer's protocol. Twenty-four to 48 h after transfection, cells were used for Western analysis and for assays of phagocytosis. Transfection efficacy, measured by trans-fecting Cy3 conjugated SiRNA targeted to luciferase (Upstate USA, Inc.), was 90–97% [Shiratsuchi and Basson, 2004].

RESULTS

Potential Role of PI-3K

Since PI-3K is translocated from the cytosol to the membrane when activated, we assessed PI-3K translocation in THP-1 macrophages exposed to extracellular pressure. Pressure-induced PI-3K translocation from a cytosolic to a membrane fraction (Fig. 1A). The relative enrichment of these fractions is demonstrated by the parallel blot for Na-K ATPase. Conver-sely, PI-3K inhibition by pretreatment with the PI-3K inhibitor LY294002 (40 μM) for 60 min prior to exposure to pressure significantly reduced pressure-induced phagocytosis by primary human monocytes compared to the effect of pressure on monocytes treated with a DMSO vehicle control (Fig. 1B). Basal phago-cytosis was not altered by LY294002. Pressure also increased THP-1 macrophage phagocytosis as reported previously [Shiratsuchi and Basson, 2004, 2005] and LY294002 inhibited pressure-induced phagocytosis, but did not alter basal phagocytosis (Fig. 1C), similar to observations in primary human monocytes.

Potential Role of Akt

Exposure to pressure increased Akt phos-phorylation. Pretreatment with either the PI-3K inhibitor LY294002 or Akt inhibitor IV inhibited basal Akt phosphorylation by $89.8 \pm 1.7\%$ ($P < 0.001$, n = 9) or $74.3 \pm 6.2\%$ ($P < 0.001$, n = 7), respectively (Fig. 2). Each inhibitor prevented the enhancement of Akt phosphorylation by pressure (Fig. 2A,B). Pres-sure-stimulated phagocytosis by primary mono-cytes was blocked by pretreatment with Akt inhibitor IV, although Akt inhibitor IV reduced basal phagocytosis (Fig. 2C). Like LY294002, Akt inhibitor IV pretreatment did not affect basal phagocytosis, but completely abrogated pressure-stimulated phagocytosis by THP-1 macrophages (Fig. 2D).

To further examine the contribution of Akt activation by pressure to pressure-induced phagocytosis, Akt expression was inhibited using SiRNA targeted to Akt. Three Akt iso-forms have been identified with distinct sub-strates and functions [Dufour et al., 2004; Gonzalez et al., 2004; Irie et al., 2005]. Akt1 and Akt2 are expressed in most cell types, while Akt3 is expressed at lower levels in most tissues [Zinda et al., 2001; Song et al., 2005]. Isozyme-targeted Akt SiRNA was transfected into THP-1 macrophages as described previously [Shirat-suchi and Basson, 2004, 2005]. Transfection with Akt1 SiRNA reduced Akt1 protein levels by 50%, while reducing Akt2 levels by only 15% (Fig. 3A, left). Conversely, Akt2 SiRNA did not change total Akt1 appreciably, but reduced Akt2 protein by 45% compared to cells trans-fected with non-targeted (NT1) SiRNA (Fig. 3A, right). Akt3 SiRNA transfection reduced Akt3 protein expression 40% (Fig. 3B). Transfection with Akt1 SiRNA (Fig. 3C) or Akt3 SiRNA (Fig. 3D) affected neither basal nor pressure-stimulated THP-1 macrophage phagocytosis compared to cells transfected with NT1 SiRNA. In contrast, Akt2 SiRNA transfection blocked pressure-stimulated THP-1 macrophage pha-gocytosis, without affecting basal phagocytosis (Fig. 3C).

