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Proteomic Analysis of Shear Stress-Mediated Protection from TNF-alpha in Endothelial Cells

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Abstract

Previous studies have shown that physiological levels of shear stress can protect endothelial cells (ECs) from apoptotic stimuli. Here we differentiate between acute and chronic protection and demonstrate the use of proteomic technologies to uncover mechanisms associated with chronic protection of endothelial cells. We hypothesized that changes in abundance of proteins associated with the TNF-alpha signaling cascade orchestrate shear stress-mediated protection from TNF-alpha when cells are pre-conditioned with shear prior to the exposure of apoptotic stimuli.

Detection of cleaved caspase 3 through Western blot analysis confirmed chronic shear-stress mediated protection from TNF-alpha. In the presence of the nitric oxide synthase (NOS) inhibitor, LMNA, chronic protection remained. Treatment with a *de novo* protein synthesis inhibitor, cycloheximide, eliminated this protective effect. Isotopic labeling experiments, coupled with liquid chromatography tandem mass spectrometry, (LC MS/MS) of isolated components of the TNF-alpha pathway revealed that CARD9, a known activator of the NF-κB pathway, was increased (60%) in sheared cells versus non-sheared cells. This result was confirmed through Western blot analysis. Our data suggests that *de novo* formation of proteins is required for protection from TNF-alpha in endothelial cells chronically exposed to shear stress, and that CARD9 is a candidate protein in this response.

Keywords

endothelium; shear stress; apoptosis; proteomics

Introduction

Maintenance of the vascular endothelium is a tightly regulated process. The balance of pro- versus anti-angiogenic factors dictates whether an endothelial cell will participate in capillary growth, or possibly contribute to vessel regression through apoptosis (3, 7). Knowledge of mechanisms involved in determining endothelial cell fate has therapeutic implications for diseases such as cancer, heart disease, and diabetes (5).

The hemodynamic force of shear stress has been shown to preserve endothelial cell integrity by protecting against apoptotic stimuli, including H_2O_2, serum deprivation, and tumor necrosis factor alpha (TNF-alpha) (10, 11, 18), however most studies have focused on the acute administration of shear forces (8, 9, 11-13). Nitric oxide production, a well-characterized process during acute shear stress exposure, has been demonstrated to contribute to this immediate shear-induced protection, specifically from TNF-alpha

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stimulation (9, 12). Increased levels of nitric oxide have been shown to inhibit apoptosis of endothelial cells by the S-nitrosylation-inactivation of caspases, cysteine proteases required to execute the apoptotic signaling pathway (12).

On the contrary, little is known about the protective mechanism of prolonged exposure to physiological shear. The focus of shear stress-induced protection has primarily been on the rapid modulation of downstream signaling events, usually involving the intrinsic mitochondrial pathway of apoptosis. The fact that TNF-alpha can initiate apoptosis via both the mitochondrial intrinsic pathway as well as the more upstream extrinsic pathway, suggests that pathway inactivation induced by both acute and chronic shear may occur in the proximal signaling pathway of the tumor necrosis factor receptor 1 (TNFR1). Following activation of TNFR1, proteins containing “death domains” interact with the receptor and trigger binding of important signaling proteins, such as Fas-associated death domain protein, or Fadd, which is responsible for activation of caspase 8. In the current studies we demonstrate that chronic shear confers protection from TNF-alpha through a NO-independent mechanism that results in de novo protein synthesis.

Using a mass spectrometry-based method to isolate and quantify TNF-alpha associated signaling proteins, we identified a protein, CARD9, which is a candidate for the protection conferred to endothelial cells by chronic shear.

Materials and Methods

Rat Endothelial Cell Culture

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

Seven week-old Sprague Dawley rats were anesthetized with an intraperoneal injection of pentobarbital (0.1 mL/100g), 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 120mM, KCL 4.7mM, $CaCl_2$ 3.0mM, $MgCl_2$ 1.43mM, $NaHCO_3$ 25mM, KH_2PO_4 1.17mM, glucose 11mM, and EDTA 0.03mM) and digested in 2% collangenase type 1 in a 37° water bath for 1 hr. The digests were centrifuged at 500×g for 10 min. The resulting pellet was resuspended in complete media (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 media was changed to modified eagle's media (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 media for 1 week until returning to complete media and grown to confluence. All cells were passaged three times prior to treatment.

Shearing Protocol

Rat vascular endothelial cells (VECs) grown in standard 100mm × 20mm cell culture plates were 85-90% confluent at the time of shearing. All cells were sheared in unrecirculated culture media using a modified cone plate viscometer as described by Sdougos et al. that has been adapted for 100 × 20mm cell culture dishes (29). The cone's fixed 0.5 degree angle was rotated at a constant speed to produce equal levels of shear across the plate. VECs were sheared at 10dynes/cm^2 and 30dynes/cm^2 of laminar flow for 18hrs, or at pulsatile flow (5-15dynes/cm^2) for 18hrs. Shearing was performed in a standard incubator with the environment controlled at 5% CO_2, 20% O_2, and at 37°C.

