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Extracellular SOD modulates canonical TNFα signaling and α5β1 integrin transactivation in vascular smooth muscle cells

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Abstract

TNFα activates NADPH oxidase 1 (Nox1) in vascular smooth muscle cells (VSMCs). The extracellular superoxide anion ($O_2^{\bullet-}$) produced is essential for the pro-inflammatory effects of the cytokine but the specific contributions of $O_2^{\bullet-}$ to signal transduction remain obscure. Extracellular superoxide dismutase (ecSOD, SOD3 gene) is a secreted protein that binds to cell surface heparin sulfate proteoglycans or to Fibulin-5 (Fib-5, FBLN5 gene), an extracellular matrix protein that also associates with elastin and integrins. ecSOD converts $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2) which prevents $NO^{\bullet}$ inactivation, limits generation of hydroxyl radical ($OH^{\bullet}$), and creates high local concentrations of H_2O_2. We hypothesized that ecSOD modifies TNFα signaling in VSMCs. Knockdown of ecSOD (siSOD3) suppressed downstream TNFα signals including MAPK (JNK and ERK phosphorylation) and NF-κB activation (luciferase reporter and IκB phosphorylation), interleukin-6 (IL-6) secretion, iNOS and VCAM expression, and proliferation (Sulforhodamine B assay, PCNA western blot). These effects were associated with significant reductions in the expression of both Type1 and 2 TNFα receptors. Reduced Fib-5 expression (siFBLN5) similarly impaired NF-κB activation by TNFα, but potentiated FAK phosphorylation at Y925. siSOD3 also increased both resting and TNFα-induced phosphorylation of FAK and of glycogen synthase kinase-3β (GSK3β), a downstream target of integrin linked kinase (ILK). These effects were dependent upon α5β1 integrins and siSOD3 increased resting sulfenylation (oxidation) of both integrin subunits, while preventing TNFα-induced increases in sulfenylation. To determine how ecSOD modified TNFα-induced inflammation in intact blood vessels, mesenteric arteries from VSMC-specific ecSOD knockout (KO) mice were exposed to TNFα (10 ng/ml) in culture for 48

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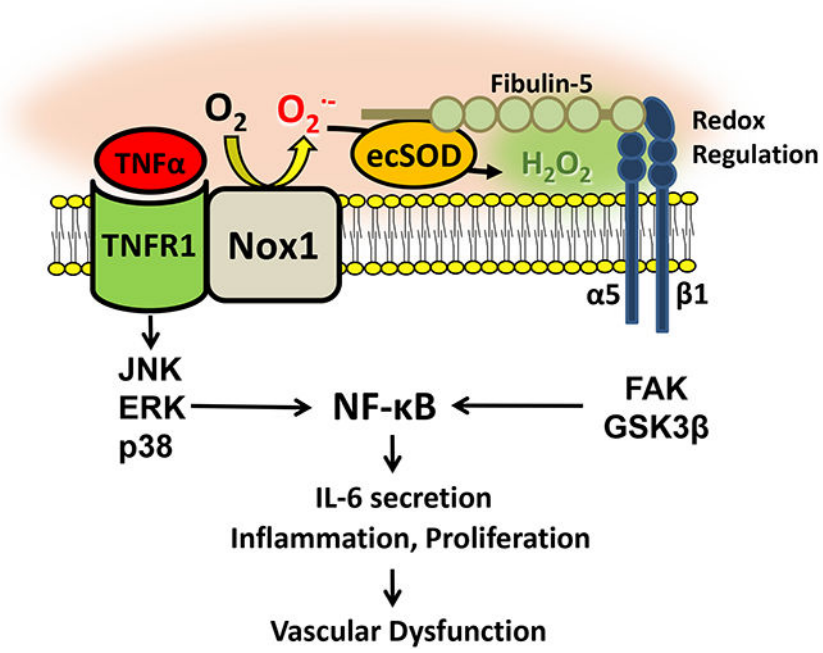
Declaration of interest

None

hrs. Relaxation to acetylcholine and sodium nitroprusside was impaired in WT but not ecSOD KO vessels. Thus, ecSOD association with Fib-5 supports pro-inflammatory TNFα signaling while tonically inhibiting α5β1 integrin activation.

Graphical Abstract



Keywords

Extracellular Superoxide Dismutase; Superoxide Dismutase 3; Tumor necrosis factor-α; Fibulin 5; Alpha5Beta1 Integrin

INTRODUCTION

Overabundance of reactive oxygen species (ROS) promotes the development of atherosclerosis, hypertension, and heart failure via numerous adverse effects that have been collectively termed “oxidative stress” [1, 2]. However, recent discussions of this topic have acknowledged that stimulus-dependent oxidation of specific targets is an important part of normal cell physiology, termed “oxidative eustress” [3]. Non-selective antioxidants support specific, adaptive vascular functions such as endothelium-dependent vasodilation. However, the relatively new concept of “reductive stress” suggests that increased availability of reducing equivalents can also disrupt housekeeping effects of ROS, impair critical signaling pathways, cause mitochondrial dysfunction, promote apoptosis, and reduce cell survival in the cardiovascular system [4]. Thus, it may not be surprising that the preponderance of clinical studies have shown no benefit of non-targeted antioxidant treatment in cardiovascular disease [5-7]. The future of antioxidant therapy is likely to depend upon targeting specific ROS in selected locations.

Serum levels of tumor necrosis factor-α (TNFα) are elevated in human hypertension [8], ischemic heart disease [9, 10], and myocardial injury [11]. TNFα produces ROS in vascular cells [12, 13] that are required for canonical signaling [14, 15]. TNFα-induced redox signaling requires extracellular superoxide ($O_2^{\bullet-}$) production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 1 (Nox1) in the plasma membrane [16, 17] of vascular smooth muscle cells (VSMCs). Downstream signals that are TNFα receptor and ROS-dependent include receptor endocytosis, activation of mitogen-activated protein kinases (MAPKs) and the pro-inflammatory transcription factor Nuclear Factor Kappa B (NF-κB).

Extracellular superoxide dismutase (ecSOD) is encoded by the SOD3 gene and is abundant in arteries, which express roughly ten times more of this protein than other tissues [18]. Based on single cell sequencing in the lung, SOD3 mRNA expression is highest in VSMCs and fibroblasts, with message being virtually undetectable in endothelial cells [19]. ecSOD is anchored to the plasma membrane which enhances cell surface activity, and it has also been shown to be endocytosed [20], suggesting a potential to modify the function of redox-active signaling endosomes [21, 22]. Surface binding occurs via either a C-terminal heparin binding domain (HBD) or by association with the N-terminal region of Fibulin-5 (Fib-5), an extracellular matrix (ECM) protein that associates with elastin and with integrins [23]. It is not known if these associations are independent of one another, or if the HBD region of ecSOD binds competitively to both sites.

The C-terminus of Fib-5 binds to integrin receptors without causing activation, and this interferes with activation by elements of the extracellular matrix [23, 24]. The integrin family includes 18 α and 8 β subunits that can be assembled into 24 distinct heterodimers. These proteins regulate cell survival, proliferation, adhesion, migration, and differentiation [25]. Integrin signaling is mediated by several mechanisms, including activation of associated cytoplasmic enzymes such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK) which control vascular remodeling [25]. In VSMCs, Fib-5 associates with alpha5beta1 (α5β1) integrins [26] which are activated by binding to the RGD motif of fibronectin [27]. It is not known if association of ecSOD with Fib-5 affects binding to integrins or controls any aspect of integrin or Fib-5 function. It is clear, however, that integrins are redox-sensitive. Their extracellular domains contain multiple disulfide bonds that are critical for control of their activation [28]. Reduction of these disulfides with dithiothreitol (DTT) facilitates fibronectin binding to α5β1 integrins [29].

Localization of ecSOD with a redox-sensitive multiprotein complex may have important physiologic consequences. ecSOD converts $O_2^{\bullet-}$ to hydrogen peroxide (H_2O_2). This enhances nitric oxide ($NO^{\bullet}$) bioavailability and reduces peroxynitrite production which may be beneficial in atherosclerosis and hypertension [24]. In addition, acceleration of $O_2^{\bullet-}$ dismutation may allow ecSOD to regulate the local environment of redox-sensitive proteins. Under pathological conditions, ecSOD can also become inactivated or converted to a peroxidase, an effect which can adversely affect vascular redox state and induce vascular dysfunction [30, 31].

We sought to determine the impact of acute loss of ecSOD (siSOD3) on TNFα signaling in VSMCs. We hypothesized that its antioxidant function would promote ROS-dependent

steps in TNFα signaling and thereby potentiate the inflammatory response. Instead, loss of ecSOD impaired canonical TNFα signaling, and was associated with a reduction in TNFα receptor expression. TNFα transactivated α5β1 integrins and this effect was enhanced by the loss of either ecSOD or Fib-5. TNFα induced oxidation of both α5 and β1 integrins, and knockdown of ecSOD increased resting integrin sulfenylation but prevented these proteins from becoming more oxidized upon exposure to TNFα. Thus, it appears that colocalization of α5β1 integrins with Fib-5/ecSOD alters local redox conditions that impact both canonical TNFα signaling and integrin transactivation.

