

**CAVEOLAE AS SPATIO-TEMPORAL COMPARTMENTS FOR ROS/RNS  
GENERATION AND NITROXIDATIVE STRESS SIGNALING**

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May, 2014

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## ABSTRACT

### CAVEOLAE AS SPATIO-TEMPORAL COMPARTMENTS FOR ROS/RNS GENERATION AND NITROXIDATIVE STRESS SIGNALING

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May, 2014

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During inflammatory conditions excessive production of reactive oxygen (ROS) and nitrogen species (RNS), peroxynitrite, is implicated in the development of vascular pathologies. Our previous studies showed that both NADPH oxidase enzyme complexes and eNOS localize to endothelial caveolae microdomains. Additionally, caveolae internalization has been shown as an activating mechanism for enzyme eNOS. However, roles of caveolae in ROS/RNS generation and downstream signaling roles in activating endothelial cells are not well known.

**Hypothesis:** Caveolae act as, a) micro-environments in providing spatio-temporal reaction compartments for ROS/RNS generation, tyrosine nitration of proteins, b) platforms to propagate localized nitroxidative signaling in inducing endothelial cell activation and dysfunction (ICAM-1, VCAM-1 expression), and c) intracellular redox signaling endosomes to regulate adhesion molecule expression.

**Objectives:** The aim of the study was to investigate whether, a) caveolae compartmentalize ROS, regulate localized tyrosine nitration of proteins, b) nitroxidative-

signaling in the endothelium is compartmentalized in caveolae, c) dynamin-2-dependent internalization of caveolae is important for activating redox signaling, and d) caveolae compartments can be targeted to reduce endothelial ROS

**Methods and results:** Cultured primary bovine aortic endothelial cells were stimulated with TNF $\alpha$  to generate ROS/RNS. Blockade of NADPH oxidase (gp91ds-tat) or scavenging of peroxynitrite (Uric acid) inhibited TNF $\alpha$ -induced protein tyrosine nitration, activation of the NF $\kappa$ B, and upregulation of ICAM-1/VCAM-1 expression. To test the role of caveolae in this process, cultured cells were depleted of caveolin-1 (siRNA). Similar to inhibitors, TNF $\alpha$  failed to induce protein-tyrosine nitration, activate NF $\kappa$ B or enhance adhesion molecule expression in cells lacking caveolin-1. These findings were corroborated *in vivo* using Cav1KO animals. Our results show that several caveolar residing proteins were nitrated on tyrosine in response to TNF $\alpha$ . Here, immunoprecipitation of cell lysates with an anti-nitrotyrosine antibody revealed Src-family kinases (SFK) in the precipitated fraction. Moreover, SFK nitration was lost in cells depleted of caveolin-1. Given that SFK nitration is associated with enzyme activation, cells were pretreated with PP2 to inhibit SFK activity. We found that PP2 attenuated the NF $\kappa$ B and adhesion molecule pathway activated by TNF $\alpha$ . Depletion of dynamin-2 (Dyn2siRNA) or inhibiting GTPase activity (Dynasore) also showed reductions in ROS generation, NF $\kappa$ B redox signaling and ICAM-1/VCAM-1 expression. Development of caveolae targeting peptide tagged with gp91ds-tat showed inhibitions in compartmentalized ROS production.

**Conclusions:** Caveolae act as sites for ROS/RNS production where resident redox-sensitive second messengers are activated and propagate signals that regulate endothelial inflammatory phenotype. Targeting NADPH oxidase enzyme specifically in caveolae can be used a therapeutic strategy to limit vascular oxidative stress.

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A special thank goes to all my friends in the department of Anatomy and Cell biology, Cardiovascular Research Center and all other departments for showing me how there is more to life than just in laboratory.

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## LIST OF ABBREVIATIONS

ROS :	Reactive Oxygen Species
RNS :	Reactive Nitrogen Species
NADPH Oxidase :	Nicotine Amide Adenine Dinucleotide Phosphate Oxidase
eNOS :	Endothelial Nitric Oxide Synthase
ICAM-1 :	Intercellular Adhesion Molecule-1
VCAM-1 :	Vascular Cell Adhesion Molecule-1
ONOO :	Peroxynitrite
SFK :	Src Family Kinases
PP2:	4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4- d]pyrimidine
TNF $\alpha$ :	Tumor Necrosis Factor- $\alpha$
TNFR1:	Tumor Necrosis Factor- $\alpha$ Receptor1
TRADD/TRAF:	TNF Receptor Associated Death Domain/TNF Receptor Associating Factor
GTPase:	Guanosine TriPhosphatase
CVDs:	Cardiovascular Diseases
CAD:	Coronary Artery Diseases
ECs:	Endothelial cells
VLDL:	Very Low Density Lipoprotien
LDL:	Low Density Lipoprotein
OxLDL:	Oxidized Low Density Lipoprotein
LPS:	Lipopolysaccharide
Ang-II:	Angiotensin-II

IL-1:	Interleukin-1
BH <sub>4</sub> :	TetraHydroBiopterin
O <sub>2</sub> <sup>-</sup> :	Superoxide
H <sub>2</sub> O <sub>2</sub> :	Hydrogen Peroxide
SOD:	SuperOxideDismutase
iNOS:	inducible Nitric Oxide Synthase
GPx:	Glutathione Peroxidase
Trx:	Thioredoxins
PON:	Paraoxonase
Prxs:	Peroxiredoxins
HO-1:	Heme Oxygenase-1
PTPs:	Protein Tyrosine Phosphatases
PTKs:	Protein Tyrosine Kinases
MAPKs:	Mitogen-Activated Protein Kinases
ERKs:	Extracellular Signal Regulated Kinases
SAPKs:	Stress Activated Protein Kinases
NFκB:	Nuclear Factor kappa-light-chain-enhancer of activated B cells
PKC:	Protein Kinase C
JNK:	c-Jun-NH2-terminal kinase
TLRs:	Toll-Like Receptors
PTN:	Protein-Tyrosine Nitration
nY:	Nitrotyrosine
3-NT:	3-NitroTyrosine
Nox:	NADPH Oxidases
FAD:	Flavin Adenine Dinucleotide

ER:	Endoplasmic Reticulum
TGF- $\beta$ :	Transforming Growth Factor- $\beta$
VSMCs:	Vascular Smooth Muscle Cells
DUOXs:	Dual Oxidases
VEGF:	Vascular Endothelial Growth Factor
NEFA:	Non Esterified Fatty Acids
CMECs:	Coronary Microvascular Endothelial Cells
PDGF:	Platelet Derived Growth Factor
ACE:	Angiotensin-Converting-Enzyme
HOPE:	Heart Outcome Prevention Evaluation
EUROPA:	European Trial on Reduction of Cardiac Events with Perindopril in Stable Coronary Artery Disease
CSD:	Caveolin Scaffolding Domain
CBM:	Caveolin Binding Motifs
Cav1KO:	Caveolin-1 Knock Out
CCVs:	Clathrin Coated Vesicles
ApoE <sup>-/-</sup> :	Apolipoprotein E Knock Out
BAECs:	Bovine Aortic Endothelial Cells
DHE:	Dihydroethidium
FBS:	Fetal Bovine Serum
L-NAME:	L-NG-Nitroarginine methyl ester
gp91ds-tat:	gp91 docking site-Trans-activator of Transcription
SDS-PAGE:	Sodium Dodecyl Sulfate-Polyacryl Amide Gel Electrophoresis
M $\beta$ CD:	Methyl- $\beta$ -Cyclo Dextrin
CBM Peptide:	Caveolin Scaffolding Domain Binding Motif Peptide

## CHAPTER 1 INTRODUCTION AND BACKGROUND

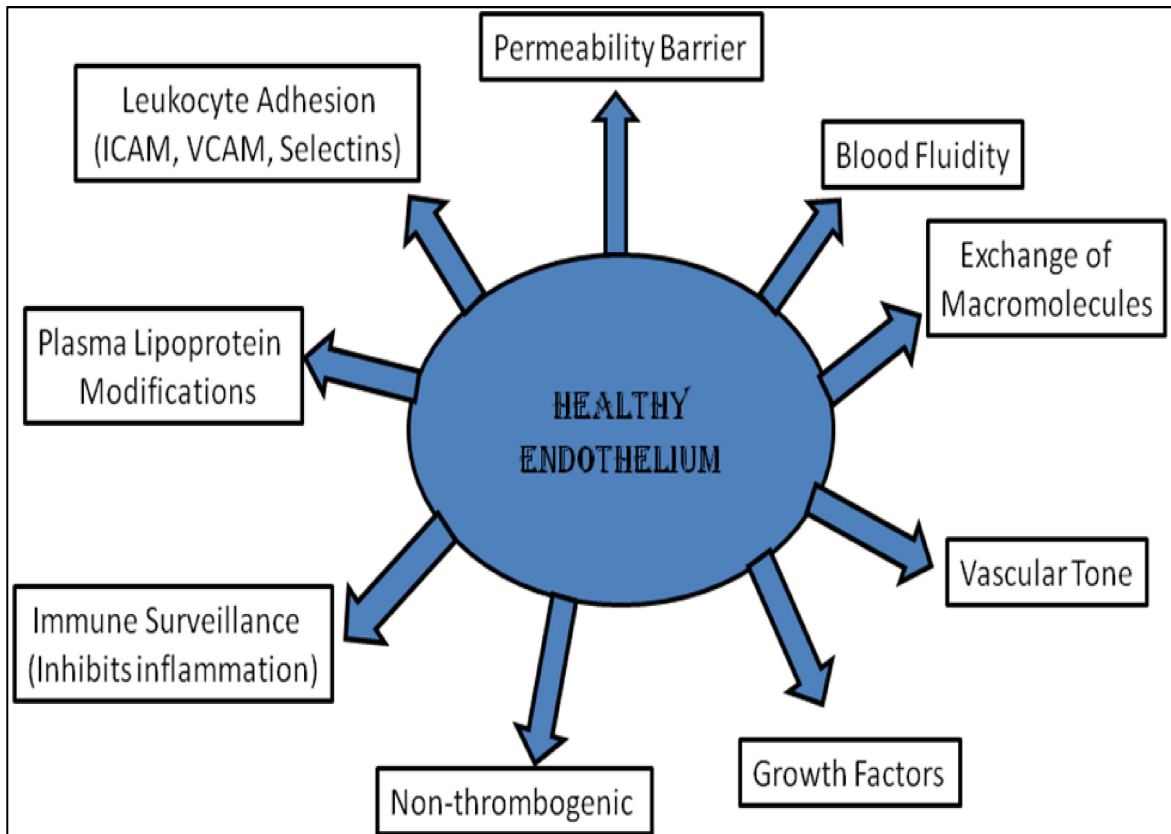
### Cardiovascular health, disease and role of endothelium

Cardiovascular diseases (CVDs) such as coronary artery disease (CAD), abdominal aortic aneurysms (AAA), heart failure and atherosclerosis are the leading cause of deaths worldwide (World Health Organization- 2010). This group of diseases constitutes the main cause of death in the Western world today. In addition, WHO expects CVDs to be the major killer globally within 15 years owing to both its rapidly increasing prevalence in developing countries and Eastern Europe and an accumulation of metabolic risk factors, including obesity and diabetes, in the Western world.