Potential Role of p70 S6 Kinase

We next explored the potential contribution of p70S6. This kinase is downstream of Akt and has several phosphorylation sites. p70S6K Thr389 phosphorylation most closely correlates with p70S6K activity and is sensitive to rapa-mycin. We examined the effect of pressure on p70S6K activation in primary monocytes. Expo-sure to pressure significantly enhanced phos-phorylation of 70S6K Thr389 by 40% ($P < 0.05$,
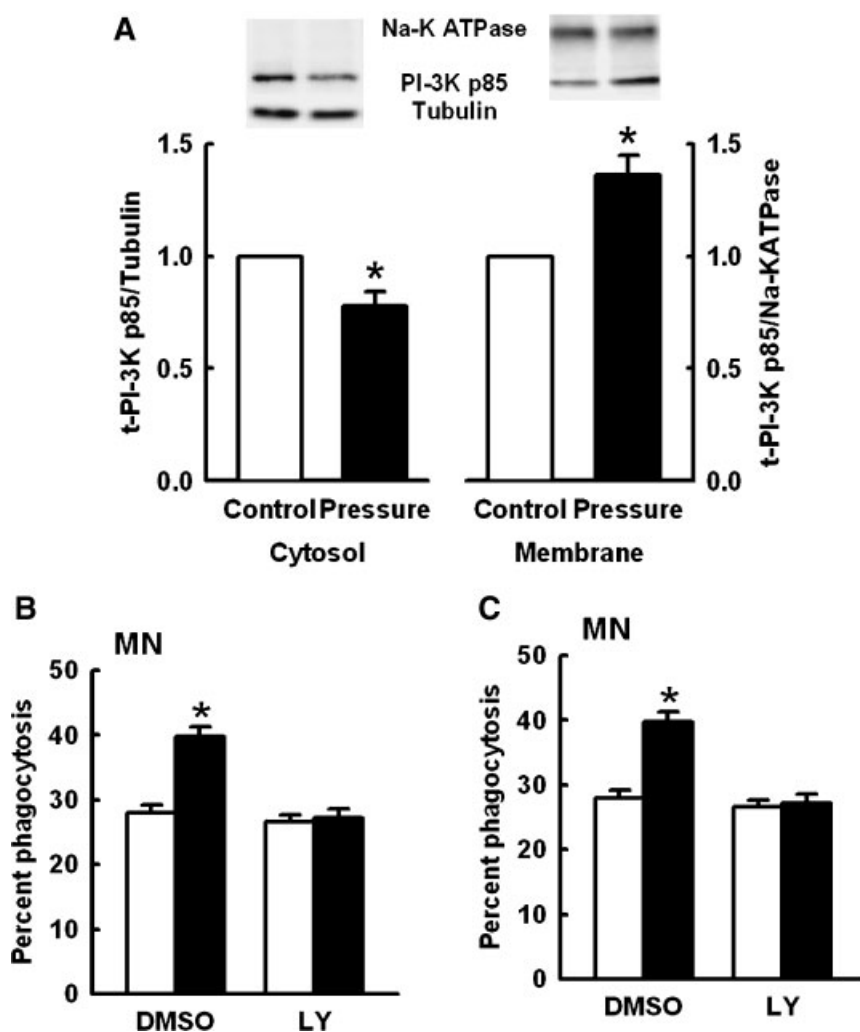
Fig. 1. Studies of PI-3K and pressure. **A**: Translocation of PI-3K p85 by pressure. The top panel represents a typical Western blot for PI-3K p85, and Na-K ATPase and tubulin as loading controls. Relative enrichment of these fractions is demonstrated by the parallel blot for Na-K ATPase. The graph summarizes densitometric results expressed as mean $\pm$ SE of the ratio of PI-3K p85 to tubulin or Na-K ATPase, normalized to ambient pressure control. Pressure decreased cytosolic (left) (*$P < 0.05$, n = 5, paired t-test) and increased membrane PI-3K p85 expression (right) (#$P < 0.01$, n = 5, paired t-test). **B**: Effect of LY294002 on primary monocyte (MN) phagocytosis under ambient and increased pressure conditions. Primary monocytes were incubated with serum-opsonized fluorescent-labeled latex beads (5 beads/cell) under ambient pressure, or 20 mmHg increased pressure conditions for 2 h. Cells were then washed with PBS, and fixed. The number of intracellular latex beads was counted using a fluorescent microscope. Results are expressed as mean $\pm$ SE of percent phagocytosis in monocytes pretreated with LY294002 (40 μM) or DMSO vehicle. Open bars represent ambient pressure and closed bars represent increased pressure. Pressure increased monocyte phagocytosis (*$P < 0.001$, n = 3, paired t-test). Pretreatment with LY294002 blocked pressure-stimulated phagocytosis without affecting basal phagocytosis. **C**: Effect of LY294002 (LY) on THP-1 macrophage phagocytosis under ambient and increased pressure conditions. Results are expressed as mean $\pm$ SE of percent phagocytosis in THP-1 macrophages pretreated with LY294002 (40 μM) or DMSO vehicle. Pressure increased THP-1 macrophage phagocytosis (*$P < 0.05$, n = 5, paired t-test). LY294002 prevented pressure-induced phagocytosis but did not affect basal phagocytosis.