METERIALS AND METHODS

Cell Culture

Primary aortic VSMC were isolated from C57/BL6 mice by explantation. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Vanderbilt University Institutional Animal Care and Use Committee. Mice were euthanized using carbon dioxide (CO_2) and thoracic aortas were excised, cleaned of adherent fat and connective tissue, and the endothelial cell layer removed by passing a pin through the lumen. The vessels were then cut into 1-2 mm square sections in a cold physiological salt solution, placed in a culture dish and maintained in an incubator at 37 °C in a humidified 5% CO_2 atmosphere. After 7-10 days, the vessel segments were removed and the VSMCs that migrated out of the explants were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1X minimum essential medium non-essential amino acids, 1X vitamin, and 25 mM HEPES. HEK293T cells were obtained from the American Tissue Culture Collection and maintained at 37 °C in 5% CO_2 in DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin.

siRNA Transfection

Mouse-specific siRNA (negative control; D-001210-01, SOD3; M-059104-01) was purchased from Dharmacon (Lafayette, CO). siRNA (100 nM) was incubated with Lipofectamine 2000 (Life Technologies) in serum-free medium for 15 min. The resultant complex of siRNA-Lipofectamine 2000 was added to the cells in DMEM containing 5% FBS and maintained for 3 days before performing experiments.

Western Blotting

Cells were serum-deprived (0.5% serum) for 3 hours before TNFα (10 ng/mL) stimulation for the indicated time periods. Protein extracts (40 - 50 μg) were separated by electrophoresis on a polyacrylamide gel and transferred to nitrocellulose membranes via standard methods. Nonspecific binding was inhibited with blocking buffer (LI-COR) for 1 hour at room-temperature. Membranes were then incubated with primary antibodies in Tris-buffered saline solution with Tween 20 (0.1%) overnight at 4 °C. Antibodies were as follows: p-JNK (#9255), JNK (#9252), p-p38 (#9216), p38 (#9212), p-ERK (#9106), ERK (#9102), p-IκB (Ser32, #2859), p-FAK (Tyr925, #3284), FAK (#3285), p-Akt (#4051), Akt (#9272), p-GSK3β (#14630), ILK (#3856), Integrin α5 (#98204), Integrin β1 (#34971) obtained from Cell Signaling Technology (Danvers, MA), ecSOD (#AF4817) and VCAM

(#AF643) from R&D Systems (Minneapolis, MN), PCNA (#05-347, MilliporeSigma, Burlington, MA), iNOS (#610328, BD Transduction Laboratories), Fibulin-5 (#60081-1-Ig, Proteintech, Rosemont, IL), Tubulin (Vanderbilt Antibody Core, Nashville, TN). After incubation with fluorescent secondary antibodies for 2 hours, signals were developed using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE) and quantified via densitometry (Image Studio). Results were normalized to the indicated proteins and expressed as arbitrary units.

NF-κB activity

NF-κB induction was measured by replication-deficient adenovirus containing a luciferase reporter gene driven by NF-κB transcriptional activation. VSMCs were infected for 24 hours followed by exposure to TNFα (10 ng/mL) in serum-free DMEM for 6 hours. siRNA was maintained for 2 days prior to treatment with adenovirus. Blocking antibodies were incubated for 1 hour prior to TNFα exposure. Blocking antibodies were as follows: Control rat IgG (556968), Integrin α4 (553153), Integrin α5 (553318), Integrin αv (553241), Integrin β1 (555002), Integrin β3 (553343) from BD Biosciences (Franklin Lakes, NJ). Luciferase activity (relative light units) was measured in reporter lysis buffer, according to the protocol of the manufacturer (Promega, Madison, WI) and normalized to protein concentration (BCA protein assay).

Interleukin-6 (IL-6) and soluble TNFR1 (sTNFR1) ELISA

Media from VSMC was collected following a 24 hr TNFα exposure and stored at −80 °C. IL-6 and sTNFR1 were quantified using an enzyme-linked immunosorbent assay (ELISA) kits (IL-6 (eBioScience, San Diego, CA), TNFRSF1A (Cat.# MRT10, R&D Systems, Minneapolis, MN)) according to the manufacturer's specifications.

Sulforhodamine B (SRB) assay

VSMCs were grown on clear-bottom 96-well plates and transfected with siRNA for 3 days. The cells were then treated with TNFα for 24 hours, and cell viability measured by SRB. Cells were fixed with 5% cold trichloroacetic acid (TCA) for 1 hour at 4 °C, washed with water, air-dried at room temperature overnight, stained with 0.057% SRB solution in 1% acetic acid for 30 min, rinsed with 1% acetic acid, and air-dried at room temperature. After drying, 10 mM Tris base (pH 10.5) was added and the optical density was measured at 510 nm in a microplate reader (FLUOstar Omega, BMG Labtech).

Sod3-Fib-5 immunoprecipitation

Wild-type human SOD3 (NM_ 003102.4, Clone ID: OHu19471, pcDNA3.1^{+}-hSOD3-DDK Flag tag) and Fibulin-5 (NM_006329.3, Clone ID: OHu17817, pcDNA3.1^{+}-FBLN5-HA) were obtained from GenScript (Piscataway, NJ). Modifications to the SOD3 plasmid were made using the QuikChange kit (Agilent, Santa Clara, CA). The modified SOD3 clones are detailed in Fig. 5. HEK293T cells were transfected using Lipofectamine 2000. For a 10 cm plate, cDNA (10 μg/μl) was incubated with 25 μl Lipofectamine 2000 in serum-free DMEM for 15 min and added to the cells in DMEM containing 10% FBS for 1 days before performing IP. Cells were exposed to lysis buffer (50mM Tris base, 150mM NaCl, 1mM

EDTA, 10% glycerol, 1mM DTT, 1% Triton X-100, 0.1% Na-DOC, 0.1% SDS, 10mM β-glycerophosphate, 20mM para-nitrophenyl phosphate, 2mM sodium pyrophosphate, 1mM Na_3 VO_4, 5mM NaF, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) at pH 7.4) for 1 hour with nutation at 4°C, and centrifuged for 30min at 20,000g. Supernatants were pre-cleared with protein-G sepharose beads for 1h at 4°C and cleared-supernatants were incubated with antibody (2 μg) for 1.5h, then incubated with protein-G sepharose for 1h. Beads were washed with lysis buffer, resuspended in SDS sample buffer, boiled and the associated proteins were then analyzed by western blot. Antibodies for IP and western blot were used as follows; Flag (#F1804, MilliporeSigma), HA (#3724, Cell Signaling Technology).

Integrin Sulfenylation

A cell permeable, biotin-labeled Cys-OH (sulfenic acid) trapping probe (3-(2,4-dioxocyclohexyl) propyl, DCP-Bio1; MilliporeSigma) was used to quantify protein sulfenylation. VSMCs were grown on 10 cm-plate and incubated with DCP-Bio1 (125 μM) for 1 hour in DMEM containing 0.5% FBS before exposure to TNFα (10ng/ml) for 3 min. Cells were then lysed (50 mM Tris, pH 7.0, 50 mM NaCl, 2 mM EDTA, 1 mM NaF, 1mM Na_3VO_4, 2.5 mM Sodium pyrophosphate, 1% Triton-X, 100 μM NEM, 100 μM IAA, protease inhibitor, 100 μM PMSF). DCP-biotin labelled proteins were pull-downed with streptavidin beads overnight, washed, and analyzed by western blot with anti-Integrin α5 and β1.

Animals

Floxed SOD3 mice were obtained from Dr. David Harrison (Vanderbilt University) and mated to animals expressing myosin heavy chain Cre (B6.FVB-Tg(MyH11-icre/ERT2)1Soff/J, Jackson Laboratories, Bar Harbor, ME). 10-14 weeks old male mice received IP injections of 100 μl Tamoxifen (20 mg/ml) or vehicle (Corn Oil) for 5 consecutive days. Mesenteric vessel segments were harvested 7-10 days after the last injection.

Wire Myography

First- or second-order mesenteric resistance artery branches (~100 μm inner diameter) were excised, cleaned of fat and connective tissues and cut into 1-2 mm length-rings in an ice-cold physiological salt solution (PSS) consisting of the following: 130 mmol/L NaCl, 4.7 mmol/L KCl, 1.18 mmol/L KH_2PO_4, 1.18 mmol/L $MgSO_4{\cdot}7H_2O$, 1.56 mmol/L $CaCl_2{\cdot}2H_2O$, 14.9 mmol/L $NaHCO_3$, 5.6 mmol/L glucose, and 0.03 mmol/L EDTA. Mesenteric rings were incubated in DMEM containing 0.5% FBS with or without TNFα (10 ng/mL) for 2 days in an incubator at 37 °C with 5% CO_2. Rings were subsequently mounted in wire myographs (Danish Myo Technology A/S, Aarhus, Denmark) containing warmed (37 °C), oxygenated (95% O_2/5% CO_2) PSS and allowed to equilibrate for at least 45 min under a passive force of 2-2.5 mN. Arterial integrity was assessed by stimulation with 120 mmol/L KCl and, after contraction reached a plateau, the rings were washed. Subsequently, the rings were stimulated with phenylephrine (PE, 10^{-6} mol/L), followed by relaxation with acetylcholine (ACh, 10^{-6} mol/L). More than an 80% relaxation response to ACh was taken as evidence of an intact endothelium. Contractile responses were assessed by cumulative exposure to PE or norepinephrine (NE; 10^{-9} to 10^{-5} mol/L). Endothelium-

dependent relaxation was performed on PE-contracted (10^{-6} mol/L) rings by cumulative addition of ACh (10^{-9} to 3×10^{-5} mol/L) and endothelium-independent relaxation was tested using sodium nitroprusside (SNP, 10^{-9} to 10^{-5} mol/L).