Western Blotting

For detection of PARP, cleaved caspase 3, IKB, phospho-IKB, Tradd, Fadd, and CARD9, cells were lysed in RIPA buffer (200mM Mannitose, 70mM Sucrose, 10mM HEPES, 1M HEPES, 1mM EDTA) plus protease inhibitors. Cell lysates were gently sonicated on ice and centrifuged for 1hr (100,000×g) and the supernatant containing the cytosolic fraction was recovered. Total protein was determined using a detergent-compatible assay (Bio-Rad, Hercules, CA). Proteins were loaded at 30μg per well and were separated by SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane (Bio-Rad) and were blocked for at least 2 hr in TBS, 0.3% BSA, and 0.1% Tween 20. Blots were then incubated with a monoclonal antibody against cleaved caspase 3 (clone Asp175-5A1) (Cell Signaling, Danvers, MA) in a 1:1000 dilution, a monoclonal antibody against PARP (clone C-2-10) (Sigma-Aldrich, St. Louis, MO) in a 1:1000 dilution, a polyclonal antibody against IKB (#9242) (Cell Signaling, Danvers, MA) in a 1:1000 dilution, a monoclonal antibody against phospho-IKB (#2859) (Cell Signaling, Danvers, MA) in a 1:1000 dilution, a polyclonal antibody against Fadd (ab24533) (Abcam Inc., Cambridge, MA) in a 1:500 dilution, a monoclonal antibody against Tradd (610572) (BD Transduction Laboratories, Franklin Lakes, NJ) in a 1:500 dilution, or a polyclonal antibody against CARD9 (ab55950) (Abcam Inc., Cambridge, MA) in a 1:500 dilution. Washed blots were then incubated with a secondary antibody conjugated to horse radish peroxidase and visualized with SuperSignal West Dura chemiluminescence substrate detection system (Pierce, Rockford, IL).

TNF-alpha Complex Isolation

The method used to isolate TNF-alpha signaling proteins has been previously described by Freed et al. (15). Briefly, 4.0×10^6 sheared and non-sheared VECs were rinsed with PBS prior to administration of biotinylated TNF-alpha (10μg) in serum-free media. The TNF-alpha was allowed to bind for 30 min at 37°C. Dithiobis[succinimidyl-propionate] (DSP) dissolved in DMSO was then added to the cellular media to a final concentration of 1mM. Cross-linking reactions were allowed to continue for 30 min at room temperature. Cells were then scraped using a rubber policeman, pelleted, and lysed using mammalian protein extraction reagent (M-PER) (Pierce, Rockford, IL). Affinity chromatography was carried out using the ProFound Pull-Down Biotinylated Protein: Protein Interaction Kit with slight modifications (Pierce, Rockford IL). 50μl of streptavidin mixture was placed in an eppendorf tube and equilibrated with Tris buffered saline. Tubes were centrifuged at 1,250×g for 30-60 sec and supernatant was aspirated off. Cell lysate containing biotinylated protein complexes was then added and allowed to incubate for 2 hrs at 4°C. The solution was then centrifuged and unbound proteins aspirated off. Multiple washes were performed using 0.75M NaCl. Elution buffer provided by the kit was then added along with 10mM dithiothreitol and allowed to incubate at room temperature for 1 hr. The contents were then transferred to a mini-spin column and centrifuged allowing separation of the complex proteins and streptavidin beads.

^{18}O Labeling of Peptides for Quantitation

Isolated TNF-alpha complex proteins were blocked by the addition of 20mM iodoacetamide (IAA) for 45min at 37°C in the dark. Samples were then transferred to 100μl vivaspin concentration spin columns (Sigma-Aldrich, St. Louis, MO). Directions from the manufacturer were followed to remove contaminants such as DTT, NaCl, and IAA. Total protein was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were then dried to zero volume using a vacuum centrifuge. The proteins were resuspended in 20μl of 200mM ammonium bicarbonate solution, and sonnicated in a water bath for 10min. Trypsin, (Fisher, Hanover Park IL) was added at a ratio of 1:50 enzyme:target protein in either $H_2{}^{16}O$ or ≥ 95% $H_2{}^{18}O$ (Cambridge Isotope Laboratories, Andover, MA) and allowed to digest for 36hr at 37°C. Samples were labeled in both the

forward and reverse direction ($^{18}O/^{16}O$, control/experimental). Digestion was stopped by the addition of 0.1% (v/v) formic acid and the two digests pooled in a 1:1 ratio. Peptides were loaded onto a Varian C18 tip (Lake Forest, CA) equilibrated in 0.1% formic acid. The peptides were loaded onto the column and washed in 10× column volume of 0.1% formic acid. Peptides were eluted by 10× column volume of solvent B (95% acetonitrile, 0.1% formic acid). The eluent was reduced to dryness using a vacuum centrifuge and the peptides resuspended in 8μl solvent A (5% acetonitrile, 0.1% formic acid) for analysis by mass spectrometry.

Mass Spectrometry

Mass spectrometric data was collected on a Thermo Fisher LTQ XL instrument. Peptides were loaded onto a 6cm C18 column packed with 5 micron C18 resin (Phenomenex, Cheshire, UK) stationary phase using a autosampler and a surveyor pump delivering two mobile phase solvents A and B at 100μl/min through a 1:200 split to a 2μl injection loop. A 2μl 'full loop' injection was made in solvent A and the sample eluted from the column by the introduction of solvent B: 0-150min 0-35% B, 150-185min 35-60% B, 185-230min 60-100%B, 230-240min 100%B, 240-250min 100-0%B, the column was left to equilibrate for 60min in 100% A before the data collection was terminated. The ions eluted from the column were electrosprayed at a voltage of 1.8kV. The capillary voltage was 45V and the temperature kept at 200°C. No auxillary or sheath gas was used. Helium was used in the ion trap, which was also used as a collision gas for fragmentation of ions. The target value for the ion trap was 3×10^4 ions in MS mode and 1×10^4 in MS/MS mode.