The basic design of large arteries and veins consists of an outer adventitia, (composed of fibroblasts, immuno-modulatory cells, resident progenitor cells, vasavasorum endothelial cells, nerves, elastic and collagen fibers), a medial layer composed of smooth muscle cells and innermost single cell layer of endothelial cells (ECs) that line the vessel lumen, called “endothelium”. The endothelium lines the entire circulatory tree and serves important functions related to vascular homeostasis (Figure 1) (Gonzalez et al., 2003; Kaperonis, Liapis, Kakisis, Dimitroulis, & Papavassiliou, 2006; Ross, 1993). The important functions of endothelium include, 1) formation of permeability barrier between circulating blood and interstitium, 2) maintaining the anticoagulant state of blood (Arnout, Hoylaerts, & Lijnen, 2006), 3) control the exchange of fluid and macromolecules between blood and the tissues (Minshall & Malik, 2006), 4) maintenance of vascular tone by generation of vasoactive compounds (Furchgott & Zawadzki, 1980), 5) production of growth factors important for the physiological functioning of the vessel wall, 6) modification of plasma lipoproteins, 7) inhibition of

inflammatory responses (Ley & Zarbock, 2006) and, 8) regulation of immune responses by expression of Selectins (E- and P-Selectins) and adhesion molecules (ICAM-1/VCAM-1) to facilitate rolling, adhesion and transmigration of leukocytes during inflammatory conditions. Initiation and progression of many CVDs is dependent on dysfunction in endothelial cells. Therefore, the endothelium plays the most important role in cardiovascular health. Since atherosclerosis is the first step in development of other cardiovascular diseases, it makes the understanding of process of atherosclerosis very essential for developing therapies to correct it.

The early phase of disease is characterized by recruitment of inflammatory cells to the artery wall. Hypercholesterolemia and hypertension being two major risk factors cause focal endothelial activation in large- and medium-sized arteries. High plasma levels of the cholesterol-rich very low-density lipoprotein (VLDL) and LDL rise, the lipoproteins infiltrate the artery wall to an extent that exceed the capacity for elimination and are retained in the extracellular matrix (Skalen et al., 2002). This accumulation sets off a cascade of events resulting in formation of lesions or plaques, which gradually impinge on the vessel lumen, impeding blood flow (Figure 2).



**Figure 1: Schematic showing important physiological functions performed by healthy endothelium.** Vascular endothelial layer is important for maintaining homeostasis as well as first line of defense against noxious stimuli.

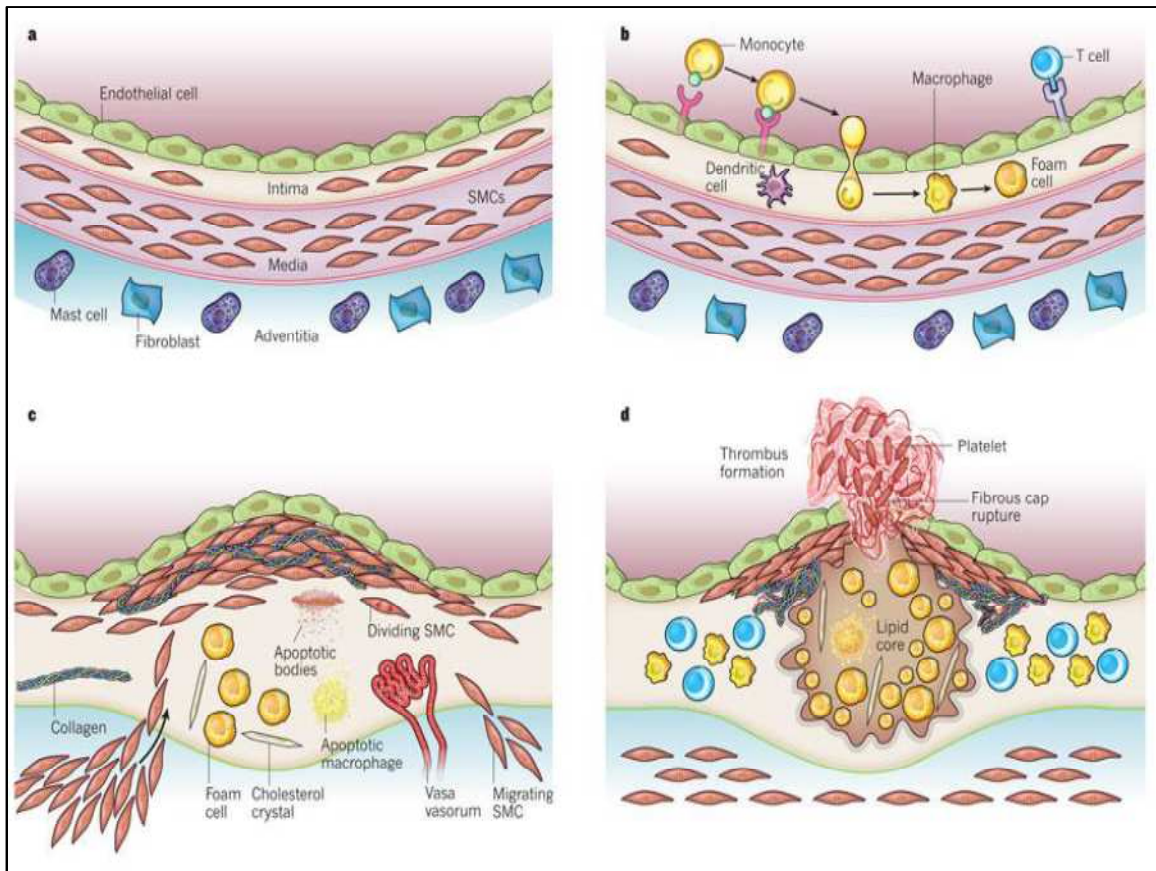
Atherosclerotic lesions, termed atheroma or atherosclerotic plaques, typically present as asymmetric focal thickening of innermost layer of the artery, the intima. The lesions are composed of connective tissue elements, cells like vascular smooth muscle and endothelial cells along with extracellular lipid droplets. The atheroma is preceded chronologically by so-called fatty streaks, which are sites of accumulation of lipid droplets and immune cells. Lipid-laden macrophages (termed “foam cells” because of the bubbly appearance of lipid droplets throughout their cytoplasm) dominate these fatty streaks, which also contain T cells, dendritic cells, and mast cells.

Atherosclerosis appears to be an inflammatory response to lipoprotein retention. LDL modification, through enzymatic attack or non-enzymatic oxidation in the intima, leads to release of bioactive phospholipids that can activate endothelial cells. Activated endothelial cells express several types of leukocyte adhesion molecules, which cause rolling of monocytes and lymphocytes on the vascular surface to adhere at the site of activation (Figure 2)(Cybulsky et al., 1991; Cybulsky & Gimbrone, 1991; Watson, 1997).

Once adherent to the endothelium, leukocytes migrate into the underlying intima in response to cause increased expression of adhesion molecular and inflammatory genes by endothelial cells and may, like LDL promote inflammation. These events tend to happen preferentially at sites of hemodynamic strain or hemodynamic flow patterns typical for atherosclerosis-prone segments (low average shear/laminar shear but high oscillatory shear stress) (Nakashima, Raines, Plump, Breslow, & Ross, 1998).

The plaque activation in response to inflammation, results in thrombosis and manifestation into clinical symptoms. There is a progressive reduction of luminal size in response to plaque obstructing blood flow. The thrombus formation requires damage to

the plaque surface initiated via activated macrophages, T cells, and mast cells. These cells produce several molecules that can destabilize lesions: pro-inflammatory cytokines like TNF $\alpha$ , proteases, coagulation factors, oxidative radicals and vasoactive molecules. These agents inhibit the formation of stable fibrous caps, attack cap collagen, and initiate thrombus formation, resulting in thrombosis and ischemia.



**Figure 2: Diagrammatic representation of series of events showing atherosclerosis.** A) Normal vessel lumen with healthy endothelium with underlying smooth muscle cells and adventitial layer of cells, B) inflammation induced endothelial activation resulting in leukocyte rolling-adhesion and sub-endothelial transmigration, C) the transmigration of cells in sub-endothelial layer results in foam cell formation, further accumulation of LDL, its oxidation (OxLDL), and d) initiation of cascade of cellular events leading to plaque formation, rupture and thrombosis (Libby, Ridker, & Hansson, 2011).