Statistical Analysis

Data values are expressed as mean ± standard error of the mean (SEM), and 'n' represents the number of individual experiments in cultured cells. Graphs were generated by Graph Pad Prism 9 (GraphPad Software, San Diego, CA). Statistical differences were assessed by unpaired *t*-test or one-way ANOVA. *Post hoc* comparisons were performed using Dunnett's analysis to compare all groups. A *P* value less than 0.05 was considered statistically significant. Contractions were recorded as changes in tension (mN) from baseline, expressed as a percentage of the response to 120mM KCl. Relaxations were expressed as a percentage of the contraction produced by PE in each ring. Return to baseline tension before addition of PE was considered to be 100% relaxation. Concentration-response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 9), and two pharmacological parameters were obtained: the maximal effect generated by the agonist (or E_{max}) and EC_{50} (molar concentration of agonist producing 50% of the maximum response).

RESULTS

ecSOD modulates Canonical TNFα Signaling

To characterize the role of ecSOD in TNFα signaling, we first assessed the effect of siRNA targeting of SOD3 on TNFα-induced MAPK activation. We quantified phosphorylation of JNK, p38, and ERK, and as previously reported, all were enhanced by TNFα (10 ng/ml). Knockdown of SOD3 reduced p-JNK and p-ERK following TNFα exposure but had no impact on phosphorylation of p38 (Fig. 1).

Another important response of VSMCs to TNFα is NF-κB activation. We assessed phosphorylation of IκBα which tonically suppresses NF-κB activation. Phosphorylation of IκBα (p-IκB) causes it to dissociate from NF-κB and become degraded, resulting in NF-κB activation. Decreased ecSOD protein expression resulted in a small but significant increase in p-IκB under resting conditions. However, phosphorylation in response to TNFα was markedly reduced (Figs. 2A and B). We confirmed altered activation of NF-κB by TNFα using a luciferase reporter assay. siSOD3 significantly decreased basal NF-κB activity as well as activation in response to TNFα (Fig. 2C). We also assessed induction of interleukin-6 (IL-6) secretion by TNFα. The abundance of IL-6 in the culture media was measured after 24 hours of exposure to TNFα. TNFα increased secretion of IL-6 into the media in Control and SOD3 knockdown cells but SOD3 siRNA significantly reduced the amount of IL-6 secreted under both resting and stimulated conditions (Fig. 2D).

We next explored the effect of TNFα on VSMC proliferation. Cell viability as quantified by the SRB assay was enhanced by TNFα following a 24 hr exposure, and this effect was impaired by SOD3 knockdown (Fig. 3A). Proliferating Cell Number Antigen (PCNA) protein expression was also increased by TNFα and this effect was once again diminished by siRNA targeting SOD3 (Fig. 3B and C). Consistent with the pro-inflammatory effects

of TNFα, inducible nitric oxide synthase (iNOS) and vascular cell adhesion molecule (VCAM) expression were increased following 24 hours of exposure, and these effects were also attenuated by SOD3 knockdown (Fig. 3B, D and E). Collectively, these data suggest that siSOD3 reduced TNFα-mediated signaling. We therefore assessed TNFα receptor (TNFR) protein expression in VSMC treated with siSOD3 and observed significantly reduced abundance of both TNFR1 and TNFR2 protein (Fig. 3F). Oxidants can activate cell surface proteases such as ADAM17 [32] which can cleave and cause shedding of the extracellular portion of TNFα receptors [33]. We reasoned that promotion of this effect by ecSOD knockdown might explain the reduced abundance of full-length TNF receptors in siSOD3-treated cells. Soluble TNFR1 was quantified by ELISA in culture media after 24 hrs of exposure to TNFα or vehicle. siSOD3 treatment did not increase the abundance of TNFR1 in the media under either control or stimulated conditions. In contrast, there was significantly less soluble TNFR1 in media from siSOD3-treated cells that were exposed to TNFα (Suppl. Fig. 1). This is likely to reflect the reduced overall abundance of the receptor and does not support an increase in shedding as the fundamental cause of this difference.

ecSOD and Integrin Signaling

ecSOD binds to the cell surface via both heparin sulfate proteoglycans [20] and Fibulin-5 [23]. Fibulin-5 also interacts directly with integrin receptors [34]. This places ecSOD in close proximity to integrins, which can be redox regulated [35], but the ability of ecSOD to impact integrin signaling has not been previously explored. As an initial test of the potential role of this protein association in TNFα signaling we knocked down Fibulin-5 (siFBLN5) and determined the impact on NF-κB activity and FAK phosphorylation. Like siSOD3, siFBLN5 reduced both resting and TNFα-induced NF-κB activity (Fig. 4A). Resting p-FAK was modestly but significantly increased but TNFα activation of FAK was enhanced (Fig. 4B). The ability of the siRNAs to reduce protein expression was confirmed (Figs. 4C and D) and these experiments also revealed that suppression of the abundance of each protein impacted expression of the other. Fibulin-5 protein levels were significantly reduced by siSOD3 treatment and ecSOD protein was approximately doubled by siFBLN5 (Fig. 4C and D). These observations support the concept that the interaction of these two proteins is physiologically important.

A human mutation in the C-terminal, heparin binding region of SOD3 (R213G) decreases binding to the cell surface and results in an approximately 10-fold increase in plasma levels of the enzyme. Heterozygotes for this mutation have increased risk of ischemic heart disease [36]. ecSOD associates with the C-terminal portion of Fibulin-5, primarily with a cationic stretch (RKKRRR) from aa210-215 [23], while the N-terminal portion of Fibulin-5 binds to integrins in an RGD-dependent manner [34]. The region of ecSOD that binds to Fibulin-5 has not been identified. To determine if Fibulin-5 might compete with heparin for association with the C-terminal region of ecSOD we deleted the C-terminal portion of the protein (aa190-222) and co-expressed this, or an R213G mutant, with full length Fibulin-5. Association was assessed by immunoprecipitation (IP) and Western blotting (Fig. 5). These C-terminal mutations did not interfere with ecSOD-Fibulin-5 binding. Neither did deletion of the N-terminal portion of ecSOD (aa1-54) that precedes the enzymatically active region of the protein [37]. Thus, it appears that it is the functional region of ecSOD that binds to

Fibulin-5. This raises the possibility of a functional interaction between the proteins that extends beyond co-localization.

Based on the impact of siFBLN5 on signaling and the established ability of that protein to bind to integrins we sought to determine if ecSOD modulates integrin activation by TNFα. We assessed the effect of siSOD3 and TNFα on downstream effectors of integrin signaling [38]. TNFα increased phosphorylation of FAK (p-FAK), protein kinase B (p-Akt) and glycogen synthase kinase 3β (p-GSK3β) which are downstream targets of ILK (Fig. 6). siSOD3 significantly increased both resting and TNFα-induced phosphorylation of FAK and GSK3β while Akt signaling was unaffected. These data suggest that ecSOD participates in tonic suppression of integrin signaling and may also limit integrin activation by TNFα.

Integrin receptor activation is associated with increased NF-κB activity [39]. To determine which integrin protein contributes to altered NF-κB activation following exposure to siSOD3, we utilized the luciferase reporter system with blocking antibodies targeting the extracellular domain of integrin receptor proteins known to be expressed in VSMCs. Blockade of α5 integrins significantly impaired TNFα-induced NF-κB activation while anti-α4, αv, β1, or β3 antibodies had no effect (Fig. 7). Fibulin-5 has been shown to bind to the fibronectin receptor, α5β1 integrin, in VSMCs [34]. We therefore utilized anti-α5 and β1 blocking antibodies targeting these two integrin subtypes to determine if TNFα-induced FAK and GSK3β phosphorylation could be attributed to this pathway. As in the previous set of experiments (Fig. 6) under control conditions (non-specific IgG) siSOD3 increased resting p-FAK and p-GSK3β, This effect on unstimulated cells was abrogated by an antibody to β1 integrin. The response was reduced by anti-α5 antibody, but the effect was not statistically significant. The combined increase in phosphorylation induced by the combination of siSOD3 and TNFα was significantly impaired by both integrin antibodies (Fig. 8). Collectively, these data point to an ability of ecSOD to maintain α5β1 integrins in an inactive state.

To assess the impact of FAK and ILK on NF-κB activity we utilized siFAK and siILK. Both resting and TNFα-stimulated NF-κB activity were remarkably reduced by knockdown of either FAK or ILK (Fig. 9). Thus, the observed decrement in NF-κB activation by TNFα in cells lacking ecSOD cannot be attributed to integrin activation. Rather, the ability of siSOD3 to promote integrin signaling may have masked the true extent of the disruption of NF-κB activation via TNFα receptors.