n = 5) and p70S6K Thr421/Ser424 by 30% ($P < 0.05$, n = 4) (Fig. 4A). p70S6K phosphorylation at Thr389 in THP-1 macrophages was statistically significantly increased by pressure (n = 10, $P < 0.05$) and inhibited 70% by Akt inhibitor IV compared to vehicle-treated cells (Fig. 4B). Rapamycin, a specific p70S6K inhibitor, significantly inhibited basal p70S6K Thr389 phosphorylation 78% but not basal p70S6K Thr421/Ser424 phosphorylation. Rapamycin prevented the augmentation of either p70S6K Thr389 or p70S6K Thr421/Ser424 phosphorylation by pressure (Fig. 4B). Similar to Akt inhibitor IV, rapamycin (100 nM) pretreatment
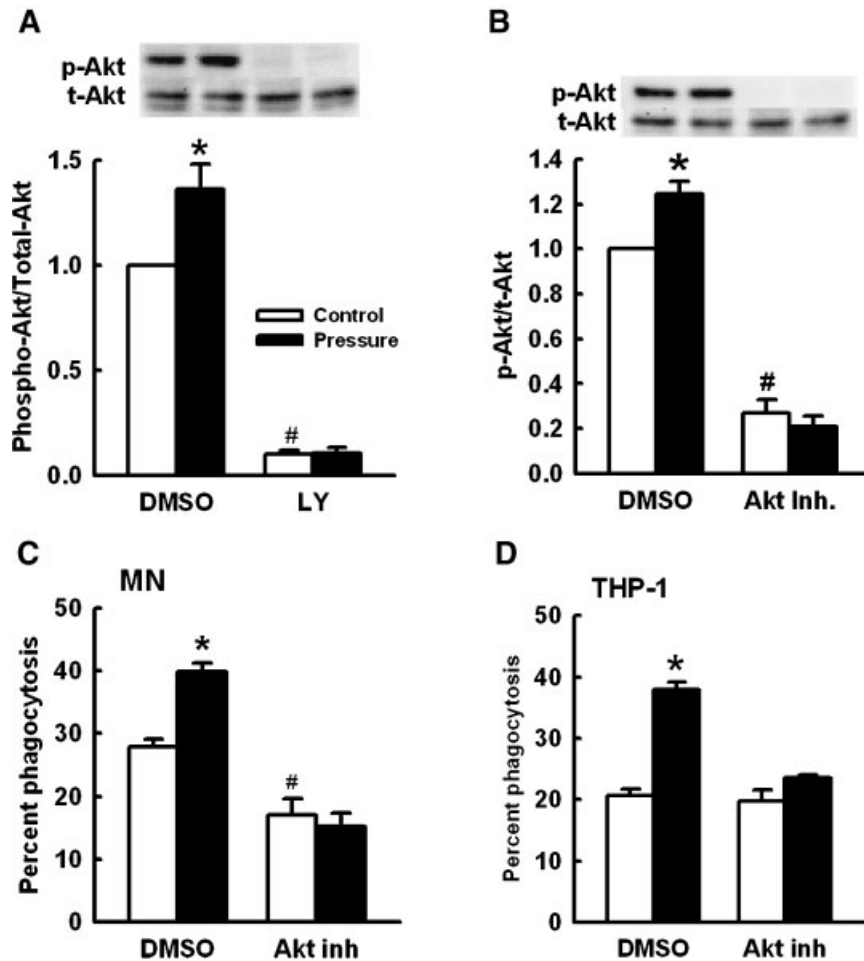
Fig. 2. Studies of Akt and pressure. **A**,**B**: THP-1 macrophages were pretreated with or without LY294002 (LY) (A) or Akt inhibitor IV (Akt inh) (B) prior to pressure exposure. The top panels represent typical Western blots for phosphorylated Akt, stripped and reprobed for total Akt as a loading control. The graphs summarize densitometric results expressed as mean ± SE of the ratio of phosphorylated Akt to total Akt, normalized to DMSO vehicle control. Open bars represent ambient pressure and closed bars increased pressure. Pressure increased Akt phosphorylation (*$P < 0.05$, paired t-test). LY294002 and Akt inhibitor IV reduced basal Akt phosphorylation 89.9% and 74% compared to cells treated with a DMSO vehicle control, respectively, and blocked the pressure effect on Akt activation. **C**: Effect of Akt inhibitor IV on primary monocyte phagocytosis under ambient and increased pressure conditions. Results are expressed as mean ± SE of percent phagocytosis in monocytes pretreated with Akt inhibitor IV or DMSO vehicle. Pressure increased monocyte phagocytosis (*$P < 0.001$, n = 3, paired t-test). Akt inhibitor IV reduced basal phagocytosis (#$P < 0.05$, n = 3, paired t-test) compared to cells treated with a DMSO vehicle control but completely abrogated pressure-stimulated phagocytosis. **D**: Effect of Akt inhibitor IV on THP-1 macrophage phagocytosis phagocytosis under ambient and increased pressure conditions. Results are expressed as mean ± SE of percent phagocytosis in THP-1 macrophages pretreated with Akt inhibitor IV or DMSO vehicle. Pressure increased THP-1 macrophage phagocytosis (*$P < 0.01$, n = 6, paired t-test). Akt inhibitor IV completely blocked pressure-stimulated without affecting basal phagocytosis.