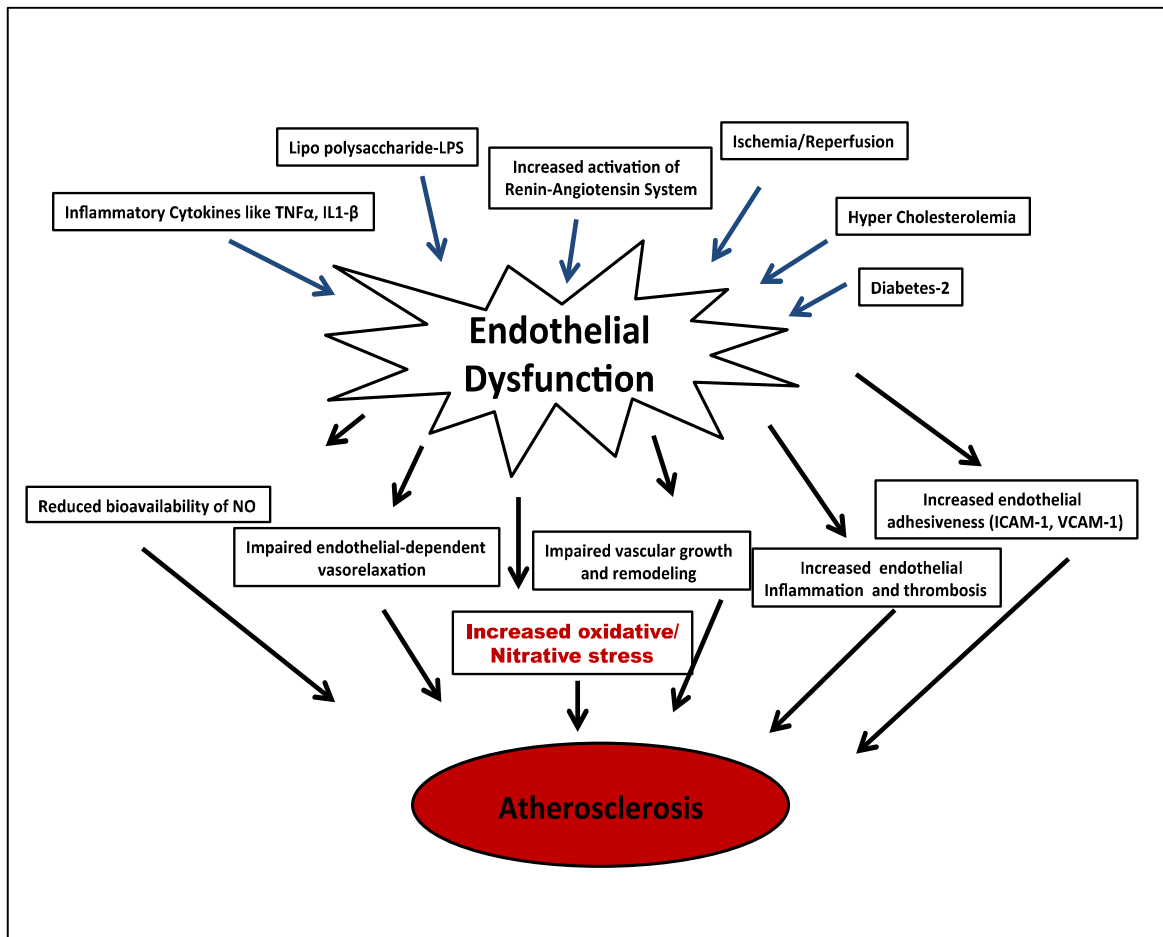
## Endothelial activation and dysfunction

Endothelial activation is a term used to denote a specific and complex change in endothelial phenotype. It is characterized most notably by an increase in endothelium-leukocyte interactions, which is essential during both physiological and pathological inflammatory responses. Activation of the endothelium occurs in response to diverse stimuli, including inflammatory cytokines, lipopolysaccharide (LPS) activation of the renin-angiotensin system, hypercholesterolemia, ischemia-reperfusion, physical trauma, and diabetes. These effectors increase the expression of cell surface adhesion molecules and selectins (E-selectin, P-selectin, vascular cell adhesion molecule; VCAM-1, Inter cellular adhesion molecule; ICAM-1), which enable leukocyte adhesion and transmigration across the endothelial barrier into the underlying tissue. This is a protective response of the blood vessel in response to inflammatory stimuli. The process is further enhanced by the release of pro-inflammatory cytokines and other factors by activated leukocytes and the endothelium itself via a feedback loop. Endothelial activation in response to excess noxious stimuli leads to a non-adaptive state called “endothelial dysfunction” which is characterized by the loss or dysregulation of the homeostatic mechanisms that operate in healthy endothelial cells. One of the other major physiological feature of endothelial dysfunction is impairment of endothelium-dependent vasorelaxation or disturbed balance between vasodilator and vasoconstrictor activity, caused by a loss of nitric oxide (NO) bioavailability in the vessel wall (Giannotti & Landmesser, 2007). Endothelial dysfunction is also an important underlying cause of pathological conditions such as, altered anti-coagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth and dysregulation of vascular remodeling (Gimbrone, 1995). Indeed, pro-inflammatory, pro-coagulant state of

the dysregulated endothelium is implicated in many pathologic conditions, including the early stages of atherosclerosis, sepsis, and hypertension and is thus a predictor of adverse cardiovascular events (Schachinger, Britten, & Zeiher, 2000; Schachinger & Zeiher, 2000).

The causative factors for the endothelial dysfunction include, smoking, high fat diet, obesity and hypercholesterolemia, diseases like, hypertension, diabetes, increased presence of oxidized-LDL, increased secretion of thrombin and inflammation. These factors lead to changes in endothelial physiology (shown in figure 3) including, 1) increased inflammation and thrombosis, 2) impaired vascular growth and remodeling, 3) a decline in NO bioavailability, 4) impaired vasorelaxation, 4) increased oxidative and nitrative stress induced by generation of reactive oxygen species (ROS) and nitrogen species (RNS) respectively.

The mechanism underlying noxious stimuli/atherogenic stimuli induced endothelial dysfunction include, altered cellular signaling via 1) increased activation of rennin-angiotensin system and Ang-II-receptor activation, 2) increased secretion of pro-inflammatory cytokines like TNF $\alpha$  and IL-1 ligand-receptor activity, 3) decreased activation, expression or lack of substrates for NO producing enzyme-eNOS, tetrahydrobiopterin (BH $_4$ ) (Pou, Pou, Bredt, Snyder, & Rosen, 1992; Wilcox et al., 1997), 4) increased activation of enzymes producing reactive oxygen species (ROS sources), 5) increased cellular and subcellular cross-reaction of reactive radicals. There is increasing amount of evidence showing that increased oxidative and nitrative stress is common underlying factors during initiation and progression of virtually all-vascular diseases.

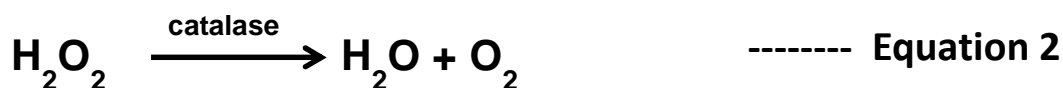
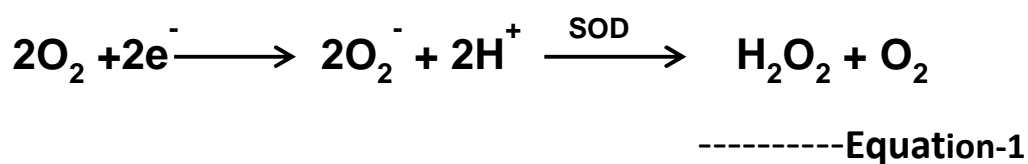


**Figure 3. Schematic showing risk factors inducing endothelial activation and dysfunction.** Increased exposure noxious stimuli causes loss of homeostatic functions of endothelium, state called as endothelial dysfunction. Increased oxidative and nitrative stress has been implicated as major players in initiation as well as progression of vascular diseases such as atherosclerosis.

## Reactive Oxygen and Nitrogen Species

Reactive oxygen species (ROS) include family of radical and non-radical oxygen species derived from molecular oxygen (Equation-1), produced in all aerobic organisms to counteract invading harmful organisms. These include, superoxide ( $O_2^-$ ), hydroxyl radical (OH $\cdot$ ), hydrogen peroxide ( $H_2O_2$ ), hypochlorous acid (HOCl), Ozone ( $O_3$ ), and singlet oxygen ( $^1O_2$ ). Excessive production of ROS results in oxidation of biological macromolecules, such as DNA, protein, carbohydrates, and lipids. The production of one ROS may lead to the generation of several other reactive species via radical chain reactions (Equation 1), starting a pathological feed-forward cycle of ROS. These ROS species can further be neutralized by endogenous enzymes like Catalase, converting  $H_2O_2$  into  $H_2O$  and molecular  $O_2$  (Equation-2).

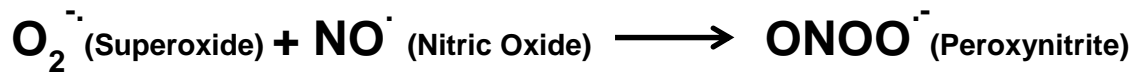
Being charged molecules, superoxide anions ( $2O_2^-$ ) does not cross cell membranes and thus are unlikely to venture far from their site of production. Therefore, the site of ROS generation has to be in proximity to the effector molecules resulting in altered cellular signaling.



In physiologic states, ROS generation is counter balanced by various cellular antioxidant enzymes like superoxide dismutase (SOD) to promote “redox homeostasis”. However, excessive/toxic ROS generation may induce an imbalance favoring oxidants over antioxidant capacity- a condition summarized by the term “oxidative stress”. The tenuous balance between free radical production and scavenging seems to be altered during pathological conditions like atherosclerosis.

Reactive Nitrogen Species (RNS) include radical and non-radical reactive nitrogen species such as, NO, nitrogen dioxide (NO<sub>2</sub>), nitrous acid (HNO<sub>2</sub>), nitrosyl cation (NO<sup>+</sup>), nitrosyl ion (NO<sup>-</sup>), peroxyxynitrite (ONOO<sup>-</sup>), peroxyxynitrous acid (ONOOH), alkyl peroxyxynitrites (ROONO). Nitric Oxide (NO), also called as gaseous signaling molecule. It is highly reactive with free radicals making it an important antioxidant. However, under hyper-physiological and pathological conditions excess superoxide can cross-react with protective NO at diffusion-controlled (Beckman, Beckman, Chen, Marshall, & Freeman, 1990) rates ( $\sim 1 \times 10^{10} \text{M}^{-1} \text{S}^{-1}$ ) to produce highly reactive and harmful, oxidizing as well as nitrating agent, peroxyxynitrite (ONOO<sup>-</sup>) (Zhou, Li, Zeng, & Huang, 2009) (Equation-3). The rate constant of superoxide dismutation by antioxidant enzyme Super Oxide Dismutase (SOD) is slower; therefore ONOO<sup>-</sup> formation cannot be prevented (Goldstein & Merenyi, 2008; Goldstein & Rabani, 2007; Goldstein, Samuni, & Merenyi, 2004; Goldstein, Squadrito, Pryor, & Czapski, 1996) Peroxyxynitrite radical generation is not only harmful by itself, but it can also induce increased activation of ROS producing enzymes via what is called as “nitrative stress”. The sites of peroxyxynitrite formation are assumed to be spatially associated with the sources of superoxide (at plasma membrane or mitochondria), due to the fact that, NO is relatively stable and highly diffusible free radical, whereas superoxide is much short lived and has restricted diffusion across

biomembranes. Therefore, localized formation of RNS may also depend on the subcellular location of ROS generators.