Quantitation Analysis

Files containing fragmentation spectra data from samples subjected to LC MS/MS were searched against the complete generic rat IPI database using MASCOT (Matrix Science, London, UK). Variable modifications allowed for included carboxyamidomethylcysteine, oxidized methionine, and a modification due to the addition of dithiobis[succinimidyl-propionate] on lysine residues (191 daltons). The peptide tolerance was set to +/− 2.0 daltons, the MS/MS tolerance was set to 0.8 daltons, and two missed cleavages were allowed. Identifications were noted when the MASCOT search results were above the 95th percentile (≥ 17). An additional criteria was set in which a protein had to be observed in at least 2 of the 5 total MS runs in order to be considered identified.

The heavy isotope of oxygen, ^{18}O, was incorporated into the peptide at the C-terminus. Where two ^{18}O atoms were incorporated into the peptide a mass difference of 4Da was seen between the peptide envelopes from the heavy and light (^{16}O) labeled samples. The intensities of the two peptides were measured by mass spectrometry and the relative abundance of each calculated (16, 19). Since every peptide was labeled, it was possible to obtain multiple ratio values for each protein, increasing the reliability of the quantitation (17, 34).

Any protein represented by a single peptide was discarded. The remaining data set was interrogated further. To be included in subsequent analysis a peptide had to match the following criteria: it must have been generated by tryptic cleavage to ensure the active incorporation of ^{18}O, manual visualization of the MS/MS spectra had to satisfy the requirement for a high signal to noise ratio, accurate protein identification, and clear visualization of the zoom scan over the entire isotopic peak mass range. Each zoom scan was assessed for quality and scans were manually excluded if: background noise was high surrounding the peptide envelope, a co-eluting peptide was suggested by overlapping envelopes, the assigned mass did not match the reported identification, or if peaks were

missing from the peptide envelope. Ratios for each peptide were then normalized to the corresponding peptide's ion score that was used to identify the protein.

Results

Role of NO and Protein Inhibition on Shear Stress-Mediated Protection

TNF-alpha treatment (10ng/ml, 12hrs) caused a significant increase in the amount of cleaved caspase 3 in endothelial cells compared to control cells in which there was no treatment. (Fig. 1A) Pre-exposure (18hrs) to laminar shear, (10 and 30 dynes/cm^2) as well as pulsatile shear, (5-15 dynes/cm^2) blocked the increase in caspase 3 cleavage caused by subsequent TNF-alpha administration.

Since nitric oxide has been previously shown to be partly responsible for shear stress protection from TNF-alpha during the acute application of shear stress, we performed Western blots against cleaved caspase 3 in cells that had been treated with the nitric oxide synthesis inhibitor N^{ω}-monomethyl-L-arginine (LNMA). There was a significant increase in the amount of cleaved caspase 3 in cells treated with LNMA (1mM for 12hrs) compared to non-treated cells ($p<0.5$). Groups treated for 12hrs simultaneously with both LNMA (1mM) and TNF-alpha (10ng/ml) also displayed significantly higher levels of caspase 3 cleavage than control cells ($p<0.5$), however no significant difference was seen between TNF-alpha-treated cells compared to TNF-alpha plus LNMA (Fig. 1B). LNMA incubation (1hr) prior to shear stress exposure did not affect the protection from TNF-alpha following shear treatment.

A significant amount of caspase 3 cleavage was observed in cells treated with the protein synthesis inhibitor cycloheximide, exposed to shear stress, and then treated with TNF-alpha, compared to control cells treated with cycloheximide. Treatment with cycloheximide completely inhibited the protective effect of shear stress on TNF-alpha-induced apoptosis indicated in Figure 1C. Figure 1D displays the representative Western blots for these experiments.

CARD9 is Increased in Cells Pre-conditioned with Shear that have undergone TNF-alpha Treatment

Mass spectrometry analysis of the isolated TNF-alpha signaling complex identified a number of proteins associated with this pathway. A total of 5 MS/MS runs were performed. In two runs sheared samples were labeled using $H_2{}^{16}O$ and in 3 runs sheared samples were labeled using $H_2{}^{18}O$ to facilitate quantification of changes in protein signaling. A combined protein list was created from all 5 runs based on a score calculated from the following formula, score = −10*log(P), where P is the probability of the spectral match being random (Matrix Science). A combined total of 106 proteins were identified from all 5 MS/MS runs. A criterion was set that required the protein to be identified from at least 2 separate MS/MS scans. From the 106 original proteins identified, 15 proteins met this criterion. Table 1 provides the list of these 15 proteins along with overall score, total peptide count, run count, and scan count. Figure 2A displays the calculated ratios of protein abundance between sheared and non-sheared conditions (sheared:non-sheared) for the 15 proteins that were quantified. One protein, caspase recruitment domain containing protein 9, (CARD9) was observed to a have a much higher ratio compared to the other 14 remaining proteins, and was determined to be significantly overexpressed in the TNF-alpha associated complex of sheared cells. The mean ratio calculated for all the proteins excluding CARD9 was 0.91. A representative mass spectra of a CARD9 peptide (KVTGKEPAR) illustrating the added mass due to addition of heavy and light oxygen isotopes is shown in Figure 2B. Table 2 displays the ratios of each protein normalized to the ion score for each peptide used to

identify the protein. To confirm these results, Western blot analysis against CARD9 was performed. Figure 3 shows that CARD9 is significantly increased in cells treated with TNF-alpha that were first pre-exposed to shear stress versus receiving TNF-alpha treatment alone.