Finally, we assessed oxidation of integrin α5 and β1 by quantification of cysteine sulfenylation (SOH) using DCP-Bio1. Both integrin α5 and β1 were sulfenylated in response to TNFα after 3 min. Sulfenylation was enhanced following siSOD3 treatment but in the setting of ecSOD knockdown TNFα no longer caused additional oxidation (Fig. 10). These data suggest that TNFα induces integrin oxidation that can be modulated by ecSOD. This may affect integrin protein structure and alter signaling.

Effect of ecSOD knockout (KO) on TNFα-induced inflammation in intact blood vessels

Analysis of the role of ecSOD knockdown on TNFα signaling in cultured VSMCs revealed a combination of reduced canonical TNFα signaling and augmented integrin activation.

To assess the overall impact of losing ecSOD in VSMCs in a more complex physiologic system we created VSMC-selective ecSOD null mice (SOD3 KO) using $SOD3^{flox/flox}$ mice expressing smooth muscle myosin heavy chain-Cre. To isolate the role of TNFα signaling, rings of first and second order mesenteric resistance arteries were placed in tissue culture and exposed to TNFα (10 ng/ml) or vehicle for 48 hours. Wire myography recording TNFα-treated vessels from WT mice displayed an increased maximal response to alpha-adrenergic stimulation induced by either phenylephrine (PE) or norepinephrine (NE). This effect was absent in SOD3 KO vessels. No differences in contractile sensitivity (EC_{50}) were observed (Fig. 11A and B). Following contraction in response to PE (10^{-6} M), relaxation was assessed in response to both the endothelium-dependent agonist acetylcholine (ACh, Fig. 11C) and the endothelium-independent nitric oxide donor sodium nitroprusside (SNP, Fig. 11D). Dilation of WT rings to both agents was impaired following TNFα exposure as reflected by a significant increase in EC_{50} (ACh: $LogEC_{50}$, WT −7.3 ± 0.08 vs. WT+TNFα −6.7 ± 0.07, $p < 0.05$; SNP: $LogEC_{50}$, WT −7.4 ± 0.08 vs. WT+TNFα −6.6 ± 0.09, $p < 0.05$). In contrast, SOD3 null vessels were partially protected from TNFα-induced loss of responsiveness to ACh ($LogEC_{50}$, KO −7.5 ± 0.07 vs. KO + TNFα −7.2 ± 0.08, $p < 0.05$; WT+TNFα −6.7 ± 0.07 vs. KO+TNFα −7.2 ± 0.08, $p < 0.05$) but completely protected from impairment of the response to SNP ($LogEC_{50}$, WT+TNFα −6.6 ± 0.09 vs. KO + TNFα −7.4 ± 0.09, $p < 0.05$). Preservation of these vasodilator responses in SOD3 null vessels is consistent with the impaired TNFα signaling observed in cultured VSMCs.

DISCUSSION

TNFα, and multiple other pro-inflammatory signaling molecules (IL-1β, angiotensin II, endothelin) induce Nox1-dependent deposition of $O_2^{\bullet-}$ into the extracellular space. In VSMCs exposed to TNFα this $O_2^{\bullet-}$ is predominantly derived from Nox1 activity [16, 17, 40] and is required for signal transduction [41]. ecSOD has commonly been viewed as a protective extracellular antioxidant enzyme. We therefore postulated that reducing the abundance of the protein would enhance the pro-inflammatory effects of TNFα. Surprisingly, siSOD3 impaired multiple indices of canonical TNFα signaling, and these effects were associated with reduced TNFR1 and TNFR2 expression. Knockdown of Fib-5, which binds ecSOD at the plasma membrane, similarly impaired signaling, suggesting that localization of ecSOD is important. Since Fib-5 also binds to integrins, the impact of siSOD3 on integrin signaling was explored. TNFα caused significant transactivation of integrins, and loss of ecSOD enhanced resting activation of α5β1 integrins as reflected by increased phosphorylation of FAK and GSK3β (phosphorylated by ILK) that was prevented by α5 and β1 siRNA or blocking antibodies. FAK and GSK3β phosphorylation were also significantly increased after TNFα treatment in siSOD3 compared to siControl cells. FAK and ILK activation appear to contribute to the overall inflammatory response to TNFα, as blockade of α5 integrins or siRNA knockdown of FAK or ILK all significantly impaired NF-κB activation. Dismutation of extracellular $O_2^{\bullet-}$ by ecSOD appears to have important redox-based effects on these integrins as TNFα caused sulfenylation of both α5 and β1 proteins and siSOD3 increased resting integrin oxidation but prevented TNFα from causing further oxidation. The ability of siSOD3 to impair TNFα signaling in cultured cells was mirrored in intact mesenteric blood vessels lacking ecSOD only in VSMCs. These arteries

were protected from *ex vivo* TNFα-induced vasomotor dysfunction. These data reveal a previously unsuspected role for ecSOD, supporting TNFR1 signaling, suppressing resting integrin activity, and limiting integrin transactivation by the cytokine.

While $O_2^{\bullet-}$ derived from NADPH oxidases is critical for normal physiological signaling, overproduction, ineffective clearance, inappropriate localization or altered metabolism of ROS can all cause oxidative stress [40]. The ability of Fib-5 knockdown to recapitulate effects of siSOD3 (Fig. 4A and B) suggests that localization of the dismutase by Fib-5 is critical for its ability to regulate TNFα signaling. This point is emphasized by the observation that while siFib-5 dramatically increased total ecSOD protein levels (Fig. 4D), signaling via both NF-κB and FAK were altered in a manner similar to ecSOD depletion. Association of ecSOD with Fib-5 is critical for localization of ecSOD to blood vessels as isolated aortae from Fib-5 null mice displayed an ~65% decrease in the abundance of the protein. This was associated with a >20-fold increase in SOD-inhibitable dihydroethidium fluorescence in Fib-5 null vessels, reflecting a dramatic increase in the abundance of $O_2^{\bullet-}$ [23]. Our observation that ecSOD interacts with Fib-5 via its enzymatically active region (Fig. 5) also raises the possibility that interaction may modify dismutase activity. This Fib-5 binding site is distinct from where ecSOD interacts with heparan/heparan sulfate proteoglycans via a C-terminal heparin binding domain. Heparin binding is physiologically relevant as a human polymorphism in this region (R231G) causes loss of association and redistribution of human SOD3 away from the cell surface, resulting in a 10-fold increase in plasma SOD concentration. Individuals with this sequence variant have an increased risk of ischemic heart disease [36].

Despite a reduced abundance of TNFR1 and 2, phosphorylation of FAK and GSK3β was potentiated by siSOD3 (Fig. 6). This suggests that FAK and ILK, downstream effectors of integrin signaling, are tonically suppressed by the presence of ecSOD. This effect is dependent upon α5β1 integrins based on the ability of selective antibodies to impair the increase in FAK and GSK3β phosphorylation following treatment with siSOD3 (Fig. 8). The observation that knockdown of either FAK or ILK reduced TNFα-mediated NF-κB activation (Fig. 9), suggests that this pathway promotes TNFα-induced inflammation. A variety of integrins, including fibronectin-binding α5β1, regulate VSMC adhesion, migration, proliferation, contraction, and differentiation [42]. Consistent with our observations, α5β1 integrins trigger FAK-dependent NF-κB activation in response to oxidized LDL [43]. Importantly, LDL activates Nox1 in VSMCs and interference with Nox1 function blocks LDL-induced proliferation [44]. Thus, ecSOD may play an important role in tonic reduction of integrin-dependent inflammation related to hyperlipidemia. It is important to note, however, that despite this integrin-dependent, pro-inflammatory effect of losing ecSOD, the overall effect is anti-inflammatory. Multiple indicators of inflammation (NK-κB activation, IL-6 production, iNOS expression) are suppressed by siSOD3 under both resting and TNFα-exposed conditions.