----- Equation 3

The first harmful effect of ONOO generation is reduction in the bioavailability of NO and thus a reduction in the protective effects of this molecule. Secondly, peroxynitrite free radical can mediate post-translation modifications like lipid peroxidation and protein nitration of important physiological cell signaling molecules (Zhou et al., 2009). The third most harmful effects are activation of ROS producing enzyme systems, thus acting as “kindling” centers for the “bonfire” of oxidative and nitroxidative stress responsible for atherosclerosis. Therefore, understanding the regulation of ROS and RNS production via different sources, their spatial proximity to other free radical sources and cellular signaling molecules involved, will provide great mechanistic insights into oxidative and nitrative stress induced vascular pathologies.

#### Role of ROS and RNS in Cardiovascular diseases

The evidence for the role of ROS/RNS induced oxidative/nitroxidative stress in atherosclerosis comes from studying the atherosclerotic lesions. The sclerotic lesions show increased levels of oxidized LDL lipids, proteins (Berliner et al., 1997), and nitrated proteins (Beckman et al., 1990) induced by increased oxidative and nitrative stress (Carr & Frei, 2001). The free radical mediate oxidation of LDL *in vitro* has also been studied extensively.

Protein tyrosine nitration has been detected in numerous tissues under normal physiological conditions (Greenacre & Ischiropoulos, 2001; Ischiropoulos, 1998). In cardiovascular system, basal protein nitration was found in all major cell types, such as myocytes, endothelial cells, fibroblasts, and vascular smooth muscle cells (Davidge, Ojimba, & McLaughlin, 1998) (Kavdia, M et al., 2013) Nitration of proteins has been observed in CVDs like inflammation, autoimmune myocarditis, heart failure, Ischemia-reperfusion injury, cardiac allograft rejection, transplant coronary artery disease, hypertension (Turko & Murad, 2002) and atherosclerosis (Patel et al., 2000).

During atherosclerosis the role of protein-tyrosine nitration has been emerging. Studies have demonstrated protein nitration in human atherosclerotic tissue (Beckman et al., 1990) (Buttery et al., 1996) (Cromheeke et al., 1999; Cuzzocrea et al., 2004; Depre, Havaux, Renkin, Vanoverschelde, & Wijns, 1999; Hunter et al., 1999; Leeuwenburgh et al., 1997; J. S. Luoma et al., 1998; P. Luoma, 1998). Nitrotyrosine immunoreactivity was detected in complex heterogeneous cellular plaques, in a relatively acellular fibrous plaques, and in myointimal plaques, resulting in plaque instability in patients (Depre et al., 1999). Presumably, tyrosine-nitrated proteins with altered function may promote atherogenesis, counteracting the well-established anti-atherogenic effects of NO. Though specific protein targets for nitration in atherosclerosis remains to be identified, but some preliminary studies in bovine atherosclerotic arteries have revealed tyrosine nitration of prostacyclin synthase in coronary arteries (Zuo et al., 2005), myosin heavy chain,  $\alpha$ -actinin and desmin. ONOO<sup>-</sup> induced nitration of iNOS has also been reported, therefore acting as feedback mechanism to inactivate NO production via enzyme inactivation (Qi J et al., 2013).

In spite of these observations, the mechanistic basis of activation of ROS/RNS sources, formation of spatio-temporally controlled cross-reaction of reactive oxygen (ROS) and NO to produce harmful ONOO, and its regulation are still unclear; it therefore becomes important to identify the molecular players and delineate the sequence of events in order to develop potential therapeutic targets in attenuating pathological effects of these radicals.

#### Natural Antioxidant enzyme systems

All cells are equipped with natural antioxidant enzyme systems to neutralize excess production of free radicals during pathophysiology. The balance between production of ROS/RNS and its scavenging by antioxidants keeps the individuals disease free. These anti-oxidant enzymatic systems include, 1) **Superoxide Dismutase (SOD)**: enzymes dismutates superoxide radicals to hydrogen peroxide and oxygen. Three different isoforms of SOD have been identified: the SOD2 (MnSOD), SOD1 (CuZnSOD), and the extracellular SOD, SOD-3, 2) **Glutathione peroxidase (GPx)**: antioxidant enzyme that effectively reduces hydrogen peroxide and lipid peroxides to water and lipid alcohols, respectively. It exists in 4 different isoforms, GPx1-4. Mice genetically engineered to lack GPx1 exhibit a pronounced susceptibility to myocardial ischemia–reperfusion injury. In patients with coronary artery disease, the activity of red blood cell GPx1 is inversely associated with the risk of cardiovascular events. The GPX/glutathione system is thought to be a major defense in low-level oxidative stress, 3) **Catalase**: catalyzes the reaction of hydrogen peroxide to water and molecular oxygen. The overexpression of catalase has a protective effect on the cardiovascular system such as delayed development of atherosclerosis, 4) **Thioredoxin reductase (Trx)**: an enzyme that participates in thiol-dependent cellular reductive processes. It is recognized as critical protective system acting via direct (antioxidant) and indirect (regulation of signal

transduction) effects. Trx peroxidase scavenges ROS (such as H<sub>2</sub>O<sub>2</sub>) and also ONOO<sup>-</sup>. The thioredoxin system may effectively regenerate proteins that were inactivated by oxidative stress, 5) **Paraoxonase (PON)**: The PON family of enzymes (PON1-4) seems to contribute to vascular antioxidant defense and protect against coronary artery disease. Deletion of the PON1 gene increases oxidative stress in mouse macrophages and aortae, and apoE<sup>-/-</sup> mice overexpressing PON1 developed fewer atherosclerotic lesions. PON2-deficient mice with an apoE<sup>-/-</sup> background developed more atherosclerotic lesions, whereas PON2-overexpressing mice were protected against those lesions, 6) **Peroxiredoxin (Prxs)**: are highly efficient peroxynitrite reductases and recent evidence suggests that they regulate peroxide-mediated signaling cascades, 7) **Heme oxygenase (HO)-1**: HOs catalyze the conversion of heme into carbon monoxide, Fe<sup>2+</sup>, bilirubin, and biliverdin. There are three human isozymes of HO, HO1-3. Cardiac-specific expression of HO-1 protects against inflammation and oxidative damage in hearts subjected to ischemia and reperfusion injury *in vivo* (Yet et al., 2001). ECs isolated from HO-1<sup>-/-</sup> mice showed an oxidative stress phenotype *in vitro* (Poss & Tonegawa, 1997). It is also known to increase endothelial cell SOD expression, 8) **Small non-protein anti-oxidants**: In addition to antioxidant enzymes, various small-molecular-weight redox-active compounds are found in biological systems. These include endogenous products of metabolism such as glutathione, uric acid and bilirubin, and compounds ingested such as antioxidant vitamins (mainly vitamins C and E) and polyphenolic compounds. Glutathione is the major determinant of the intracellular redox environment, whereas uric acid and bilirubin can scavenge ROS species in the extracellular space. Direct *in vivo* evidence for a protective effect of these compounds against oxidative stress is currently limited, others include, a) **Vitamin C (L-ascorbate)** is considered the most efficacious water-soluble antioxidant in human plasma. This

molecule effectively scavenges various reactive oxygen and nitrogen species and regenerates  $\alpha$ -tocopherol from its radical species. In addition, L-ascorbate chemically stabilizes BH<sub>4</sub>, b) **Vitamin E** contains tocopherols and tocotrienols, with  $\alpha$ -tocopherol being the most active form. Molecules with vitamin E activity are excellent scavengers of lipid peroxides, which are generally thought to be their major antioxidant action, c) **Dietary polyphenolic antioxidants** are also likely to assist in preventing ROS-induced damage.

**Peroxynitrite** can also be neutralized/scavenged naturally or therapeutically, whereas the efficacies of such systems and drugs are limited. One of the harmful effects of peroxynitrite includes, oxidation or nitration of enzymes catalyzing peroxynitrite scavenging itself. **Peroxiredoxins** especially Peroxiredoxin V in humans is known to neutralize peroxynitrite at a rate slower than the rate of peroxynitrite formation. Another antioxidant enzyme (Bryk R et al., 2000), **Glutathione peroxidase** has also been shown to readily serve in reduction of peroxynitrite to nitrite *in vitro*, whereas *in vivo* effects are not known (Sies H et al., 1997). Some of the physiological modulators include, thiol-based antioxidants, such as **mercaptoalkylguanidines**, **N-acetylcysteine** and **dihydrolipoic acid (Szabo C et al., 2003)**. Another compound widely used to neutralizing peroxynitrite is **Ebselen**. Ebselen showed protective effects in several models of inflammation and reperfusion injury (Klotz & Sies, 2003) and also improved endothelial function in diabetic blood vessels and kidneys. **Tempol** is a stable nitroxide radical that has been successfully shown to protect cells from nitroxidative stress conditions. Tempol acts by catalytically diverting peroxynitrite/carbon dioxide reactivity from nitration to nitrosation reactions (Fernandes DC et al., 2005). Some of these protective effects are due to its antioxidant functions. Several marketed drugs such as **Cabergoline** and **simvastatin** have been described to have peroxynitrite scavenging