A Fadd to Tradd Signaling Switch and NF- κB Activation by IKB-α Is Not Observed in Cells Pre-conditioned with Shear Stress and Exposed to TNF-alpha

Since CARD9 has been shown to interact with Bcl-10 causing activation of the NF-κB pathway, Western blot analysis (Figure 4A) was performed against both Fadd and Tradd, docking proteins for the TNF-alpha receptor that are known to direct signaling either towards the apoptotic pathway or the NF-κB pathway, respectively. Figure 4B summarizes these results. Treatment with TNF-alpha caused a significant increase in the amount of Fadd protein expressed compared to the non-treated control, however this increase was attenuated when cells were exposed to shear prior to TNF-alpha treatment. No significant increase in Tradd was observed in any of the treatment groups. Proteins involved in the NF-κB pathway downstream of Tradd activation were also measured via Western blot. Phosphorylated IκB-α: total IκB-α expression was relatively quantified and no significant difference was observed between any of the four treatment groups as shown in Figure 4C.

Discussion

The results of this study suggest that endothelial cells preconditioned with shear stress are protected from TNF-alpha-induced apoptosis and that, unlike acute shear exposure, this protection is not mediated by nitric oxide production, but instead relies on *de novo* synthesis of proteins to suppress the apoptotic actions of TNF-alpha. In these experiments, proteins associated with TNF-alpha signaling were isolated and subjected to isotopic labeling and mass spectrometry analysis in order to examine differences in protein expression in sheared versus non-sheared cells. An increase in the protein CARD9, a known activator of the NF-κB pathway, was observed in sheared cells exposed to TNF-alpha. Proteins involved in the TNF-alpha-induced NF-κB pathway were examined in order to test the hypothesis that CARD9 was causing a switch in TNF-alpha signaling from the known Fadd-driven apoptotic pathway to the Tradd-activated inflammatory pathway (21). No evidence of NF-κB activation in cells preconditioned with shear followed by TNF-alpha treatment was found.

It has been demonstrated in previous studies that endothelial cells are protected from apoptotic stimuli when simultaneously being exposed to acute shear stress (11). In the current study we have demonstrated a similar protection in endothelial cells that were pre-exposed to a chronic shear at a lower level of 10 dynes/cm^2, a higher level of 30 dynes/cm^2, as well as pulsatile pattern, (5-15 dynes/cm^2) indicating this protection exists under a variety of shear conditions.

TNF-alpha is a cytokine known to be involved in systemic inflammation which can have a direct impact on surrounding endothelium (24). Levels of TNF-alpha in vivo can increase rapidly, for instance during sepsis or in chronic inflammatory diseases (22). In the current study we used 10ng/ml of TNF-α to study shear protective effects. Other studies have used 10ng/ml of TNF-alpha to induce apoptosis in cultured endothelial cells and more importantly to study the mechanisms used to counteract this effect (32). Although this amount is higher than one would find in vivo during a healthy state, for purposes of our studies, 10ng/ml of TNF-alpha produced apoptosis that was able to be quantified.

Dimmeler and colleagues have suggested a role for nitric oxide (NO) in the acute shear-induced protection mechanism against TNF-alpha (10). It is widely accepted that acute exposure of endothelial cells to shear stress (within seconds) results in an immediate

increase in NO production (26, 33). Studies reported by Dimmeler have shown that shear-induced protection from TNF-alpha is attenuated with treatment of the eNOS inhibitor LNMA (10). In that particular study, TNF-alpha was administered at the onset of shearing, whereas in the current study cells were first pre-exposed to shear for 18hrs prior to TNF-alpha treatment. In our experiments we did not observe any attenuation of shear stress-protection with LNMA treatment. We attribute this discrepancy to temporal differences in protection mechanisms. Since the increase in NO due to shear occurs so rapidly, the robust increase in NO may be responsible for immediate protection from TNF-alpha by nitrosylating, and therefore inhibiting, caspases, cysteine proteases that render the cell apoptotic (23). While NO may be involved in immediate short-term protection, 18hrs of shear is a sufficient time to increase transcription of regulatory proteins such as CARD9 that are capable of “shunting” cellular activation of signals between pathways . Alternatively, 18hrs of shear stress may specifically upregulate anti-apoptotic, or survival proteins, which protect against TNF-alpha exposure. In the current studies we focused on these long term regulatory mechanisms. Of particular relevance is the *in vivo* situation in which endothelial cells in perfused versus non-perfused vessels may be exposed to cytokines such as TNF-alpha. In this scenario, the endothelium has been subjected to blood flow-induced shear stress prior to cytokine exposure. For this reason, cells in the present study were first preconditioned with shear prior to TNF-alpha treatment. It should be noted that experiments performed in which LNMA- treated cells were administered TNF-alpha at the onset of shear resulted in a loss of shear stress protection and therefore supported the previous findings of Dimmeler and colleagues. (data not shown)

In the current study, endothelial cells incubated with the protein inhibitor cycloheximide prior to conditioning with shear stress, were not protected against subsequent TNF-alpha administration. This is indicated by the significant increase in cleaved caspase 3, suggesting that protection may be attributed to shear stress-induced protein expression. Although the fact that the protection conferred by chronic shear stress requires de novo protein expression is not surprising, there is a lack of studies indicating the role of this de novo process as well as the associated mechanisms. Interestingly in these experiments in which protein synthesis was inhibited, progressive levels of cell stress (TNF-alpha, shear stress, and TNF-alpha +shear) resulted in increasing activation of caspases. (Figure 1C) The increase of cleaved caspase 3 in the TNF-alpha group can be attributed to induction of the apoptotic pathway leading to caspase 3 activation as previously described (25). More interesting is the increased apoptosis measured in the shear stress group alone. It is well accepted that laminar shear maintains endothelial cell integrity by inducing expression of various growth factors (2). In the presence of a protein inhibitor such as cycloheximide, increases in these protective proteins were inhibited leading to an unmasking of detrimental effects of shear. This finding strengthens our hypothesis that shear stress induces protective protein expression to ward off the deleterious effects of TNF-alpha as well as shear stress itself.