The need for localization of ecSOD is likely related to the redox chemistry of $O_2^{\bullet-}$ and it's extremely short half-life. The presence of ecSOD suppresses resting oxidation of both α5 and β1 integrins, so what oxidant is responsible for increased oxidation in its absence (Fig. 10)? Spontaneous dismutation to H_2O_2 occurs by second-order kinetics with a rate constant

in the ~10^6 $M^{-1}s^{-1}$ range [45]. Dismutation becomes first-order and is accelerated to ~2 x 10^9 $M^{-1}s^{-1}$ by ecSOD [46]. Modeling of $O_2^{\bullet-}$ metabolism in mitochondria estimated that the presence of manganese SOD (SOD2 gene) in that compartment reduces the half-life of $O_2^{\bullet-}$ from ~100msec to ~35μsec, and its diffusion distance from ~50μm to ~400nm while also producing higher local concentrations of H_2O_2 [47]. Similarly, reduced ecSOD expression would be anticipated to extend the sphere of influence of $O_2^{\bullet-}$ outward from Nox1 while simultaneously reducing the local concentration of H_2O_2. Superoxide is itself capable of oxidizing cysteine thiols, although the precise chemical mechanism remains under significant debate [48]. Increased $O_2^{\bullet-}$ may also promote formation of metabolites of $O_2^{\bullet-}$ that are highly capable of oxidizing thiols. Enzymatic dismutation of $O_2^{\bullet-}$ limits its ability to reduce iron to the ferrous (Fe^{2+}) state, which in the presence of H_2O_2 can participate in Fenton chemistry to produce hydroxyl radical ($HO^{\bullet}$) [49]. $O_2^{\bullet-}$ is membrane impermeant but may cross via anion channels [50, 51]. Thus, intracellular effects of altered ecSOD abundance cannot be ruled out. However, increased sulfenylation of α5 and β1 integrins after knockdown of ecSOD is likely to be occurring at extracellular sites as these proteins are rich in extracellular cysteine residues, many of which are engaged in disulfide bonding. Although H_2O_2 is uncharged it is highly polar and therefore also has relatively limited membrane permeability [52] but it readily crosses the plasma membrane via aquaporin channels [53]. The complex, ROS-dependent chemistry that somehow results in integrin oxidation highlights how much remains to be learned about subcellular localization of $O_2^{\bullet-}$, $NO^{\bullet}$ and H_2O_2 and their interdependent roles in redox signaling.

The ability of siSOD3 to reduce the inflammatory response of cultured VSMCs to TNFα was recapitulated by the protective effect of VSMC-specific ecSOD KO on the response of intact blood vessels to TNFα *in vitro*. We previously demonstrated that the impact of a 48hr exposure to TNFα in tissue culture was primarily to impair VSMC responsiveness to vasodilators with a more modest impact on the endothelium [50]. This result was confirmed in the current work. In WT vessels, TNFα augmented the maximal contractile responses to both PE (α1) and NE (α1 and α2) to a similar degree. TNFα also impaired the vasodilator response to both ACh and SNP. These effects were almost completely abrogated in VSMC-specific SOD3 null mice, although these vessels still exhibited a small but significant rightward shift in the dose-response to ACh. This likely demonstrates that modest endothelial dysfunction was present in both groups (Fig. 11). Using tissue culture to expose vessels to TNFα allowed isolation of the TNFα response which would not be possible in intact animals where TNFα triggers a complex secondary inflammatory response. This work confirms the signaling defect characterized in cultured cells in a much more complex system.

Collectively, these data demonstrate a novel and potentially critical role of $O_2^{\bullet-}$ and ecSOD in supporting “housekeeping” TNFα signaling and providing tonic suppression of integrin signaling and associated VSMC inflammation. A fundamental assumption of efforts to treat “oxidative stress” has been that oxidants play a dominantly deleterious role in pathologic states. Goals of therapy have been to inhibit over-activated pro-inflammatory signaling pathways and to scavenge overproduced or misplaced ROS that are downstream of this signaling. The failure of these efforts to produce significant clinical benefit in cardiovascular disease has been frustrating and has been attributed either to failure to achieve adequate

suppression of ROS levels, or to target the correct ROS. The current work highlights the need for better understanding of the intricacies of ROS signaling. Overwhelming the housekeeping functions of critical antioxidant systems may mitigate otherwise beneficial effects of ROS scavenging. While there is real potential to modulate TNFα signaling via control of redox-dependent reactions, optimization of this approach may require targeting of ROS in a site- and oxidant species-specific manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

GRANT SUPPORT

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ABBREVIATIONS

ecSOD	extracellular superoxide dismutase
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
Fib-5	Fibulin-5
GSK3β	glycogen synthase kinase-3β
IL-6	interleukin-6
ILK	integrin-linked kinase
INK	c-Jun N-terminal kinase
MAPK	mitogen activated protein kinase
NADPH	reduced nicotinamide-adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
Nox	NADPH oxidase
ROS	reactive oxygen species
siRNA	small interfering ribonucleic acid
SOD3	extracellular superoxide dismutase
TNFα	tumor necrosis factor-α
TNFR	tumor necrosis factor-α receptor
VSMC	vascular smooth muscle cell

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HIGHLIGHTS

- ecSOD (SOD3) knockdown blocks canonical, pro-inflammatory TNFα signaling in VSMCs.
- SOD3 knockdown (siSOD3) activates α5 and β1 integrin signaling.
- TNFα or siSOD3 increases sulfenylation (oxidation) of α5 and β1 integrins.
- The enzymatic region of ecSOD binds to fibulin-5, which modifies TNFα signaling.
- Smooth muscle-specific ecSOD KO prevents TNFα-induced vascular dysfunction.

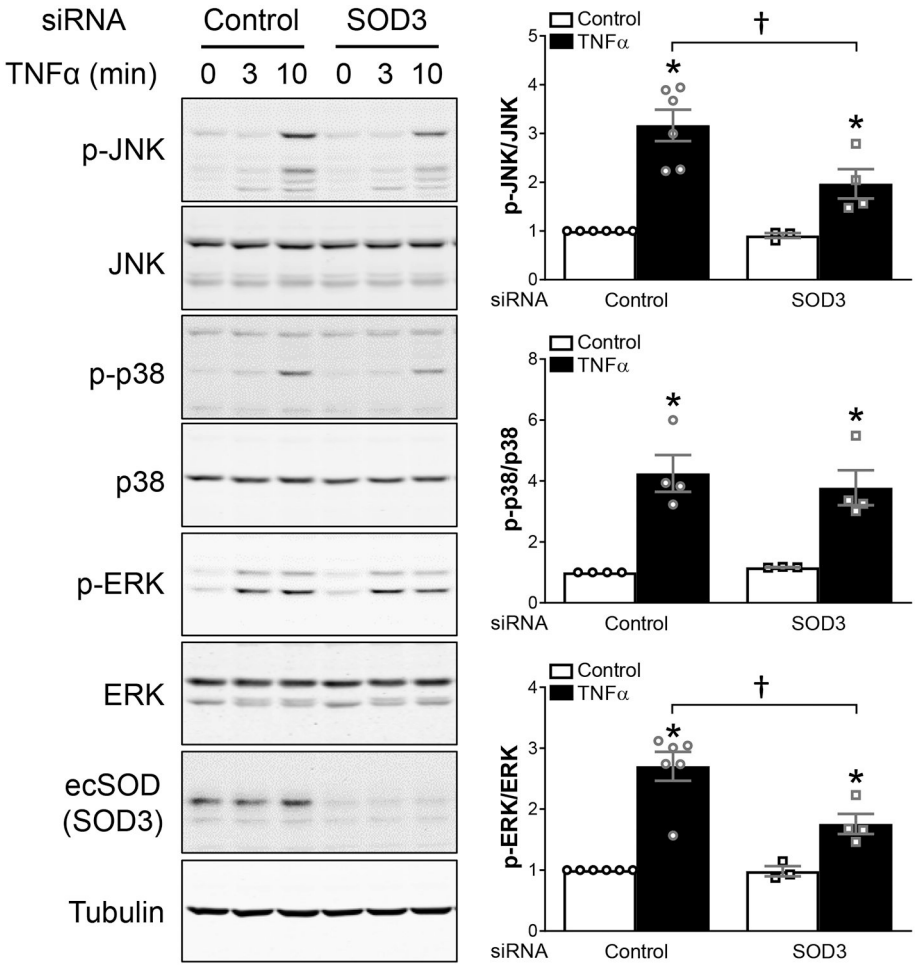

Figure 1.

Knockdown of ecSOD by siRNA (siSOD3) attenuates TNFα (10 ng/mL)-mediated MAPK activation, p-JNK (JNK phosphorylation) and p-ERK (ERK phosphorylation) in VSMC. Bar graphs show the quantification of protein expression in 10 min exposure of TNFα. Results are presented as mean ± SEM in each experimental group after normalization to control (siControl, 0 min TNFα) levels. *p<0.05 compared to control in control siRNA (siControl), †p<0.05 (n=3-6).

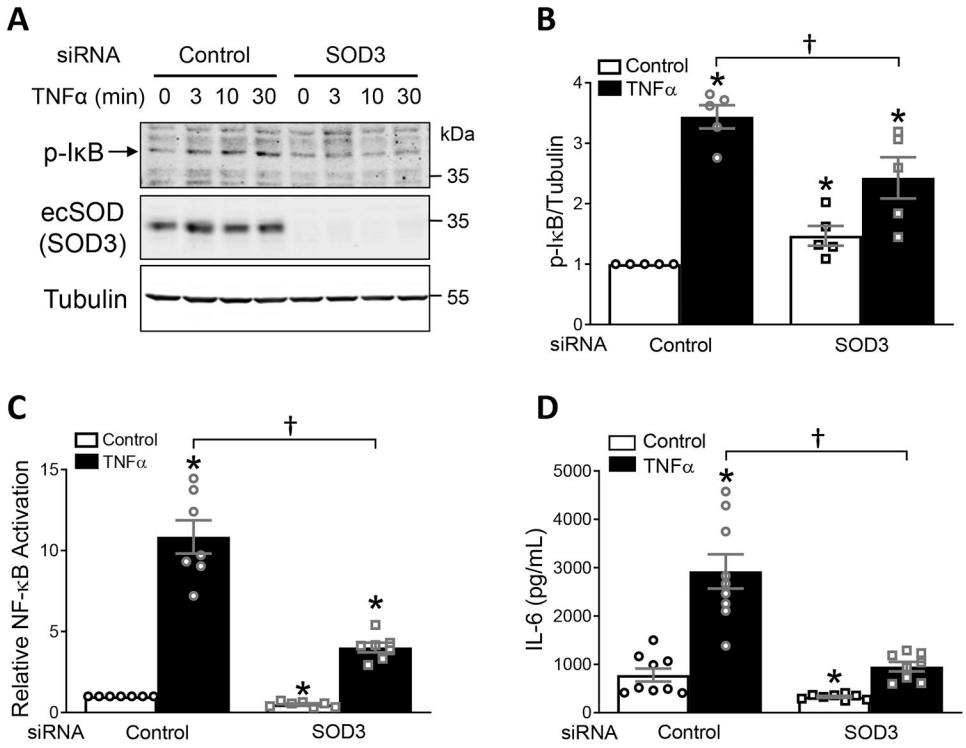

Figure 2.