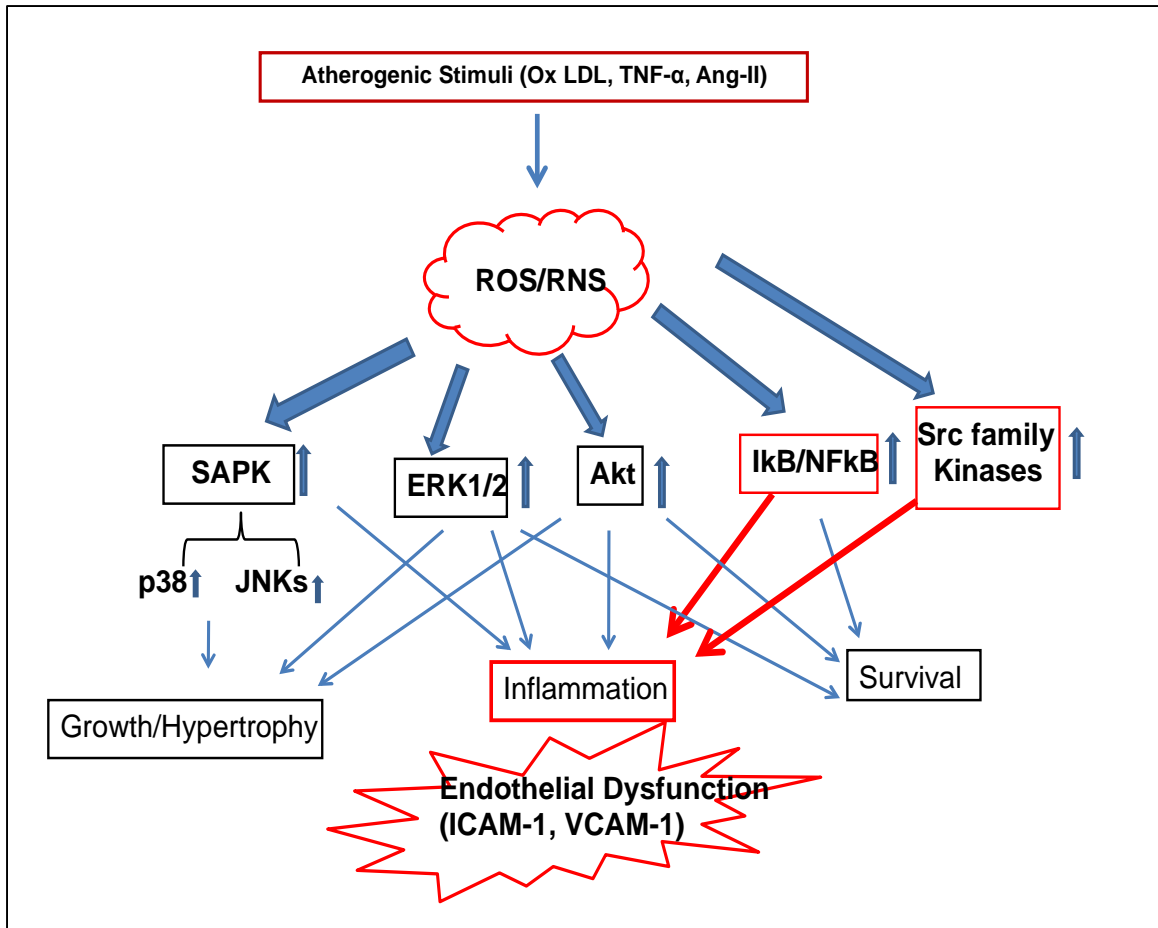
activities, although in most cases they do not react directly with peroxynitrite but with its secondary radicals (Diaber A et al., 2005). Numerous other molecules present in natural or dietary products, such as carotenoids, polyphenol oligomers and **epicatechin**, have shown to effectively protect cells from peroxynitrite (Schroeder P et al., 2003). However, caution is exercised in interpreting pharmacological responses obtained from these agents, as they tend to react slowly with peroxynitrite itself and so react with other reactive species instead, including peroxynitrite derived radicals.

#### ROS/RNS induced oxidative/nitrative or nitroxidative signaling

Oxidative stress occurs when redox homeostasis within the cells is altered. This imbalance is due to either an overproduction of ROS or a deficiency in antioxidant defense mechanisms. Once generated, ROS/RNS intersect with the known and well-established pathways governing cell growth and physiology to induce specific oxidative and nitrative or “nitroxidative signaling”. This leads to highly specific acute and chronic changes in cell phenotype. The oxidative and nitrating radicals have direct protein targets wherein exposure of these proteins leads to an altered redox state.

**ROS induced Redox Signaling:** Different redox-active species react with selected cellular targets to modulate cell signaling. NO is known to bind the heme group of an array of enzymes to modulate their activity (e.g., binding of NO to the heme of sGC activates the enzyme and this signals the vasorelaxation of blood vessels). Similarly,  $O_2^{\cdot-}$  reacts with iron-sulfur clusters of key cellular proteins to alter their activity e.g., destabilization of the iron-sulfur cluster of mitochondrial aconitase by  $O_2^{\cdot-}$  blocks the enzyme activity, resulting in the inhibition of mitochondrial respiration. Another important aspect underlying redox signaling is the reversible, covalent modification of specific

cysteine thiol residues that reside within active and allosteric sites of proteins, which results in alteration of protein function. Various redox modifications of cysteines alter protein function with those mediated by  $H_2O_2$  of particular relevance to cellular signaling. Although,  $H_2O_2$  is a mild oxidant, hence relatively inert with most bio-molecules, it can still oxidize cysteine residues in proteins to produce disulfide (R-S-S-R) bridge formation via sulfenic acid (R-SOH) (Denu & Tanner, 1998). In addition to oxidation, thiols are subjected to S-nitrosation/S-nitrosylation, which describes the covalent binding of NO to cysteine thiols to form protein-S-nitrosothiols (R-S-NO). Some of the intracellular effector pathways involved in redox signaling include, a) Phosphatases, such as protein tyrosine phosphatases (PTPs), b) Protein tyrosine kinases (PTKs) leading to activation of Mitogen Activated Protein Kinases (MAPKs) like Extracellular signaling regulated kinases (ERKs), Receptor tyrosine kinases (RTKs) and inactivation of protein tyrosine phosphatases (PTPs), Src-family kinases, c) Serine/Threonine Kinases and phosphatases like Jun-N-terminal kinase (JNK), p38 MAP kinase; also known as Stress Activated Protein Kinases (SAPKs), Akt and c)  $NF\kappa B$  and AP1 transcription factors (**Figure 6**). Some of the important redox signaling mediators pertinent to our study is discussed in great detail.



**Figure 4. Flow diagram showing ROS and RNS induce activation of variety of signaling cascades important for cellular physiological functions.** Flow diagram based on available data in all the cell types discussed above showing activation of Stress activated proteins kinases (SAPKs), NFκB pathway, MAPKs, PI3K/Akt and Src family kinase signaling in response to increased oxidative stress and nitrative stress. SFK activation in response to RNS in endothelial cells is not known and also Information about nitration targets of ONOO- are not known.

**a) Phosphatases:** Phosphatases like protein tyrosine phosphatases (PTPs) are a family of enzymes responsible for removing phosphate groups from phosphorylated amino acid residues on proteins to counteract the signaling actions of protein kinases, which can also be activated in response to oxidative modifications. The PTPs are characterized by the presence of cysteine residue, which is important for phosphatase activity, but is also susceptible to oxidation, resulting in inactivation of PTPs.

**b) Protein kinases:** The presence of cysteine residues in functional centers of kinases renders them sensitive to redox control.  $H_2O_2$  induced oxidation of Src kinase at Cys-245 in SH2 domain and Cys-487 in the kinase domain, results in activation of the enzyme, leading to MAP kinase signaling.  $H_2O_2$  treatment or cyclic strain-induced ROS production activates Src in endothelial cells, leading to activation of MAP kinase signaling, whereas, the oxidative inactivation of PTPs is considered to represent an initial event leading to receptor tyrosine kinase trans-activation. In  $H_2O_2$  treated endothelial cells, activation of Src kinase is necessary for the trans-activation of the EGF receptor and resultant stimulation of the JNK or PI3K/Akt/eNOS pathways that act to promote or protect against oxidative stress-induced endothelial cell death, respectively (Sato K et al., 2001). Oxidative activation of PKC $\alpha$  by NADPH oxidase-derived ROS in endothelial cells is essential for VCAM-1-dependent transendothelial migration of leukocytes (Abdala-Valencia & Cook-Mills, 2006).