In the current study we hypothesized that changes in the TNF-alpha associated signaling proteins are responsible for the shear-induced protection. A major challenge commonly encountered in proteomic analysis is the ability to identify changes in TNF-alpha associated signaling proteins responsible for the shear-induced protection compared to the significantly abundant cytoskeletal proteins, for instance, actin and vimentin(6). Due to this dynamic range issue a common approach is to isolate or “pull-out” the proteins of interest prior to quantification (36). Many of these methods are successful at isolating intracellular signaling complexes but do not include the extracellular receptor:ligand complex. We have recently reported a method in which we identified ~70% of the proteins known to be involved in the apoptotic TNF-alpha pathway (15). In the current study, this same methodology was used to now quantitate differences in some of these TNF-alpha associated proteins in sheared versus non-sheared cells.

The strategy used to quantitate such differences in protein expression involved the use of isotopic labels, specifically a heavy isotope of oxygen or ^{18}O. This method, which has been previously used in proteomic studies (20, 27, 28, 31), relies on the incorporation of either the normal ^{16}O or the heavier ^{18}O molecule onto the end of a peptide following enzymatic reaction with trypsin. Although this allows for quantification of two identical peptides from two separate samples, not all proteins are quantifiable by this method. For instance, as with other labeling techniques, ^{18}O-labeling adds more complexity to protein identification(14). In addition, the algorithms used to analyze the mass spectra and calculate the ratios of labeled:unlabeled peptides rely heavily on high resolution spectra. In the current study we identified several abundant cytoskeletal proteins as associated with the TNF-alpha signaling complex. The larger size of abundant cytoskeletal proteins and thus increased number of tryptic peptides favors these proteins to be analyzed, in comparison to the significantly smaller and less abundant signaling proteins. It is therefore not surprising that we were able to identify and quantify abundant cytoskeletal proteins in 5/5 mass spectrometry runs.

With the strict criteria used in this study only 15 TNF-alpha associated proteins were quantified. Of these 15 proteins, only CARD9 was an outlier of the normal distribution of the calculated protein abundance ratios. Zoomquant analysis indicated that CARD9 was increased by approximately 50% in sheared cells treated with TNF-alpha compared to non-sheared cells also treated with TNF-alpha. Western blot analysis confirmed the presence of CARD9 in the TNF-alpha-biotin pull-down and verified the increase in CARD9 observed by MS in preconditioned sheared endothelial cells following TNF alpha treatment.

Studies done on CARD9 have shown interaction with the protein BCL10, an anti-apoptotic protein belonging to the Bcl family. Formation of the CARD9-BCL10 complex has been correlated to the activation of NF-κB (4). Multiple lines of evidence have demonstrated an anti-apoptotic role of NF-κB. For instance studies have shown that inhibition of NF-κB or IKK with specific antibodies can trigger apoptosis within the cell (1, 35). Wang and colleagues showed the importance of NF-κB in regulating apoptosis, demonstrating that activation of NF-κB inhibited apoptosis by blocking the activation of caspase 8, an upstream caspase activated by Fadd.

The fact that phosphorylated IKB-α: total IκB-α expression was not significantly altered in cells protected from TNF-alpha does not rule out the possibility of NF-κB activation. It is well accepted that NF-κB is sequestered in the cytoplasm and not allowed to enter the nucleus to initiate transcription until its regulatory subunits (IκB) are targeted for ubiquitination via phosphorylation (30). The protein complex responsible for this phosphorylation is the IκB kinase (IKK) signalosome. This signalosome as well as its target, IκB, has multiple subunits that are under constant regulation. Our results suggest that NF-κB activation by CARD9 is not occurring via phosphorylation of IκB-α, however it does not rule out the possibility that activation may be occurring via some other mechanism.

Our results clearly show that shear protection of endothelial cells induces two distinct mechanisms which evolve over time; an acute NO dependent mechanism followed by a chronic NO independent mechanism inducing protein synthesis. In situations in which perfusion to capillaries is compromised inhibition of blood vessel growth and even microvascular rarefaction can occur. The aim of this study was to investigate the mechanisms by which shear stress provides endothelial cells the capability to withstand chronic apoptotic stimuli, specifically from TNF-alpha. The present study confirmed that pre-exposure to the mechanical force of shear stress due to flow can protect endothelial cells from TNF-alpha-induced apoptosis and that this protection occurs in a nitric oxide-independent fashion. An increase in CARD9, a protein known to be involved in the NF-κB survival pathway, was observed both by isotopic labeling coupled with mass spectrometry

analysis, as well as by Western blot. We can conclude that a “signaling switch” is not occurring between the apoptotic Fadd route and the cell survival route that involves Tradd. Further functional validation studies are required to define the specific anti-apoptotic mechanism involving CARD9 using siRNA knockdowns in the protective response to chronic shear stress.