abrogated pressure-induced primary monocyte phagocytosis (Fig. 4C) as well as THP-1 macrophage phagocytosis (Fig. 4D). Further studies using THP-1 macrophages also demonstrated that exposure to pressure significantly increased activation of mTOR (mammalian target of rapamycin), a kinase that activates p70S6K. Rapamycin pretreatment increased basal mTOR activation by 20.6 ± 8.1% ($P < 0.05$, n = 9) and reversed the pressure effect ($P < 0.05$, n = 9) (Fig. 5A). Akt activation by pressure was not affected by rapamycin (Fig. 5B). To further examine whether mTOR activation by pressure might be responsible for pressure-stimulated phagocytosis, SiRNA targeted to mTOR was transfected into THP-1 macrophages. mTOR SiRNA transfection reduced total mTOR expression by 42.1 ± 3.3% ($P < 0.001$, n = 5) (Fig. 5C)
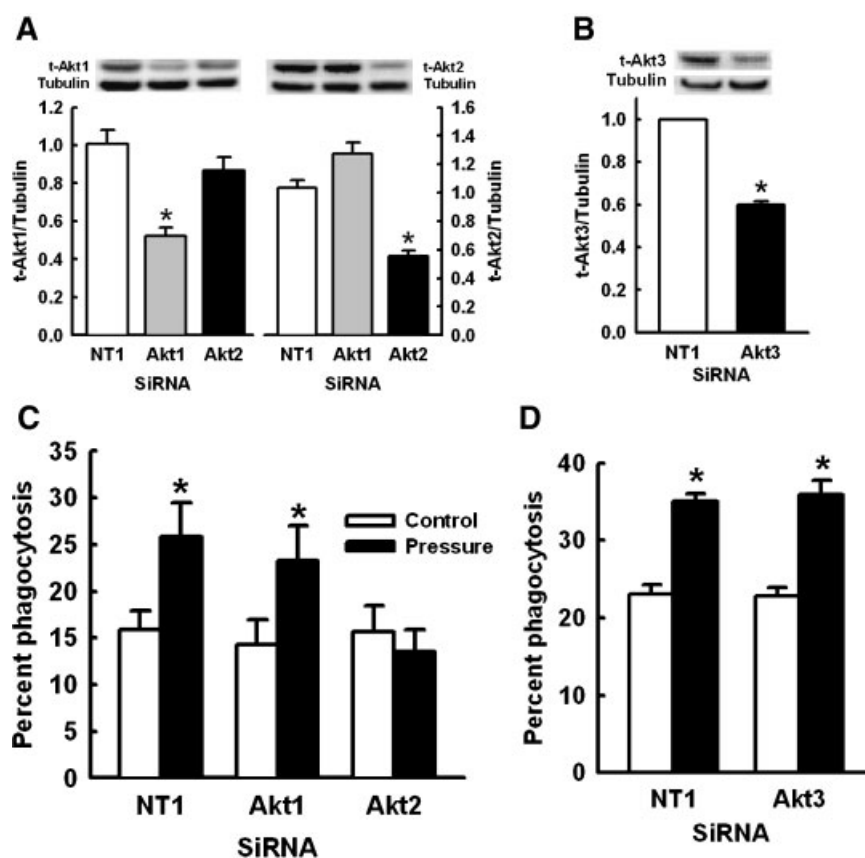
Fig. 3. Transfection with Akt SiRNA into THP-1 macrophages. **A**: Specificity of SiRNA. The top panel represents a typical Western blot probed with antibodies against total-Akt1 (left), total-Akt2 (right) and tubulin as a loading control. The graph summarizes densitometric results expressed as mean ± SE of the ratio of total-Akt1 (left) or total-Akt2 (right) to tubulin, normalized to NT-transfected cells. Akt1 SiRNA reduced Akt1, but not Akt2 protein expression (*$P < 0.001$, n = 12, paired t-test). Akt2 SiRNA decreased Akt2, but not Akt1 expression (*$P < 0.01$, n = 10, paired t-test). **B**: Inhibition of Akt3 protein expression by Akt3 SiRNA. The top panel represents a typical Western blot probed; the graph summarizes densitometric results expressed as mean ± SE of the ratio of total-Akt3 to tubulin, normalized to NT-transfected cells. Akt3 SiRNA significantly reduced Akt3 protein expression (*$P < 0.05$, n = 4, paired t-test). **C**: Effect of pressure on phagocytosis in cells transfected with Akt1 SiRNA or Akt2 SiRNA. Results are expressed as mean ± SE of percent phagocytosis in THP-1 macrophages transfected with NT1, Akt1, or Akt2 SiRNA. Open bars represent ambient pressure and closed bars represent increased pressure. Pressure increased phagocytosis by THP-1 macrophages transfected with NT1 SiRNA. Transfection with Akt1 SiRNA did not affect basal or pressure-stimulated phagocytosis. Akt2 SiRNA did not affect basal, but blocked pressure-stimulated phagocytosis. *$P < 0.05$ compared to NT1 SiRNA transfected cells at ambient pressure (n = 8, paired t-test). **D**: Effect of pressure on phagocytosis in cells transfected with Akt3 SiRNA. Results are expressed as mean ± SE of percent phagocytosis in THP-1 macrophages transfected with NT1 SiRNA or Akt3 SiRNA. Open bars represent ambient pressure and closed bars represent increased pressure. Pressure increased phagocytosis by THP-1 macrophages transfected with NT1 SiRNA. Pressure increased THP-1 macrophage phagocytosis (*$P < 0.01$, n = 6 compared to corresponding controls, Students' t-test). Transfection with Akt3 SiRNA did not affect basal or pressure-stimulated phagocytosis.

and completely blocked pressure-stimulated THP-1 macrophage phagocytosis (Fig. 5D).

To examine whether p70S6K activation by pressure might play a role in pressure-induced THP-1 macrophage phagocytosis, SiRNA targeted to p70S6K was designed. Transfection with p70S6K SiRNA reduced total p70S6K protein expression $40 \pm 0.05\%$ (n = 8, $P < 0.001$) compared to cells transfected with NT1 SiRNA (Fig. 6A). Pressure-induced

phagocytosis of THP-1 macrophages was completely blocked but basal phagocytosis was not affected by 70S6K SiRNA (Fig. 6B) similar to what we had observed after transfection with Akt2 SiRNA (Fig. 3B).