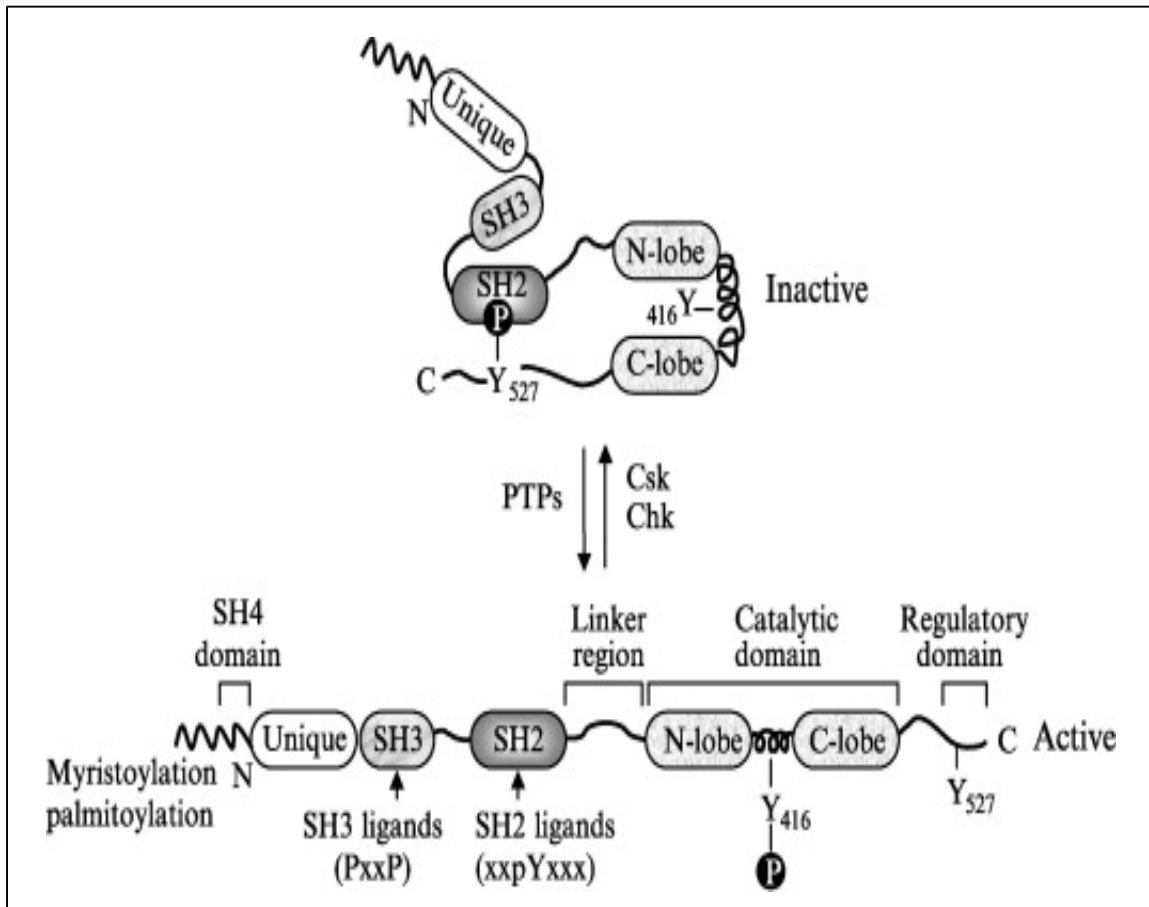
**Mitogen-Activated Protein Kinases (MAPKs):** The mitogen-activated protein kinases (MAPKs) are the most important family of serine-threonine kinases, upon which many other signaling pathways converge. MAPKs are activated by dual phosphorylation at a specific tripeptide motif, mediated by a conserved protein kinase cascade involving MAPK kinases (MKK or MEK) and MAPK kinase kinases (MKKK or MEKK) (Dong,

Davis, & Flavell, 2002). Upstream signaling pathways triggering MKKK activation mainly depends on the activation of growth factor receptors and small G-proteins such as Ras, Rac and Cdc42. The four major groups of MAPKs are extracellular signal-regulated protein kinase (ERK1/2), p38 MAPK (alpha, beta, gamma, delta), c-Jun NH2-terminal kinase (JNK1, 2 and 3) and big MAP kinase (BMK or ERK5). MAP kinases become activated upon cell exposure to a wide range of stimuli, notably including oxidants such as H<sub>2</sub>O<sub>2</sub> and generated ROS in endothelial cells. These MAPKs have also been implicated in up-regulation of ICAM-1 resulting in endothelial dysfunction (Tamura et al., 1998) (Tamura DY et al., 1998).

**Src Family Kinases:** Src family nonreceptor tyrosine kinases (SFKs) are present in essentially all metazoan cells, where their regulated activation by diverse growth factor, cytokine, adhesion, and antigen receptors is critical for generating an appropriate cellular response to external stimuli (Brown & Cooper, 1996) (Thomas & Brugge, 1997). The nine members of the Src family include Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes, and Yrk. Src kinases share a conserved domain structure consisting of consecutive SH3, SH2, and tyrosine kinase (SH1) domains. All family members also contain an SH4 membrane-targeting region at their N-terminus, which is always myristoylated and sometimes palmitoylated (Koegl, Zlatkine, Ley, Courtneidge, & Magee, 1994). The SH4 region is followed by a 'unique' domain of 50–70 residues, which is divergent among family members. A hallmark of SFKs is a short C-terminal tail, which bears an autoinhibitory phosphorylation site (Tyrosine 527 in Src) (Cooper & King, 1986). Phosphorylation of tyrosine 527 indirectly regulates the enzymatic activity of Src kinase by inducing conformational changes of the protein. When tyrosine 527 is phosphorylated, the phosphoresidue can form an intramolecular bond with the SH2 domain, forcing Src

Kinase into a closed, inactive position, indirectly decreasing the activity of the kinase domain (Nada, Okada, MacAuley, Cooper, & Nakagawa, 1991). Src family members require phosphorylation within a segment of the kinase domain termed the activation loop for full catalytic activity. In Src, this autophosphorylation site is Tyrosine 416 (Smart et al., 1981) (Smart *et al.*, 1981). Phosphorylation of tyrosine 416 modulates the activity of the enzyme by directly regulating the kinase domain; addition of a phosphate to tyrosine 416 increases the enzymatic activity of the protein (Xu, Doshi, Lei, Eck, & Harrison, 1999; Xu, Harrison, & Eck, 1997). *In vivo*, Src kinases are phosphorylated on either Tyr 416 (in their active state) or Tyr 527 (in the inactive state). The inactivating phosphorylation on Tyr 527 is carried out by the Src-specific kinase Csk or its homolog Chk. Phosphorylation of the C-terminal tail promotes assembly of the SH2, SH3, and kinase domains into an autoinhibited conformation maintained by intimate interactions among these domains (Figure 5) (Sicheri & Kuriyan, 1997) (Xu et al., 1997).

Different oxidative agents have been demonstrated to stimulate SFKs by inducing tyrosine phosphorylation events and activate signaling pathways such as NFκB (Abe, Takahashi, Ishida, Lee, & Berk, 1997) (Staal et al., 1994). However, it is not yet clear whether ROS cause direct activation of tyrosine kinase or inhibition of tyrosine phosphatase activity. Because all tyrosine phosphatases have reactive cysteine residues in their active site, it has been proposed that inhibition of these enzymes by oxidants may produce increases in tyrosine phosphorylation. In this regard, PKC-mediated phosphorylation in response to oxidative stress may be the result of inhibition of protein phosphatase 1 and 2A activity. Therefore, oxidants may regulate protein tyrosine phosphorylation by modifying the cascade and play a role in modulating several biochemical events that control cell growth, differentiation, and inflammation.



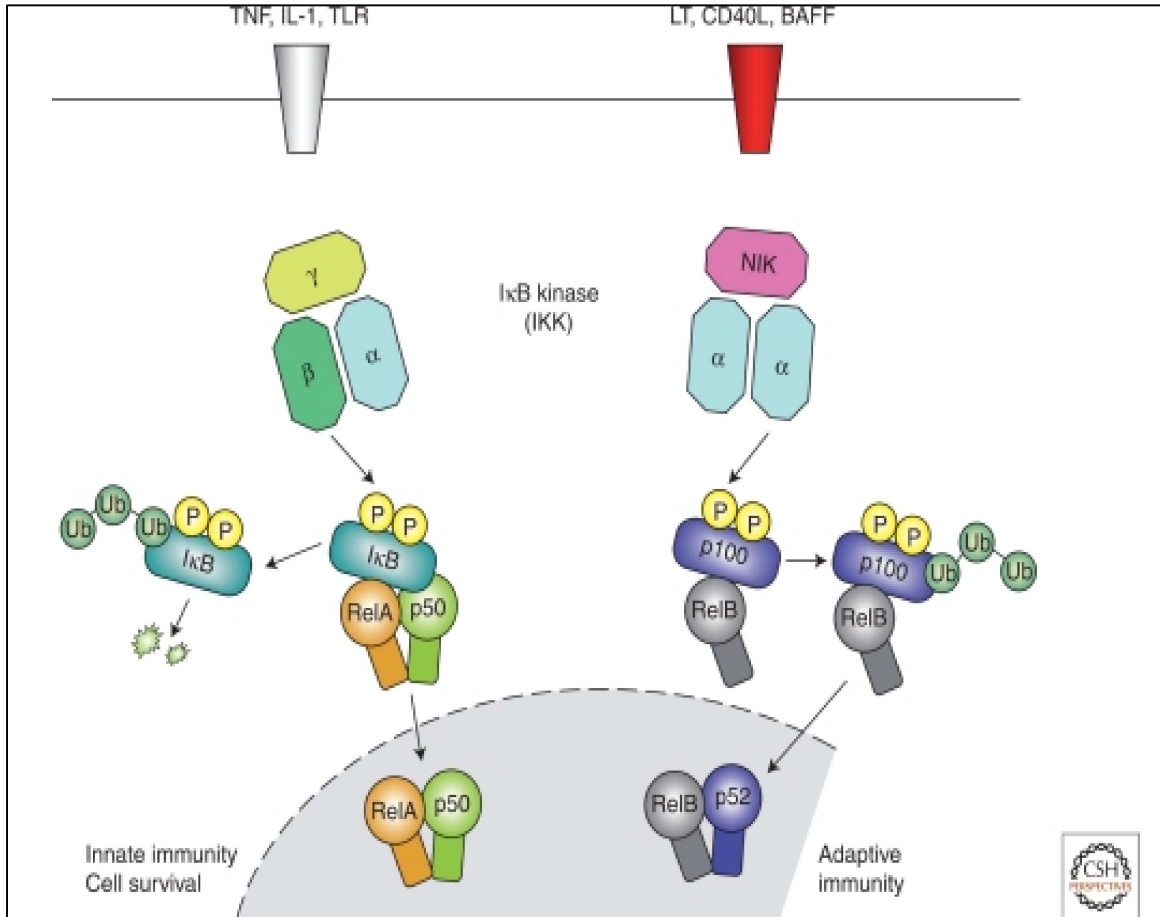
**Figure 5. Schematic model of Src-family kinase regulation.** Inhibitory phosphorylation (pY<sub>527</sub>) leads to an intramolecular interaction with the SH2 domain docking site and autoinhibition. Dephosphorylation of the inhibitory site leads to enzyme activation (phosphorylation of Y<sub>416</sub>) and release of the substrate-binding site (SH2/SH3 domains) (Minetti M et al., 2002).