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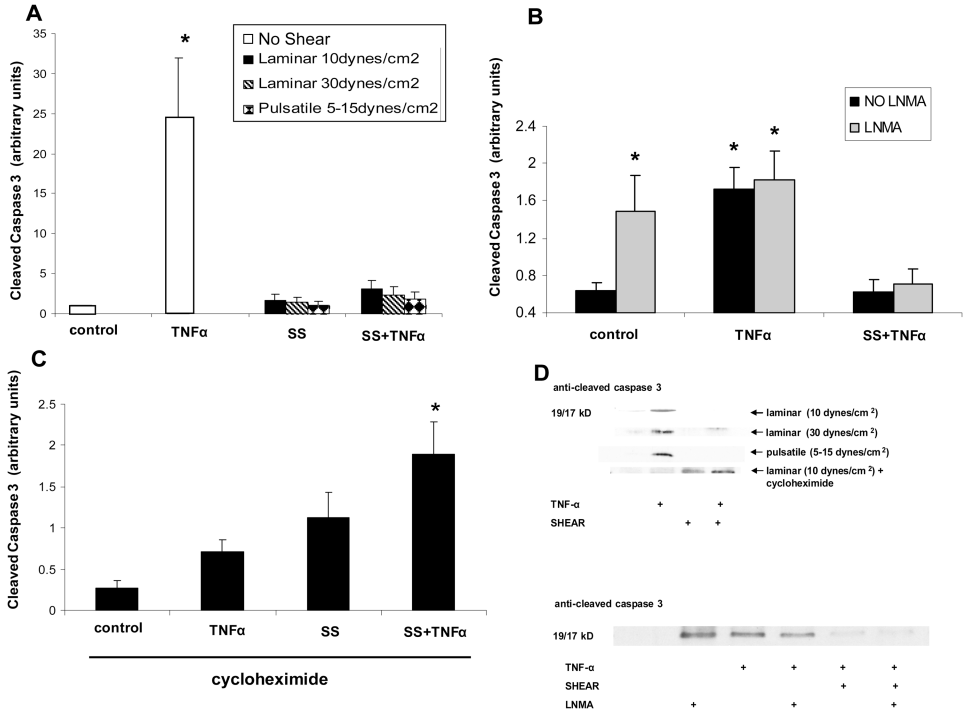

Fig. 1.

A, Quantitative densitometry comparing cleaved caspase 3 and poly-ADP-Ribose-Polymerase (PARP) protein levels in non-treated control cells, cells treated with 10ng/ml of TNF-alpha for 12hrs, cells exposed to shear for 18hrs (10 dynes/cm^2), and cells first exposed to shear (10 dynes/cm^2 for 18hrs) then treated with 10ng/ml of TNF-alpha for 12hrs (mean+/−SEM, n=5). B, Cells treated with 1mM LNMA for 12hrs, cells treated with 10ng/ml of TNF-alpha for 12hrs, cells treated with 1mM LNMA and 10ng/ml TNF-alpha for 12hrs simultaneously, cells first exposed to shear (10 dynes/cm^2 for 18hrs) then treated with 10ng/ml of TNF-alpha for 12hrs, and cells first incubated with 1mM LNMA for 1hr then exposed to shear (10 dynes/cm^2 for 18hrs) followed by TNF-alpha treatment (10ng/ml for 12hrs) (mean+/−SEM, n=5). C, Control cells treated with cycloheximide alone, (0.36μmol/L, 18hrs) cells first incubated with cycloheximide (0.36μmol/L) followed by TNF-alpha, (10ng/mL, 12hrs) cells incubated with cycloheximide (0.36μmol/L) followed by shear stress exposure, (10 dynes/cm^2 for 18hrs) and cells incubated with cycloheximide (0.36μmol/L) then exposed to shear stress (10 dynes/cm^2 for 18hrs) followed by TNF-alpha treatment (10ng/mL, 12hrs) (mean+/−SEM, n=6). D, Representative images of Western blots for each experiment A-C.

(*) Significant difference vs. no treatment group, $p<0.05$.

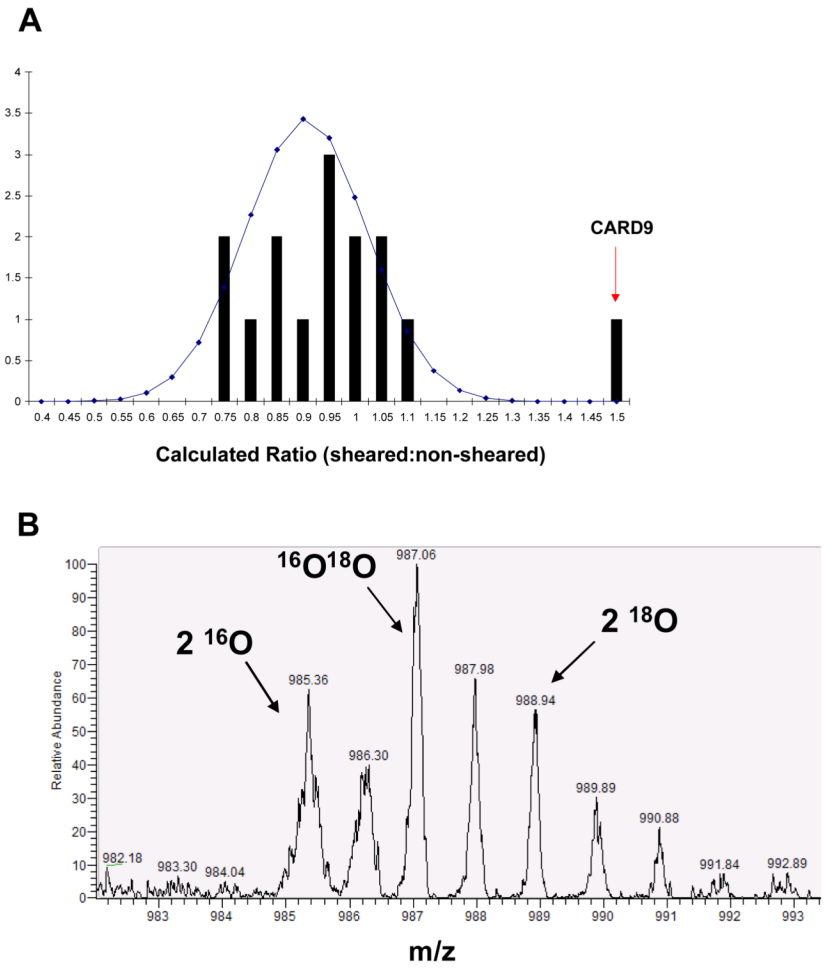

Fig. 2.