TNFα-mediated inflammation is attenuated by ecSOD knockdown. **A**. IκB phosphorylation (p-IκB) at rest is increased slightly by siSOD3 but the increment in phosphorylation induced by TNFα is diminished. **B.** Bar graphs reflect analysis of western blot data at zero and 10 min. *p<0.05 compared to a no TNFα control following Control siRNA. †p<0.05 (n=5). **C**. TNFα-induced NF-κB activation is decreased in SOD3 knockdown in VSMC. Results are presented as mean ± SEM in each experimental group after normalization to control. *p<0.05 compared to Control in Control siRNA. †p<0.05 (n=7). **D**. IL-6 production in response to TNFα (10 ng/ml, 24 hrs) is reduced by SOD3 knockdown in VSMCs. *p<0.05 compared to a no TNFα control following Control siRNA. †p<0.05 (n=8-9).

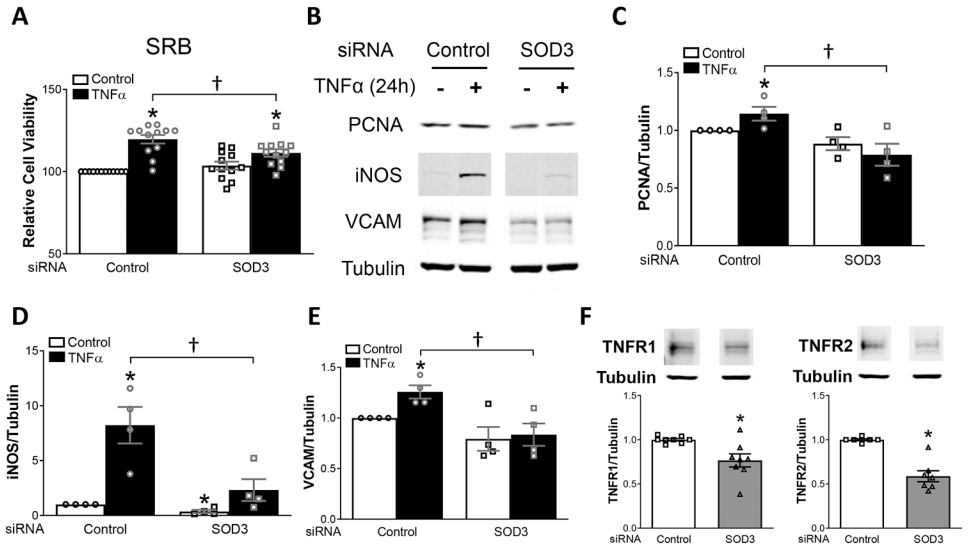

Figure 3.

Impaired responsiveness to TNFα following ecSOD knockdown. **A-C**. siSOD3 reduced TNFα-mediated cell viability as measured by SRB assay (**A**; n=12) and relative abundance of PCNA protein expression (**B** and **C**). **B**. Representative western blot shows expression of PCNA, iNOS, and VCAM after 24 hours of exposure to TNFα. **C-E**. Bar graphs show the relative protein abundance after normalization to that of tubulin (n=4). Results are expressed as mean ± SEM in each experimental group after normalization to control levels. *$p <$ 0.05 compared to Control in siControl, †$p < 0.05$. **F**. Expression of TNFRs is attenuated by siSOD3. Representative western blot images show expression of TNFR1 and TNFR2 in VSMC. Bar graphs show the relative abundance of receptors after normalization to tubulin. *$p < 0.05$ compared to siControl (n=7-8).

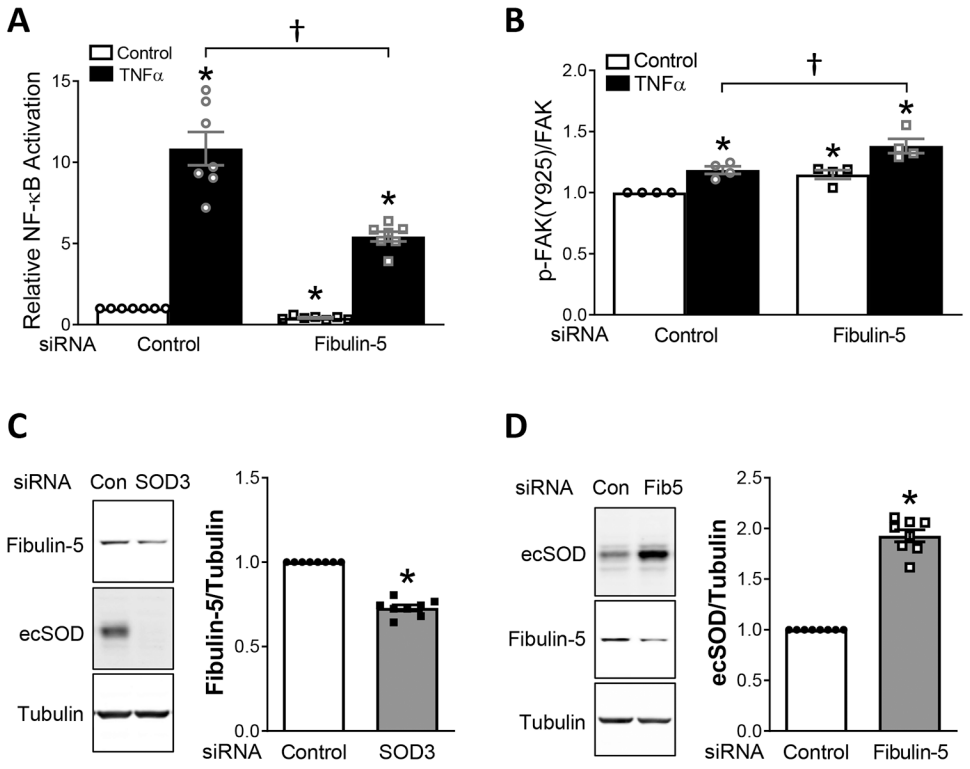

Figure 4.

Fibulin-5 modulates TNFα signaling. **A**. TNFα-induced NF-κB activation is decreased in Fib-5 knockdown in VSMC. Data are presented as mean ± SEM in each experimental group after normalization to control. *p<0.05 compared to Control in Control siRNA. †p<0.05 (n=7). **B**. Downstream signaling protein of integrin, FAK, is increased by Fib-5 knockdown. FAK phosphorylation (p-FAK at Y925) indicates FAK activation in response to TNFα (10 ng/mL, 10 min). *p<0.05 compared to Control in Control siRNA. †p<0.05 (n=4). **C** and **D**. Fib-5 and ecSOD expression affect each other in knockdown condition. With ecSOD knockdown, Fib-5 protein expression is attenuated (C), whereas ecSOD protein expression is potentiated by Fib-5 knockdown (D). *p<0.05 compared to Control siRNA (n=8).

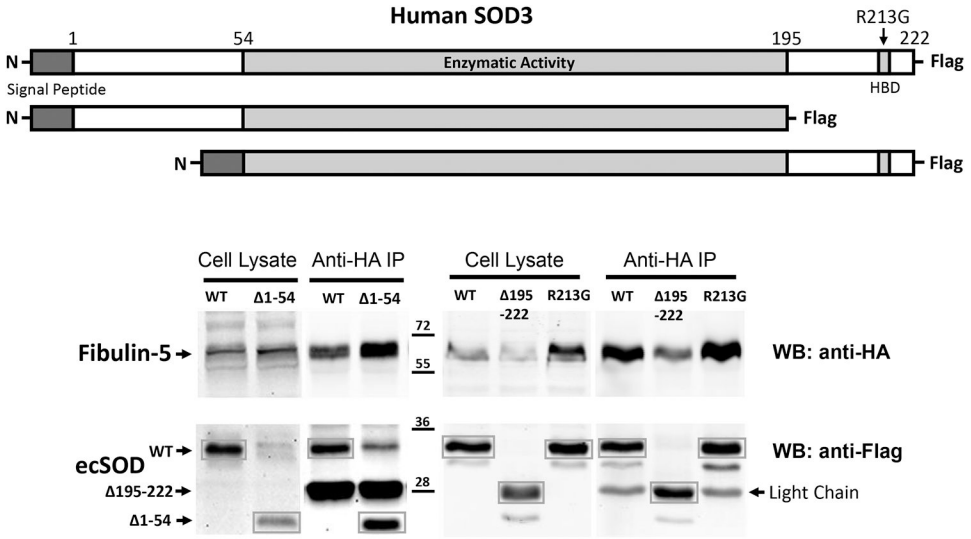

Figure 5.