**c) Transcription factors:** An important mechanism through which ROS and RNS alter vascular cell functions through redox-dependent alteration of transcriptional output. In mammalian cells, numerous transcription factors are considered to be subject to oxidant and NO control, including NF- $\kappa$ B, Nrf2, AP-1, IF-1, and p53 (H. Chen, Song, & Chan, 2009; X. L. Chen, Varner, et al., 2003). The NF $\kappa$ B is an oxidant sensitive transcription factor activated during inflammatory conditions and has been shown to drive transcription of adhesion molecule genes involved in development of dysfunctional endothelial phenotype.

**NF- $\kappa$ B Signaling Pathway:** NF $\kappa$ B proteins are a family of transcription factors and are of central role in the control of inflammatory and immune-response genes (Ghosh & Hayden, 2008; Vallabhapurapu & Karin, 2009). The mammalian NF $\kappa$ B proteins consist of five different related family members that bind as homodimers or heterodimers to a 10-base pair kB sites. All of these family members have a Rel-homology (RHD) domain that is essential for DNA binding and dimerization. The three Rel members of the family, RelA (also known as p65), RelB, and cRel, have a C-terminal transcription activation domain (TAD) that serves to positively regulate gene expression. The two other mammalian NF- $\kappa$ B proteins are synthesized as larger p105 and p100 precursor proteins, which have C-terminal ankyrin repeats that inhibit DNA binding until partially processed by proteasome to the smaller p50 and p52 products, respectively (Hatada EN et al., 1992) (Figure 6). In addition, these proteins lack a TAD and therefore do not generally activate transcription unless paired as a heterodimer with one of the Rel proteins. All NF- $\kappa$ B proteins are capable of homodimerization or heterodimerization with the other NF- $\kappa$ B proteins with the exception of RelB, which can only form heterodimers. Though most NF- $\kappa$ B dimer combinations result in the regulation of similar sets of genes, the ability to interact in various homo- and hetero- dimer configurations contributes to their ability to

bind with varying affinities to  $\kappa$ B sites in distinct DNA sequences, and they thus regulate unique, as well as overlapping, gene sets. In general, NF $\kappa$ B activity is principally regulated by I $\kappa$ B proteins. Three of these proteins are considered “typical” I $\kappa$ Bs, namely I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ . These proteins bind to NF- $\kappa$ B proteins and mask their DNA binding domains. They also possess potent nuclear export signals (NES) and generally remove NF- $\kappa$ B proteins from the nucleus, and are thus inhibitory in multiple ways. Two other I $\kappa$ Bs, I $\kappa$ B $\zeta$  and Bcl-3, are considered “atypical”. They are found in the nucleus, are inducibly expressed, bind only to p50 and p52 homodimers, and under certain circumstances may act to repress or to activate these homodimers. The activity of the typical I $\kappa$ Bs is controlled through phosphorylation by upstream I $\kappa$ B kinases (IKKs) (Figure 6).

NF- $\kappa$ B transcription factor activation has been associated with endothelial cell dysfunction and vascular inflammation (Collins, 1993). Redox-sensitive activation of NF- $\kappa$ B is necessary for VEGF to promote endothelial gene expression of the mitochondrial isoform of SOD (MnSOD) or proinflammatory stimuli to induce the expression of adhesion molecules (VCAM-1, ICAM-1) (X. L. Chen, Varner, et al., 2003). H<sub>2</sub>O<sub>2</sub> involves inactivation of IKK through oxidation and S-glutathionulation of Cys-179 of the  $\beta$ -subunit of the kinase. Similarly, IKK activity and hence NF- $\kappa$ B activation is inhibited by S-nitrosylation of Cys-179. Endothelial levels of the p65 subunit of NF- $\kappa$ B are elevated in arteries from aged versus young humans and this is correlated with extent of increase in nitration of endothelial cell proteins and impairment of endothelium-dependent relaxation in aged subjects.



**Figure 6. NFκB signaling pathway.** The diagram illustrates the canonical and alternative pathways for NFκB activation. The canonical pathway is triggered by TLRs and proinflammatory cytokines such as TNF $\alpha$  and IL-1, leading to activation of RelA (p65) that regulates expression of proinflammatory and cell survival genes. These pathways are characterized by the differential requirement of IKK subunits. IKK $\beta$  regulates activation of the canonical pathway through phosphorylation of IκBs and requires the IKK $\gamma$  subunit but not IKK $\alpha$ , whereas IKK $\alpha$  is required for activation of the alternative pathway through the phosphorylation and processing of p100, the precursor of p52, but this is independent of both IKK $\beta$  and IKK $\gamma$  (Lawrence et al., 2009).

**RNS (Peroxynitrite) Induced Nitroxidative Signaling:** The harmful RNS, ONOO<sup>-</sup>, is a strong oxidant as well as nitrating agent capable of modifying most biological molecules and compounds, including amino acids such as tyrosine, tryptophan, cysteine, and methionine. If formed in sufficient amounts to overcome cellular antioxidant defenses, peroxynitrite and its secondary radicals will inflict various oxidative damages to proteins, lipids and DNA. There is now substantial evidence that these multiple pathways of peroxynitrite-mediated cytotoxicity play key roles in the development and perpetuation of a number of cardiovascular and non-cardiovascular diseases (Pacher, Beckman, & Liaudet, 2007; Szabo, Ischiropoulos, & Radi, 2007). A less well-characterized consequence of peroxynitrite relies in the modulation of cell signal transduction. Increasing experimental evidence, mainly obtained in cellular models, indeed supports such novel roles of peroxynitrite and has changed our understanding of the pathophysiological roles of this radical species. Nitration of free and protein bound tyrosine to yield nitrotyrosine is a well-established *in-vitro* reaction of ONOO<sup>-</sup>. It has been observed that not all tyrosine residues in a protein are nitrated and not all proteins are targets of nitration (Ischiropoulos, 1998; Souza, Daikhin, Yudkoff, Raman, & Ischiropoulos, 1999). It is believed that certain proteins can be preferentially targeted for nitration based not only on the composition and structure of a given target but also on intracellular concentration, localization and interaction with other molecules.

Some of the important peroxynitrite induced signaling pathways are discussed below.

a) **Protein tyrosine nitration and phosphotyrosine signaling:** Protein tyrosine nitration is a covalent modification resulting from the addition of a nitro (-NO<sub>2</sub>) group onto one of the two equivalent ortho carbons of the aromatic ring of tyrosine (Radi, 2004). The nitration of tyrosine residues in a protein is expected to change the function

of a protein. A gain-of-function or loss-of-function (Balafanova et al., 2002; Gole et al., 2000) with possible effects on steric hindrance and a distortion of the local protein structure have also been reported (Ischiropoulos, 1998). However, the inhibition of function is considered more common consequence of tyrosine nitration (Ischiropoulos, 1998) (Greenacre & Ischiropoulos, 2001). Loss or increased phosphorylation of residues resulting in, constitutively active proteins have also been reported (A. Gow, Duran, Thom, & Ischiropoulos, 1996; Kong, Yim, Stadtman, & Chock, 1996; MacMillan-Crow, Greendorfer, Vickers, & Thompson, 2000; C. Mallozzi, A. M. Di Stasi, & M. Minetti, 2001a; C. Mallozzi, M. A. Di Stasi, & M. Minetti, 2001b). Nitration and phosphorylation of tyrosine residues in proteins are two competing chemical reactions. The influence of protein tyrosine nitration (PTN) on tyrosine phosphorylation and vice versa may not be simply deleterious but play a more subtle role in modulating signal transduction in addition to phosphorylation-dephosphorylation reactions.

Widespread tyrosine nitration occurs in cells during *in vitro* exposure to peroxynitrite, affecting structural proteins, ion channels, metabolic enzymes, and proteins involved in apoptosis. The relevance of such observations *in vivo* remains, however, to be established, given that the yield of nitrotyrosine formation under conditions of elevated peroxynitrite generation *in vivo* (nitrooxidative stress) remains largely smaller than what can be achieved *in vitro* with direct peroxynitrite exposure. Indeed, only 1 to 10 residues of tyrosine per 100,000 are found nitrated in plasma proteins under inflammatory conditions, such as those observed in cardiovascular disease, although up to 10 times more 3-NT can be detected in tissues.

b) **Akt/protein kinase B:** Some amount of data exists in different cell types suggesting regulation of Akt signaling by peroxynitrite. Peroxynitrite and the peroxynitrite donor SIN-

1 triggered PKB/Akt phosphorylation and activation in skin fibroblasts (Klotz & Sies, 2003), rat hepatoma cell line (Kang et al., 2002), and neural cells (M. H. Li, Cha, & Surh, 2006), all resulting in apoptosis. Although, the mechanism for these events are not known, some groups believe this to be more of an oxidative, but not nitrative type of chemistry induced by peroxynitrite (Delgado-Esteban, Martin-Zanca, Andres-Martin, Almeida, & Bolanos, 2007). In contrast, there is evidence for peroxynitrite-induced inhibition in Akt signaling in PC12 cells (Shacka et al., 2006), and Raw 264.7 macrophages (Hellberg, Boggs, & Lapetina, 1998) and most importantly in endothelial cells (Song et al., 2007). The findings here have been applied to diabetic vessels, which show high oxidative stress and protein-tyrosine nitration. It was observed that Akt molecule in diabetic vessels undergo nitration events and are therefore considered to be central players in development of endothelial dysfunction and further disease progression. The effects seen in above examples here point towards dependence on peroxynitrite concentrations, the cell types, and the chemical microenvironment.