DISCUSSION

We have previously reported that 20 mmHg above ambient extracellular pressure (equivalent
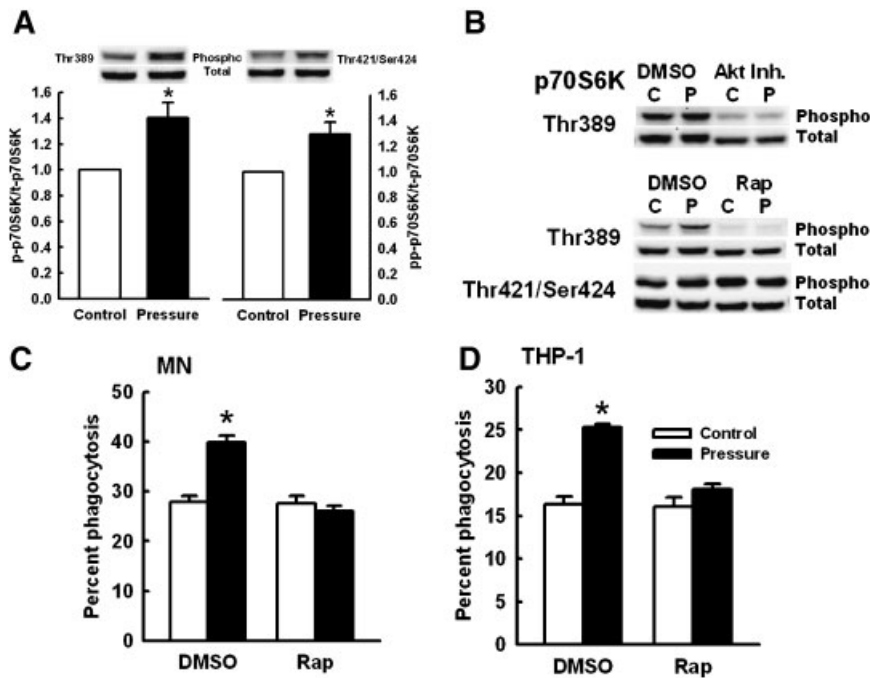
Fig. 4. Studies of p70S6K activation and pressure. **A**: Increase in p70S6K phosphorylation in primary human monocytes by pressure. The top panel represents typical Western blots probed for phosphorylated p70S6K Thr389 (left) and double phosphorylated p70S6K thr421/Ser424 (right), stripped and reprobed for total p70S6K as a loading control. The graph summarizes densitometric results expressed as mean ± SE of the ratio of phosphorylated p70S6K to total p70S6K. Pressure increased both p70S5K Thr389 (left: *$P < 0.05$, n = 6, paired t-test) and Thr421/Ser424 (right: *$P < 0.05$, n = 5, paired t-test) phosphorylation. **B**: Effects of Akt inhibitor IV and rapamycin on p70S6K activation in THP-1 macrophages. The figures represent typical Western blots of phosphorylated p70S6K Thr389 or double phosphorylated p70S6K Thr421/Ser424, stripped and reprobed for total p70S6K as a loading control. Figures are representative of at least eight similar blots. Pressure increased both p70S6K Thr389 phosphorylation (top and middle blots: $P < 0.05$, n = 9, paired t-test) and p70S5K Thr421/Ser424 (bottom blots: $P < 0.05$, n = 9, paired t-test) phosphorylation. Akt inhibitor IV (Akt inh) decreased p70S6K Thr389 phosphorylation (top: $P < 0.001$,

n = 8) and prevented the pressure effect. Rapamycin (Rap) decreased p70S5K Thy389 phosphorylation (middle: $P < 0.001$, n = 9, paired t-test) but did not affect p70S6K Thr421/Ser424 phosphorylation (bottom). Rapamycin blocked the pressure effect on p70S5K Thr389 and Thr421/Ser424 phosphorylation. **C**: Effect of rapamycin on monocyte phagocytosis. Results are expressed as mean ± SE of percent phagocytosis in monocytes pretreated with rapamycin or DMSO vehicle. Pretreatment with rapamycin did not affect basal phagocytosis but abrogated pressure-induced phagocytosis. *$P < 0.001$ compared to ambient pressure control, n = 3, paired t-test. **D**: Effect of rapamycin (Rap) on THP-1 macrophage phagocytosis. Results are expressed as mean ± SE of percent phagocytosis in THP-1 macrophages pretreated with rapamycin or DMSO vehicle. Open bars represent ambient pressure and closed bars represent increased pressure. Pretreatment with rapamycin abrogated pressure-induced but had no effect on basal phagocytosis. *$P < 0.05$ compared to ambient pressure control, n = 6, paired t-test.

to normal interstitial tissue pressure) increases serum-opsonized latex bead uptake by PMA-stimulated THP-1 macrophages, primary human peripheral monocytes and monocyte-derived macrophages by increasing p38 activation and/or inhibiting a FAK–ERK pathway [Shiratsuchi and Basson, 2004, 2005]. In this study, we demonstrated that activation of Akt2, but not Akt1 or Akt3, might contribute to the promotion of THP-1 macrophage phagocytosis through activating p70S6K in response to 20 mmHg increased extracellular pressure. Pressure translocated PI-3K from a cytosolic to a membrane fraction, and PI-3K inhibitor LY294002 and Akt inhibitor attenuated pressure-induced phagocytosis in

THP-1 macrophages as well as primary human monocytes. Inhibition of Akt2 or p70S6K by pharmacologic inhibitors or specific SiRNA abrogated pressure-induced THP-1 macrophage phagocytosis.