Site of association of ecSOD with Fib-5. Human SOD3 is modified with R213G in heparin binding domain (HBD), deletion of C-terminal 195-222 (Δ195-222) or N-terminal 1-54 (Δ1-54) peptides. These SOD3 cDNA are tagged with Flag and Fibulin-5 is tagged with HA. IP was performed with anti-HA (Fibulin-5) antibody and western blot was analyzed with anti-HA (Fibulin-5) and anti-Flag (SOD3) antibodies. Representative western blots show that all 3 SOD3 mutations interact with Fibulin-5, suggesting that Fibulin-5 binds in Enzymatic activity area (aa54-195) of SOD3.

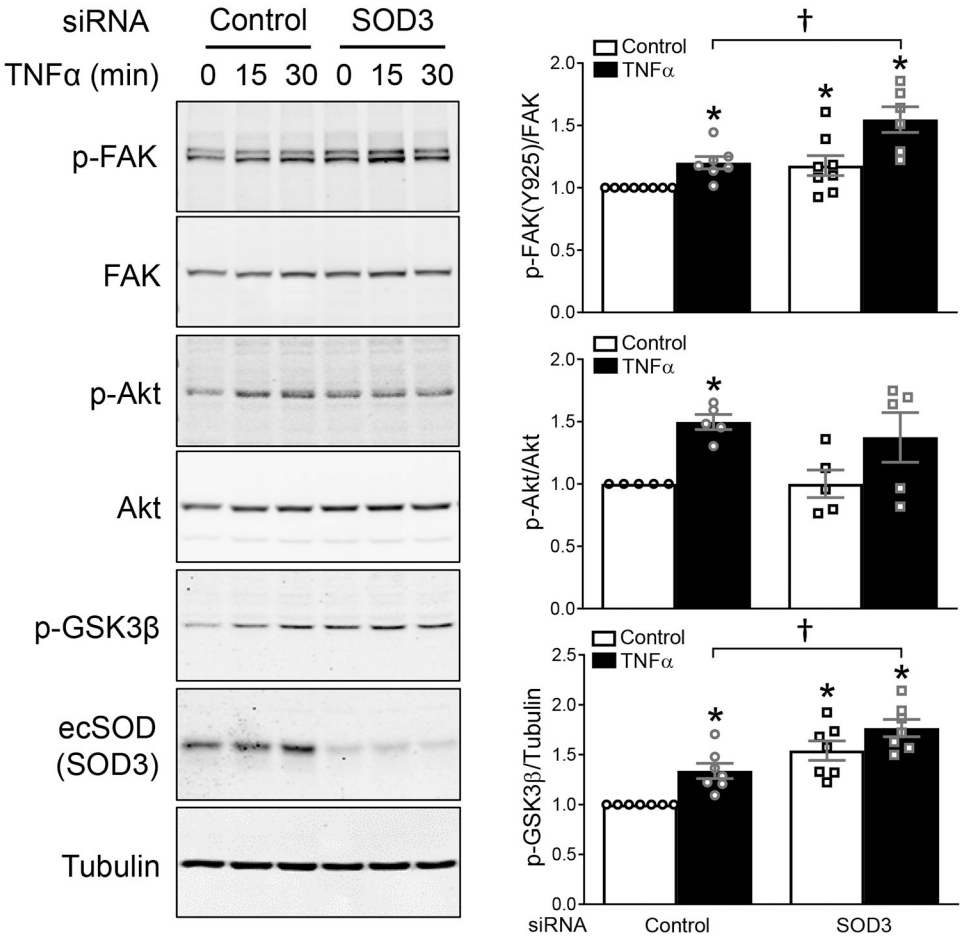

Figure 6.

Knockdown of ecSOD potentiates TNFα-mediated downstream integrin-dependent signaling. FAK activation (FAK phosphorylation; p-FAK) and GSK3β phosphorylation (p-GSK3β) after TNFα (10 ng/mL) stimulation are enhanced by siSOD3. Bar graphs show quantification of protein expression after 0 min and 15 min of exposure to TNFα. Results are presented as mean ± SEM in each experimental group after normalization to control (siControl, 0 min TNFα) levels. *p<0.05 compared to Control in control siRNA (siControl). †p<0.05 (n=5-8).

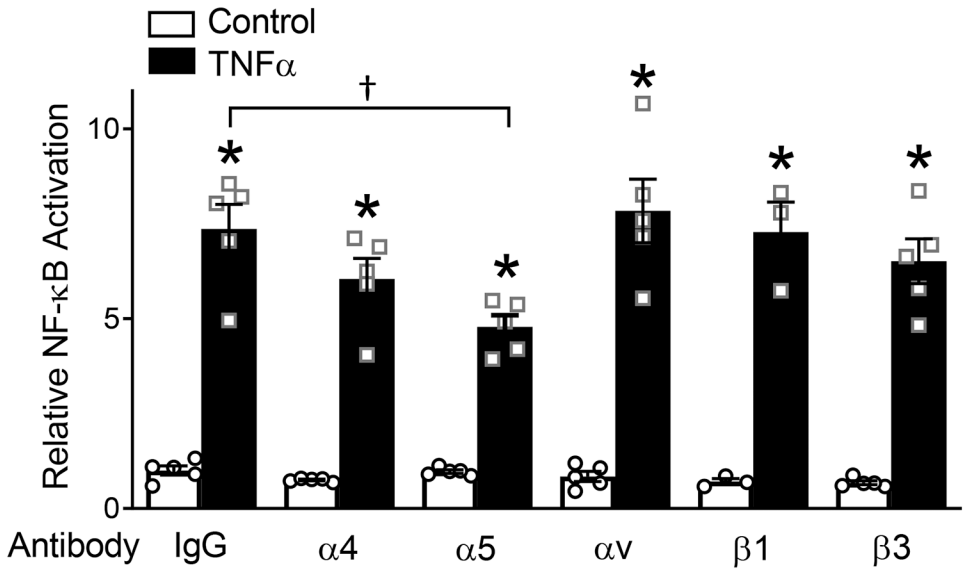

Figure 7.

TNFα-induced NF-κB activation in VSMCs is reduced by anti-α5 blocking antibody. Results are presented as mean ± SEM in each experimental group after normalization to control. *p<0.05 compared to Control (no TNFα) in IgG antibody. †p<0.05 (n=3-5).

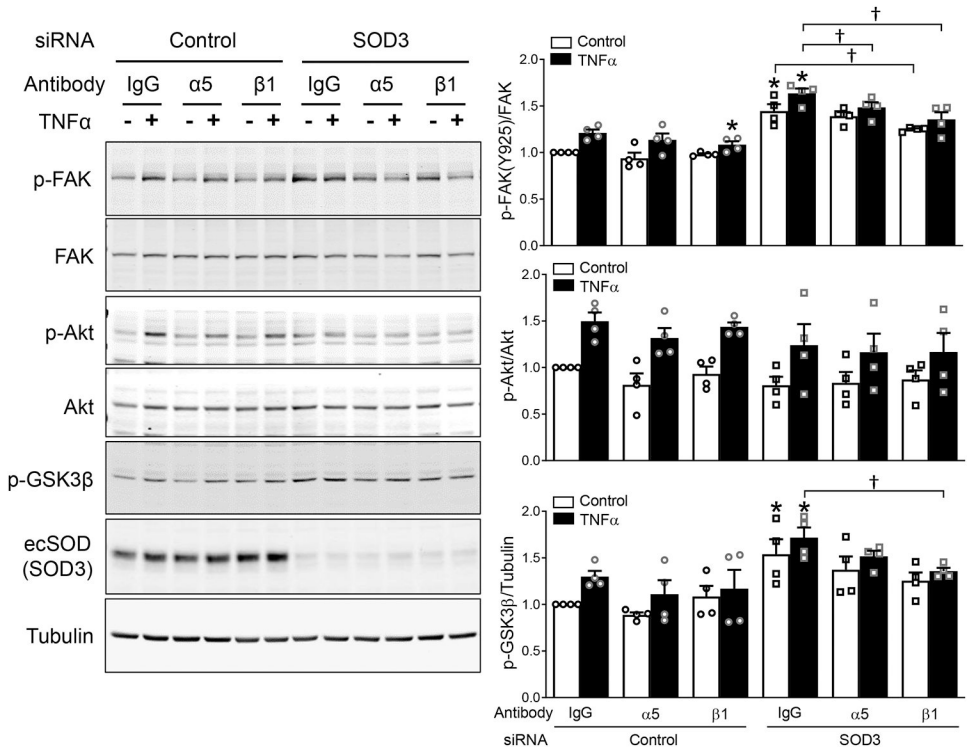

Figure 8.