A, Relative ratios of the 15 quantifiable proteins from the ^{18}O isotopic labeling experiment plotted as a ratio histogram under a normal distribution. One of the quantifiable proteins, CARD9, is shown as an outlier of the group. B, Normalized average ratio for CARD9 compared to the combined average ratio for all other identified proteins (top 15 - CARD9). C, Representative mass spectra showing both the heavy and light isotopic peaks from a CARD9 peptide, KVTGKEPAR, m/z of 985.57.

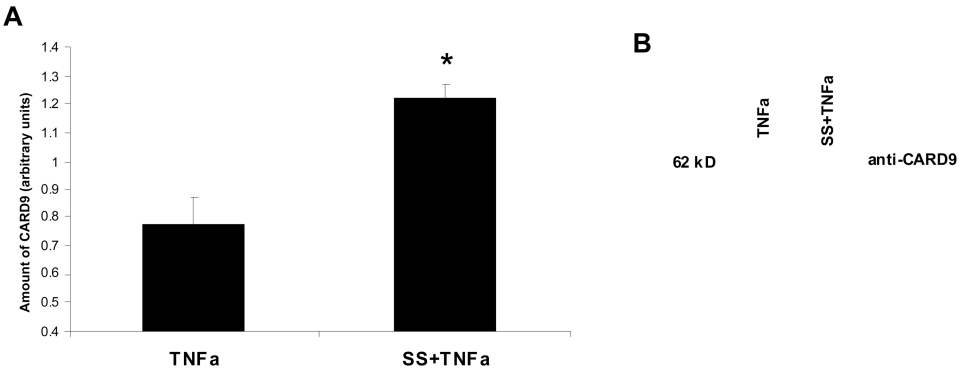

Fig. 3.

Quantitative densitometry comparing the amount of CARD9 in cells treated with TNF-alpha (10ng/mL, 12hrs) and in cells first exposed to shear stress (10 dynes/cm^2 for 18hrs) followed by TNF-alpha treatment (10ng/mL, 12hrs). (n=6). (*) Significant difference, $p<0.05$. (mean +/− SEM)

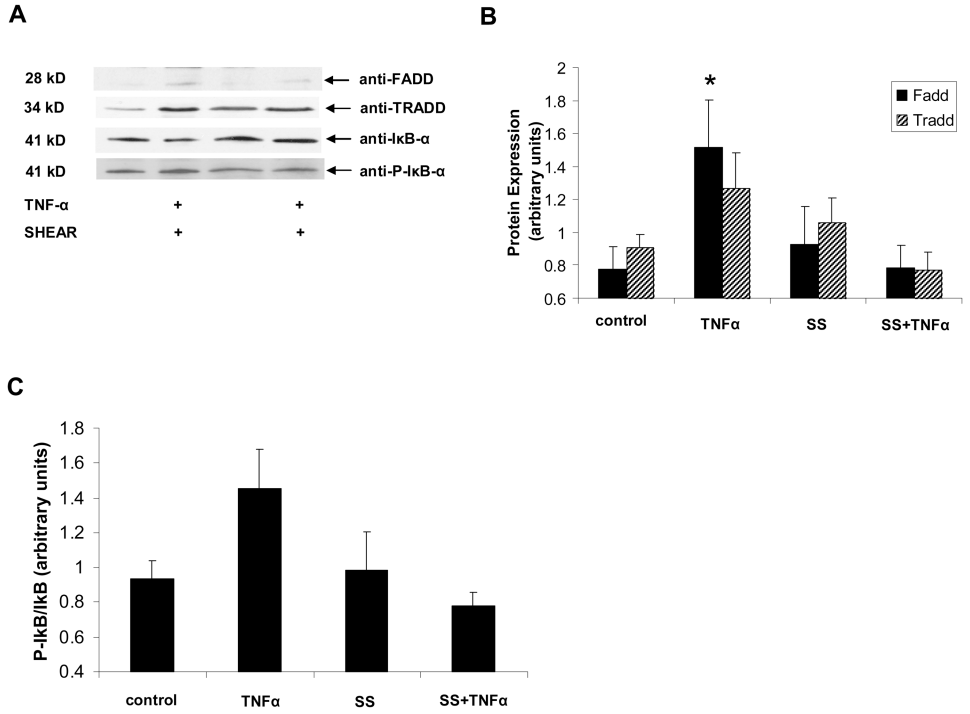

Fig. 4.

A, Quantitative densitometry comparing both Fadd and Tradd protein levels in non-treated control cells, cells treated with 10ng/ml of TNF-alpha for 12hrs, cells exposed to shear (10 dynes/cm^2 for 18hrs), and cells first exposed to shear (10 dynes/cm^2 for 18hrs) followed by TNF-alpha treatment (10ng/ml for 12hrs) (n=6). (mean+/−SEM) B, Phosphorylated IκB-α: total IκB in non-treated control cells, cells treated with 10ng/ml of TNF-alpha for 12hrs, cells exposed to shear (10 dynes/cm^2 for 18hrs), and cells first exposed to shear (10 dynes/cm^2 for 18hrs) followed by TNF-alpha treatment (10ng/ml for 12hrs). (n=6) (mean+/−SEM) (*) Significant difference vs. no treatment $p<0.05$.