Although some investigators have reported that edema formation in unconstrained inflamed or infected tissues can be associated with decreases in interstitial pressures of as much as 150 mmHg [Guyton et al., 1971; Koller et al., 1993; Wiig et al., 2003], infection or edema in closed compartments may increase tissue pressure by 5–80 mmHg [Bertram et al., 1997; Schaser et al., 2003]. In patients with colonic inflammatory bowel disease, for instance,
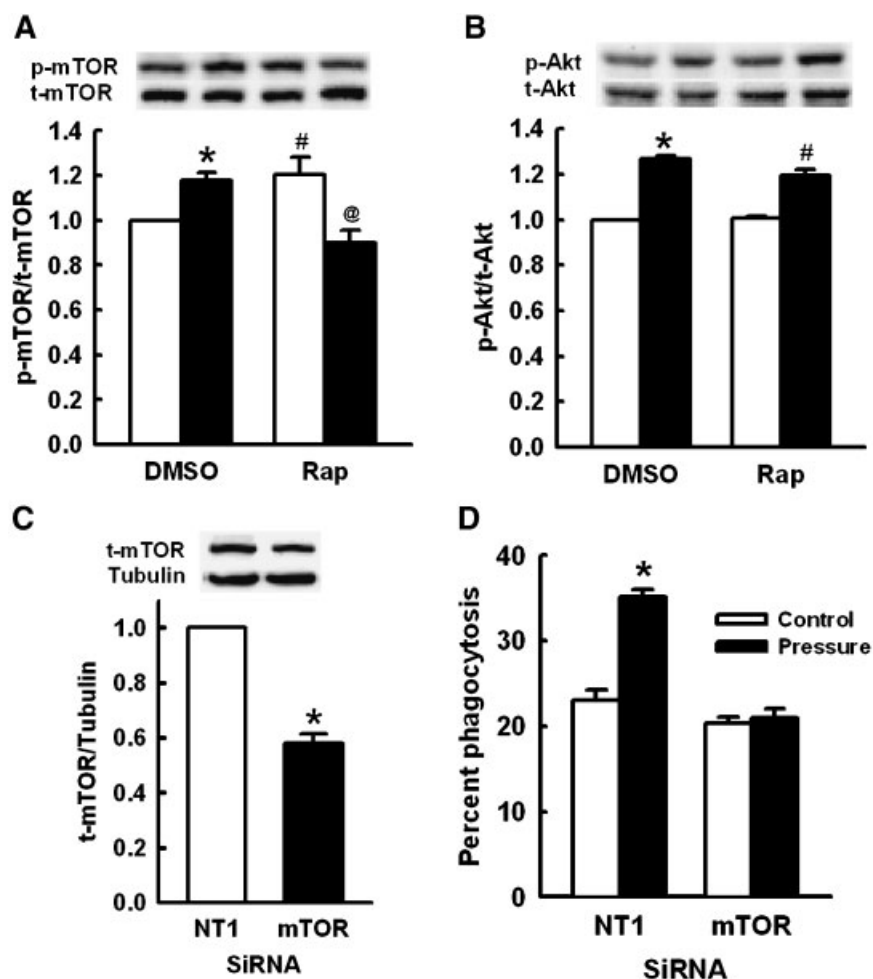
Fig. 5. Studies of rapamycin and mTOR. **A:** Rapamycin effect on mTOR activation. The top panel represents typical Western blots probed for phosphorylated mTOR, stripped and reprobed for total mTOR as a loading control. The graph summarizes densitometric results expressed as mean ± SE of the ratio of phosphorylated mTOR to total mTOR, normalized to control. Pressure increased mTOR phosphorylation (*$P < 0.005$, n = 9, paired t-test). Rapamycin pretreatment increased basal mTOR phosphorylation (#$P < 0.05$, n = 9, paired t-test) compared to DMSO-treated cells, and pressure reversed mTOR phosphorylation in Rap-treated cells (@$P < 0.05$, n = 9, compared to Rap-treated ambient pressure control, paired t-test). **B:** Rapamycin effect on Akt. The top panel represents typical Western blots probed for phosphorylated Akt, stripped, and reprobed for total Akt as a loading control. The graph summarizes densitometric results expressed as mean ± SE of the ratio of phosphorylated Akt to total Akt, normalized to control. Pressure increased Akt phosphorylation (*$P < 0.05$ compared to corresponding controls, n = 9, paired t-test). Rapamycin pretreatment did not affect basal nor pressure-induced Akt phosphorylation. **C:** Inhibition of mTOR protein expression by mTOR SiRNA. The top panel represents typical Western blots probed for total mTOR and tubulin as a loading control. The graph summarizes densitometric results expressed as mean ± SE of the ratio of total mTOR to tubulin, normalized to NT1 SiRNA transfected cells. Transfection with mTOR SiRNA reduced total-p70S6K protein expression 40% (*$P < 0.001$, n = 5, paired t-test). **D:** Effect of pressure on phagocytosis in mTOR SiRNA transfected THP-1 macrophages. Results are expressed as mean ± SE of percent phagocytosis in THP-1 macrophages transfected with NT1 or mTOR SiRNA. Open bars represent ambient pressure and closed bars represent increased pressure. Pressure increased phagocytosis by THP-1 macrophages transfected with NT1 SiRNA (*$P < 0.001$, n = 6, Students' t-test). Transfection with mTOR SiRNA blocked pressure-stimulated phagocytosis with no effect on basal.

colonic blood flow is increased 2–6 fold, causing capillary pressure to rise by 10–40 mmHg [Hulten et al., 1977; Granger and Barrowman, 1983]. It has also been reported that interstitial fluid pressures within solid tumors are 18–50 mmHg higher than those in surrounding normal tissues [Wiig and Gadeholt, 1985; Nathanson and Nelson, 1994; Zachos et al., 2001; Heldin et al., 2004; Diresta et al., 2005]. Immune cells such as macrophages and T cells infiltrate into tumors [Dirkx et al., 2006; Wang et al., 2006], and tumor-associated
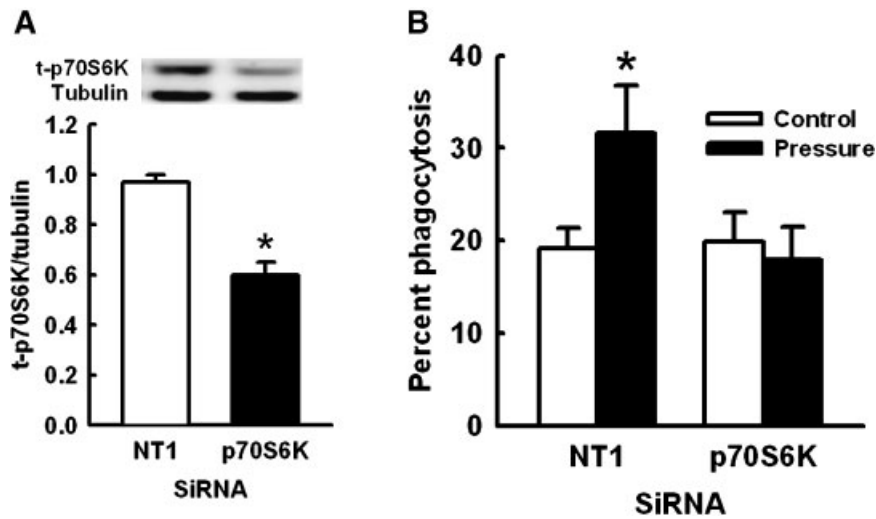
Fig. 6. Transfection with p70S6K SiRNA. **A**: Inhibition of p70S6K protein expression by p70S6K SiRNA. The top panel represents typical Western blots probed for total p70S6K and tubulin as a loading control. The graph summarizes densitometric results expressed as mean ± SE of the ratio of total p70S6K to tubulin, normalized to NT1 SiRNA transfected cells. Transfection with p70S6K SiRNA reduced total-p70S6K protein expression 40% (*$P < 0.001$, n = 8, paired *t*-test). **B**: Effect of pressure on phagocytosis by p70S6K SiRNA transfected THP-1 macrophages. Results are expressed as mean ± SE of percent phagocytosis in THP-1 macrophages transfected with NT1 or p70S6K SiRNA. Open bars represent ambient pressure and closed bars represent increased pressure. Pressure increased phagocytosis by THP-1 macrophages transfected with NT1 SiRNA (*$P < 0.05$, n = 4, paired *t*-test). Transfection with p70S6K SiRNA did not affect basal, but blocked pressure-stimulated phagocytosis.