Blocking antibodies targeting integrin α5 or β1 reduce activation of integrin-dependent signaling in response to siSOD3. Following TNFα stimulation for 10 min, ecSOD knockdown increased FAK (p-FAK) and GSK3β phosphorylation (p-GSK3β). Anti-α5 or anti-β1 antibodies reduce p-FAK and the anti-β1 antibody decreases p-GSKβ. *p<0.05 compared to TNFα in control siRNA and IgG. †p<0.05 (n=4).

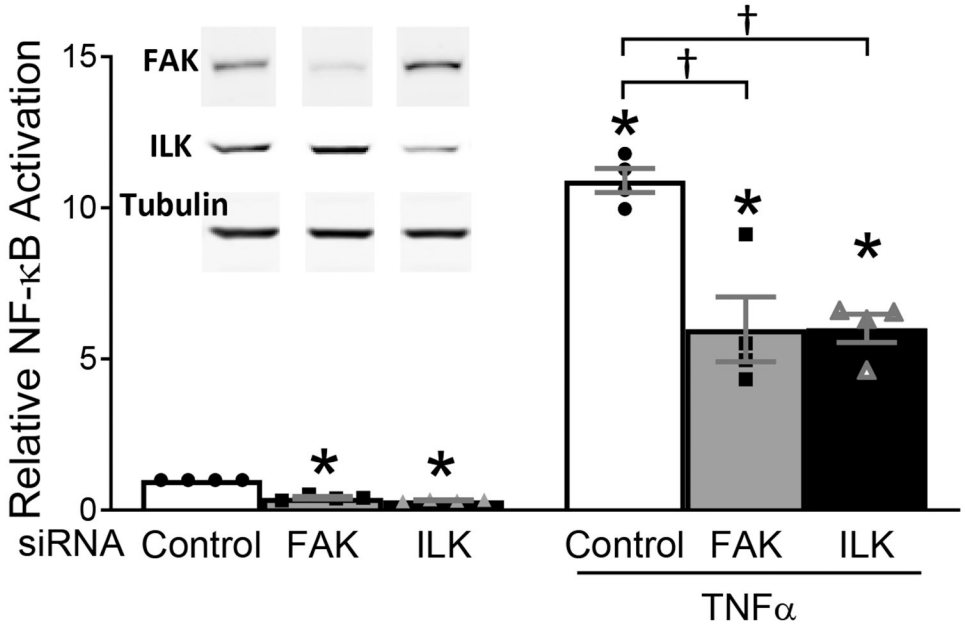

Figure 9.

Knockdown of integrin-associated signaling proteins, FAK and ILK, attenuate NF-κB activation by TNFα. Bar graphs are presented as mean ± SEM in each experimental group after normalization to control. Western blot figures show the knockdown of FAK and ILK protein expression. *p<0.05 compared to Control in control siRNA. †p<0.05 (n=4).

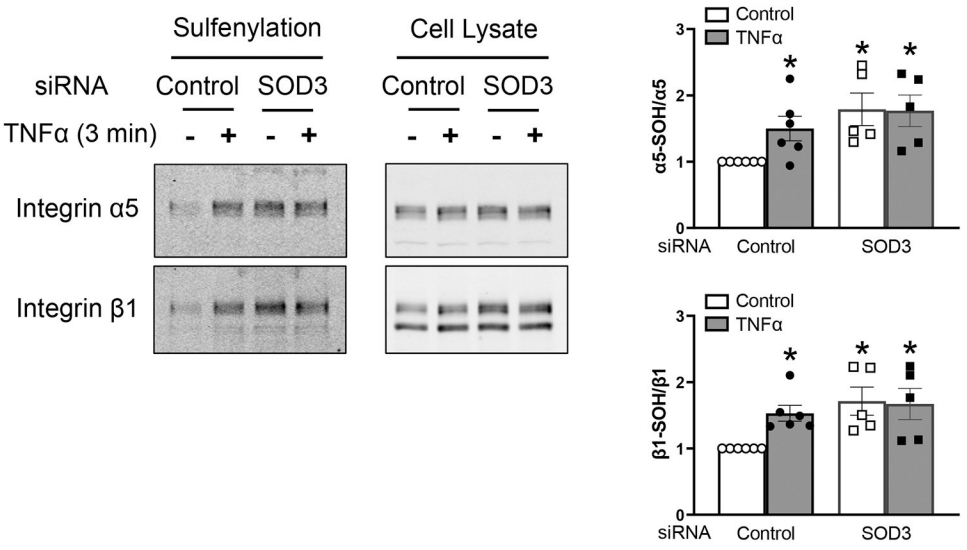

Figure 10.

Oxidation of integrin receptor proteins in response to siSOD3 and TNFα. Sulfenylation (-SOH) of integrin α5 and β1 is increased by TNFα stimulation or by siSOD3. The response to TNFα is lost in siSOD3-treatred cells. Representative western blot shows expression of sulfenylated integrin (α5 and β1) and total integrin protein after 3 min of exposure to TNFα in control or SOD3 siRNA. Bar graphs present the quantification of integrin α5 or β1 sulfenylation after normalization to each total integrin α5 or β1. Results are showed as mean ± SEM in each experimental group after normalization to control. *p<0.05 compared to Control in control siRNA (n=5-6).

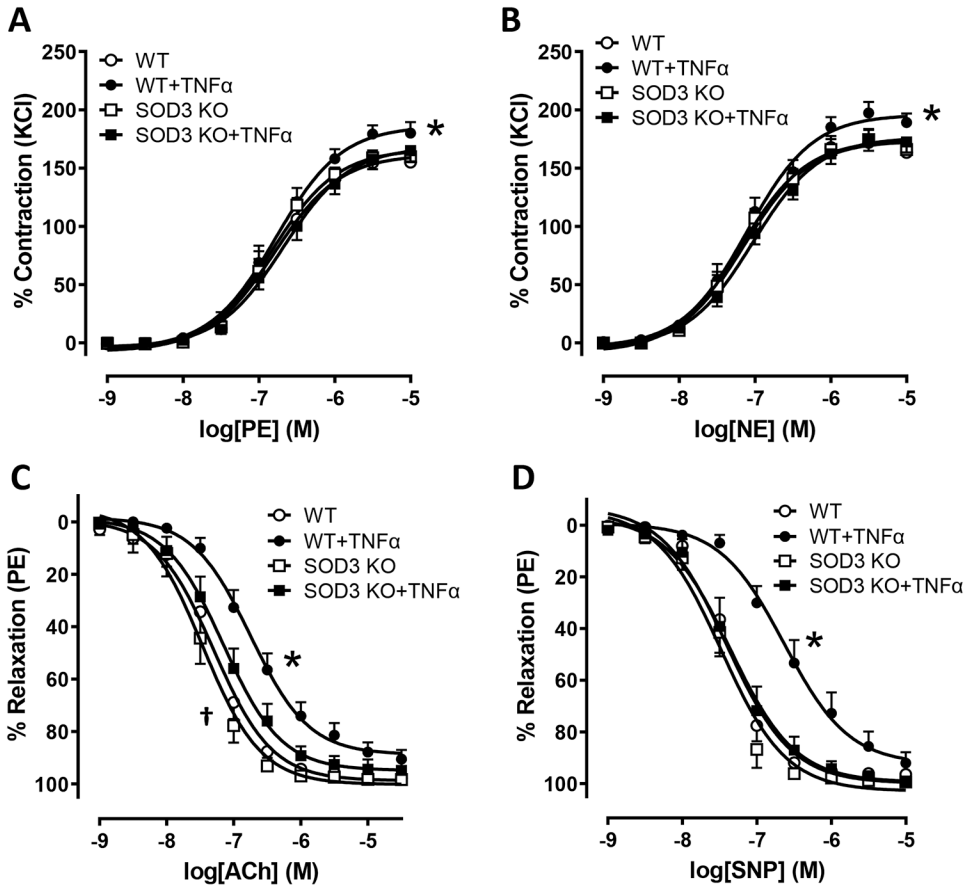

Figure 11.

TNFα-induced impairment of vascular function is prevented by SOD3 KO. Vascular reactivity was measured in mesenteric arteries from WT and VSMC-specific SOD3 KO mice. **A** and **B**. Contractile responses to PE (A) and NE (B) were enhanced by a 48hr exposure to TNFα in WT vessels (n = 5). This effect was absent in SOD3 KO vessels (n = 7-8). *$p < 0.05$ WT vs. WT+TNFα for E_{max}. **C**. Relaxation to ACh is impaired by TNFα (10 ng/mL, 2 days) in WT (n = 5) and this effect is mitigated by SOD3 KO (n = 8). *$p < 0.05$ WT vs. WT+TNFα or WT+TNFα vs. SOD3 KO+TNFα for EC_{50}, †$p < 0.05$ SOD3 KO vs. SOD3 KO+TNFα for EC_{50}. **D**. Relaxation to SNP was reduced by TNFα (10 ng/mL, 2 days) in WT (n = 5) rings and this effect was completely absent in SOD3 KO vessels (n = 7-8). *$p < 0.05$ WT vs. WT+TNFα for EC_{50} or WT+TNFα vs. SOD3 KO+TNFα for EC_{50}.