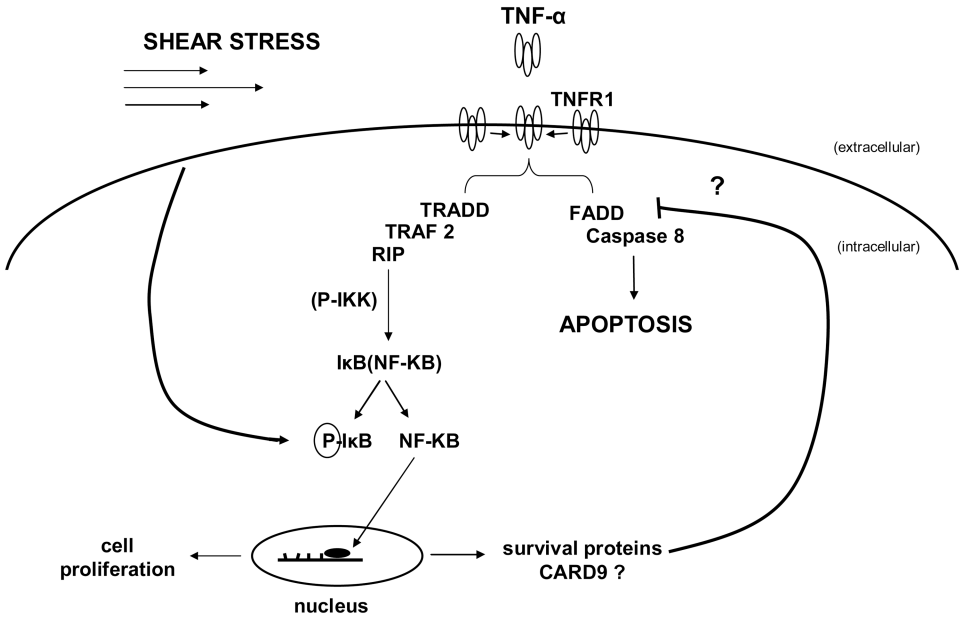

Fig. 5.

Diagram illustrating the signaling mechanism of how CARD9 may be inhibiting apoptosis from TNF-alpha. Upon binding of TNF-alpha to TNFR1, the receptor trimerizes and activates 1 of 2 signaling cascades. Activation of the FADD pathway results in further activation of a caspase cascade resulting in apoptosis. Activation of the TRADD pathway results in activation of NF-KB which signals to the nucleus to increase the number of survival proteins resulting in cell proliferation. Shear stress is known to result in phosphorylation of IκB, thus releasing NF-KB from its inhibitory state in order to signal to the nucleus. Our study suggests that CARD9 is one of the survival proteins that may be interacting with the caspase-induced pathway of TNF-alpha, thus protecting endothelial cells from apoptosis during chronic shear.

Table 1

Table of the 15 quantifiable proteins from the ^{18}O isotopic labeling experiment. Proteins are arranged by score provided from the calculation; score = -10*log(P), where P is the probability of the spectral match being random. Total peptide count, total scan count, and the number of runs (out of 5) the protein was identified, have also been added.

Name	Accession	Description	Score	Peptide Count	Run Count	Scan Count
IPI00189819	P60711	Actin, cytoplasmic 1	4.57E+12	112	5	113
IPI00230941	P31000	Vimentin	3.64E+12	101	5	102
IPI00655291	Q91XN6	tropomyosin 1, alpha isoform a	3.41E+09	12	5	13
IPI00421560	Q62744;Q6P786;Q99MI8	alpha actinin 4	1.32E+09	12	2	13
IPI00212014	P46462	Transitional endoplasmic reticulum ATPase	1.98E+08	8	2	9
IPI00188804	P02401	60S acidic ribosomal protein P2	68034880	17	3	18
IPI00206624	P06761	78 kDa glucose-regulated protein precursor	40953777	12	4	13
IPI00231615	P07150	Annexin A1	31218190	10	3	11
IPI00230914	P09895	60S ribosomal protein L5	24940369	4	3	5
IPI00189651	Q9EPY0	Caspase recruitment domain-containing protein 9	10698860	21	3	22
IPI00567369	Q63610	Isoform 1 of tropomyosin alpha-3 chain	3489430	2	3	3
IPI00421718	P47198	60S ribosomal protein L22	2880815	5	2	6
IPI00187860	Q4V8K6	Heterogeneous nuclear ribonucleoprotein C	1736017	4	2	5
IPI00212776	P62909	40S ribosomal protein S3	986614.1	5	3	6
IPI00627068	Q4KM71	Splicing factor proline/glutamine rich	629134.5	4	2	5

Table 2

Table of the quantifiable proteins excluding CARD9 along with their average ratio calculated by Zoomquant. The ratios for these 14 proteins were averaged together to give an overall protein average ratio of 0.91.

Name	Accession	Description	Average Ratio
IPI00189819	P60711	Actin, cytoplasmic 1	1.05
IPI00230941	P31000	Vimentin	0.98
IPI00655291	Q91XN6	tropomyosin 1, alpha isoform a	0.85
IPI00421560	Q62744;Q6P786;Q998	alpha actinin 4	0.81
IPI00212014	P46462	Transitional endoplasmic reticulum ATPase	0.93
IPI00188804	P02401	60S acidic ribosomal protein P2	0.97
IPI00206624	P06761	78 kDa glucose-regulated protein precursor	1.03
IPI00231615	P07150	Annexin A1	0.75
IPI00230914	P09895	60S ribosomal protein L5	0.71
IPI00567369	Q63610	Isoform 1 of tropomyosin alpha-3 chain	0.92
IPI00421718	P47198	60S ribosomal protein L22	0.88
IPI00187860	Q4V8K6	Heterogeneous nuclear ribonucleoprotein C	0.77
IPI00212776	P62909	40S ribosomal protein S3	1.09
IPI00627068	Q4KM71	Splicing factor proline/glutamine rich	0.95
		Protein Average	**0.91**