macrophages might therefore be exposed to elevated tissue pressure within tumors. Activation or inhibition of macrophages by changes in extracellular pressure may therefore affect macrophage function in such settings. As discussed above, tissue pressure may be decreased or increased by inflammation and infection, depending upon the pathophysiology involved. In addition, tissue pressures are also elevated within malignant tumors. The effects of increased pressure upon macrophage function thus may have direct implications for the function of macrophages exposed to such increased pressures in vivo. How these same signal pathways are affected by decreased pressure, as may occur in other settings of inflammation, awaits further study.

Monocytes and macrophages are sensitive to biomechanical stimuli including cyclic strain, shear and pressure and play a critical role in wound healing, infection and inflammation [Aderem, 2003; Park and Barbul, 2004]. Mechanical strain has been reported to induce class A scavenger receptor expression and immediate-early response gene expression in THP-1 cells [Sakamoto et al., 2001]. Constant applied pressure (40–130 mmHg) increases the migration of human monocytic U937 cells in a dose-dependent manner [Singhal et al., 1997]. Macrophage pro-inflammatory cytokine production is also promoted in response to very high cyclic pressure associated with joint movement ($\approx 1,000$ mmHg), the combination of cyclic strain and 75-mmHg pressure, or high pressure (over 70 mmHg) combined with stimulation by endotoxin [Yang et al., 2000; McEvoy et al., 2002]. Constant 20 mmHg extracellular pressure, equivalent to normal interstitial tissue pressure and may be more relevant to the cell biology of inflamed tissue, stimulates spontaneous macrophage TNF-α production, but inhibits LPS-induced IL-1β production and enhances THP-1 macrophage phagocytosis through activating p38 and/or inhibiting FAK–ERK pathways [Shiratsuchi and Basson, 2004, 2005]. Increased pressure (40–90 mmHg), but not cyclic strain, has also been shown to increase the uptake of aggregated IgG by mouse macrophage J774.16 cells [Mattana et al., 1996].

PI-3K is activated by stress stimuli such as growth factors, microbial infection, and hydrostatic pressure [Salh et al., 1998; Weinstein et al., 2000; Celli et al., 2001; Salvador-Silva et al., 2004; Pastukh et al., 2005], and mediates IL-8 secretion in alveolar epithelial cells in vitro

by cyclic strain [Vlahakis et al., 1999] and nuclear factor-κB activation in rat lung cells [Uhlig et al., 2004]. The contribution of PI-3K in Fc receptor-mediated phagocytosis is well documented [Defilippi et al., 1999; May and Machesky, 2001; Stephens et al., 2002; Aderem, 2003]. Pathogenic microbes including entero-pathogenic *Escherichia coli* and *Helicobacter pylori* modulate PI-3K activation to control macrophage phagocytosis [Celli et al., 2001; Allen et al., 2005]. However, invasion by *Salmonella typhimurium* in human monocytic U937 cells and contractile activities associated with macrophage phagocytosis are independent of PI-3K [Araki et al., 2003; Forsberg et al., 2003]. Although basal CR-mediated phagocytosis is independent of PI-3K, stimulation-induced CR-mediated phagocytosis does require PI-3K activation [Lutz and Correll, 2003; Liu et al., 2005]. Thus, it was not obvious a priori whether PI-3K would be required for pressure-stimulated phagocytosis in this system. We observed that exposure to extra-cellular pressure-induced PI-3K p85 translocation from the cytosol to a membrane fraction and inhibition of PI-3K by LY294002 attenuated pressure-induced serum-opsonized latex bead phagocytosis by THP-1 macrophages, but did not modulate basal phagocytosis, suggesting that PI-3K activation is required for pressure-stimulated THP-1 macrophage phagocytosis.

Akt, regulates cell growth, survival, and mobility [Datta et al., 1999; Pastukh et al., 2005; Song et al., 2005], and is activated by mechanical strain in mesangial and vascular endothelial cells, and by pressure overloading in rabbit heart [Miyamoto et al., 2004; Krepinsky et al., 2005]. Akt has been implicated as an enzyme subsequently activated by PI-3K activation in macrophage phagocytosis [Forsberg et al., 2003; Lutz and Correll, 2003; Song et al., 2004]. MSP or olidonin promote CR-mediated macrophage phagocytosis and simultaneously induce Akt activation [Lutz and Correll, 2003; Liu et al., 2005], suggesting that Akt might regulate stimulation-induced CR-mediated phagocytosis. In our system, pressure increased Akt phosphorylation in THP-1 macrophages, and Akt activation was inhibited by pretreatment with Akt inhibitor IV or the PI-3K inhibitor LY294002. Like LY294002, Akt inhibitor IV also abrogated pressure-stimulated, but not basal, THP-1 macrophage phagocytosis. Although PI-3K and Akt may not influence all

modes of phagocytosis [May and Machesky, 2001; Aderem, 2003], our data suggest that Akt activation by pressure is required for the stimulation of THP-1 macrophage phagocytosis in our system.

There are three highly homologous Akt isoforms, with a conserved domain structure that includes a pleckstrin homology domain. Each is activated by similar mechanisms [Song et al., 2005]. Akt1 and Akt2 are expressed in most cell types [Datta et al., 1999; Zinda et al., 2001], and Akt3 is expressed at the lowest levels in most tissues [Zinda et al., 2001]. Akt1 is required for cardiac myocyte growth [DeBosch et al., 2006] and essential for intestinal epithelial cell survival [Dufour et al., 2004], and mediates PI-3K-dependent effects on glucose transport in adipocytes [Kim et al., 2000]. Overexpression of Akt2 up-regulates β1 integrins in human breast and ovarian cancer cells [Arboleda et al., 2003]. Recent studies suggest opposing roles for Akt1 an Akt2 in controlling cell proliferation [Heron-Milhavet et al., 2006] and cell migration [Zhou et al., 2006]. Although isoform-specific roles for the Akt isoforms have not previously been explored in the regulation of macrophage function, our results suggest that activation of Akt2, but not Akt1or Akt3, subsequent to PI-3K activation mediates THP-1 macrophage phagocytosis stimulated by pressure and that Akt isoforms play different roles in promotion of CR-mediated phagocytosis by pressure.

mTOR, known as FRAP or RAFT, regulates protein synthesis and mitogenesis via p70/p85 S6K and 4E-BP1, the repressor of protein synthesis initiation factor IF-4E binding protein [Gingras et al., 2004; Li and Sumpio, 2005; Sarbassov dos et al., 2005]. mTOR is activated by nutrients and plays a major role in autophagy during cell death at starvation [Meijer and Codogno, 2004; Codogno and Meijer, 2005; Sarbassov dos et al., 2005]. However, it is not clear whether mTOR is involved in foreign body phagocytosis by macrophages. In our current study, mTOR phosphorylation was increased by pressure, and pretreatment with rapamycin not only blocked the increase in mTOR phosphorylation associated with pressure but also prevented pressure-induced THP-1 macrophage phagocytosis. Furthermore, inhibition of mTOR protein expression by mTOR SiRNA blocked pressure-induced phagocytosis. Thus, mTOR might mediate the pressure effect.

A major finding of this study is that the Akt2–mTOR–p70S6K pathway plays a central role in pressure-induced macrophage phagocytosis. Activation of p70S6K has been shown by strain in vascular endothelial cells [Li and Sumpio, 2005], by multiaxial strech in muscle cells [Hornberger et al., 2005] and by pressure in aged rat aorta [Rice et al., 2005]. Interaction of integrin with extracellular matrix protein induces p70S6K activation in fibroblasts [Malik and Parsons, 1996]. Fc-receptor-mediated phagocytosis in the murine macrophage cell line Raw264.7 is enhanced by Akt through ribosomal p70S6K activation [Ganesan et al., 2004]. In this study, we demonstrated that exposure to pressure significantly increased p70S6K phosphorylation at Thr389 and Thr421/Ser424 in primary human monocytes as well as in THP-1 macrophages. The PI-3K/Akt and mTOR pathways are known to activate p70S6K. Indeed, p70S6K phosphorylation was reduced by Akt inhibitor IV or rapamycin treatment, although inhibition of Akt increased basal mTOR phosphorylation. Our results also showed that rapamycin inhibited Thr389 phosphorylation of p70S6K by over 70% but did not affect Thr421/Ser424 phosphorylation. However, rapamycin blocked not only p70S6K Thr389 but also Thr421/Ser424 phosphorylation increased by pressure and abrogated pressure-stimulated phagocytosis. Although the magnitude of the activation of p70S6K Thr389 by pressure in THP-1 macrophages appeared relatively small compare to the same effect in primary peripheral monocytes, inhibition of p70S6K activation by Akt inhibitor IV or rapamycin did block pressure-induced phagocytosis in each. Furthermore, transfection with SiRNA targeted to p70S6K to reduce total p70S6K expression completely blocked pressure-induced THP-1 macrophage phagocytosis. These data suggest that pressure may promote THP-1 macrophage phagocytosis by increasing p70S6K phosphorylation.

In summary, these studies demonstrate the contribution of PI-3K, Akt, mTOR, and p70S6K activation to the regulation of serum-opsonized particle phagocytosis in THP-1 macrophages and primary human monocytes. We have previously reported that modulation of FAK and the MAPK including ERK and p38 is important for the stimulation of phagocytosis by pressure. Modulation of these signals in response to changes in extracellular pressure in settings of infection or inflammation may alter macrophage phagocytic activity in vivo, and may represent attractive targets for therapeutic intervention. The elucidation of the regulation of pressure-induced ERK inhibition and p38 activation by p70S6K and the relationship between mTOR–p70S6K and FAK-Y397 phosphorylation awaits further study.

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