c) **Mitrogen-Activated Protein Kinases (MAPKs)** such as **Extracellular signal-regulated protein kinase (ERK)** plays a central role in oxidative signaling pathways, whereas functioning in response to peroxynitrite, is limited. In endothelial cells, peroxynitrite activated ERK via direct oxidative S-glutathiolation of p21ras. Studies using natural peroxynitrite inhibitor epicatechin, showed decreases in total protein nitration but no change in ERK activation. Similarly, **c-Jun NH2-terminal kinase (JNK)** activation in response to peroxynitrite has been reported in various cell types *in vitro*, including raft cardiomyocytes, vascular endothelial cells. In the vasculature, peroxynitrite-dependent JNK activation has been shown to occur in endothelial cells exposed to laminar shear stress, which is essential to prevent the development of atherosclerosis, suggesting

peroxynitrite roles as a molecular link between mechanical stress and endothelial functioning (Go YM et al., 1999). The other signaling molecules affected by peroxynitrite in endothelial cells include, **p38 MAP kinase, Protein kinase C.**

d) **NFκB Signaling:** As described before, ROS appear to favor NFκB activation, but specific role of peroxynitrite in this regard, is incompletely understood. A series of experiments performed in human polymorphonuclear cells (PMNCs) indicated exposure to exogenous peroxynitrite, as well as induction of endogenous peroxynitrite generation by LPS, cytokines or Toll-like receptors (TLR-9) activated NFκB, leading to an increase in IL-8 secretion (Filep, Beauchamp, Baron, & Paquette, 1998). In mononuclear cells, peroxynitrite triggered NFκB activation and proteasomal degradation of IκB, a novel mechanism for NFκB activation (Matata & Galinanes, 2002). Contrary to these findings, inhibitory effects of peroxynitrite on NFκB activation are reported. Peroxynitrite inhibited both constitutive and induced NFκB activities in p19, SH-SY5Y and HEK293 cells via p65 nitration at Tyr66 and Tyr152, leading to destabilization and nuclear export (Park, Huq, Hu, & Wei, 2005). In line with these studies, short exposure of cardiac H9c2 cells or endothelial EAHY-926 and HMEC-1 cells to peroxynitrite completely inhibited IKKβ phosphorylation and NFκB activation in response to LPS or inflammatory cytokines (Levrant et al., 2005). These effects were accompanied by an increase in IKKα phosphorylation, consistent with differential regulation of IKKα and IKKβ by peroxynitrite. Together, these findings provide initial evidence for the unforeseen concept that peroxynitrite may downregulate inflammatory responses under certain conditions.

e) **Src-Family Kinases:** Peroxynitrite mediated alterations in SFK activity has been demonstrated in erythrocytes and neuronal synaptosomes. SFK members, c-Src, Lyn, and Fyn activity showed increases in response to exogenous infusions of peroxynitrite

(Minetti, Mallozzi, & Di Stasi, 2002) (Mallozzi et al., 2001a; C. Mallozzi, M. A. Di Stasi, & M. Minetti, 2001c; Serafini, Mallozzi, Di Stasi, & Minetti, 2005). However, in endothelial cells peroxynitrite induced changes in SFKs, are not worked out. Most of the information-regarding role of peroxynitrite on SFKs comes from neuronal and erythrocytes, whereas effects on endothelial cell, are not known.

The evidence discussed above shows that peroxynitrite has effects on signaling cascades, whereas information regarding mechanism of how it modulates signaling cascades and targets of peroxynitrite radical is not clear. It is plausible that some of the signaling molecules discussed above are targets of peroxynitrite and undergo nitration at tyrosine. This nitration induced activation of signaling molecules and kinases can explain changes in phosphorylation/dephosphorylation states of molecules. Based on diversity of effects of ROS and RNS on different signaling cascades, it is possible that each cell type requires special set of conditions for oxidation and nitration reactions. Also, understanding the mechanism of ROS/RNS generation under the specialized conditions in endothelial cells will be of great importance in gaining in-depth knowledge about etiology of vascular diseases involving endothelial dysfunction such as, atherosclerosis.

#### ROS and RNS Sources in endothelial cells

Given that ROS and RNS are likely to play critical signaling roles essential for the normal functioning of the blood vessel wall, an understanding of the, a) sources of ROS/RNS, b) relative patho-physiological significance of each source, c) determining their regulation via specific regulatory mechanisms is important for the development of effective therapies to reduce vascular oxidative and nitrate stress.

The major sources of ROS generation in endothelial cells include the mitochondrial electron-transport chain, NADPH oxidases, xanthine oxidase, cytochrome P450, and uncoupled endothelial NO synthase (eNOS) (J. Li et al., 2008). The coupled eNOS is the only known source of non-radical NO and most of the other harmful RNS are produced as secondary products of cross-reaction between primary NO and ROS.

The **mitochondrial electron transport chain** can produce  $O_2^{\cdot-}$  as a by-product of electron flow from molecular oxygen via this chain. Electron transport chain reactions of the inner mitochondrial membrane, monoamine oxidase in the outer mitochondrial membrane may represent another source of  $H_2O_2$ . The  $O_2^{\cdot-}$  generated from the mitochondria is implicated in diabetic vasculopathy and in ischemia/reperfusion.

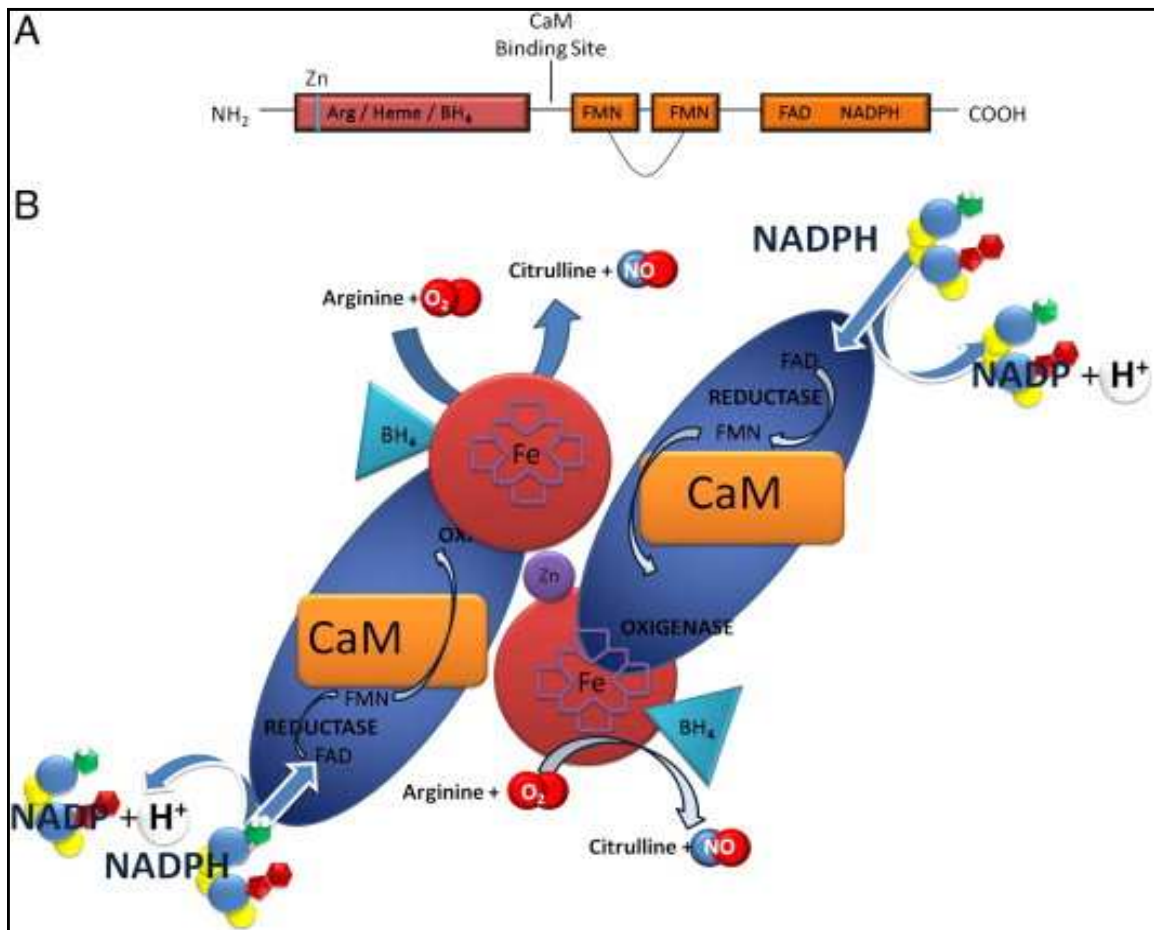
**Xanthine Oxidase** is an iron sulfur molybdenum flavoprotein exists in two different isoforms of xanthine dehydrogenase and xanthine oxidase each present in higher concentrations in endothelial cells of capillaries. Upon oxidation of xanthine or hypoxanthine to uric acid, the xanthine dehydrogenase produces NADPH whereas xanthine oxidase can generate  $O_2^{\cdot-}$ , amplifying oxidative stress originally arising from other sources. This shows that there are complex interactions among different sources of ROS. Therefore, it is increasingly clear that there are multiple sources of ROS in endothelial cells.

**Nitric oxide synthases (NOS)** is a source of both RNS as well as ROS. The enzyme Nitric Oxide Synthase (NOS) exists in three isoforms; neuronal isoform (nNOS), macrophage or inducible isoform (iNOS) which is only expressed in cells that have been exposed to inflammatory mediators or other injurious stimuli that activate the macrophages, and endothelial NOS, eNOS producing NO in vasculature (Lamas,

Marsden, Li, Tempst, & Michel, 1992). NO production by iNOS isoform has been suggested as a major mechanism by which cytokines mediate cardiac contractile dysfunction and iNOS higher protein expression has been demonstrated in development of cardiovascular diseases (Cannon, 1998; Schulz et al., 1995; Wildhirt, Dudek, Suzuki, & Bing, 1995). It generates substantially higher amounts of NO for long periods of time (Cannon, 1998; Moncada & Higgs, 1991; Zweier, Fertmann, & Wei, 2001).

eNOS produces NO via the oxidation of L-arginine to L-citrulline. The enzyme exists as homodimer with each monomer consisting of reductase (containing the binding sites for NADPH, FAD, and FMN and an oxygenase domain (containing Zn, tetrahydrobiopterin, heme and L-arginine) that are connected by a calmodulin binding hinge region. Inactive eNOS is bound to the protein caveolin and is located in small invaginations in the cell membrane called caveolae (Bucci et al., 2000). When intracellular levels of  $Ca^{+2}$  increase eNOS detaches from caveolin and is activated. Once intracellular stores of  $Ca^{+2}$  are depleted, membrane channels for extracellular  $Ca^{+2}$  are opened,  $Ca^{+2}$  binds to the protein calmodulin undergoing conformational change, thus allowing eNOS binding and NO release. Figure 7, shows different structural components of eNOS and mechanism of production of NO via transfer of electron from L-arginine to L-citrulline).

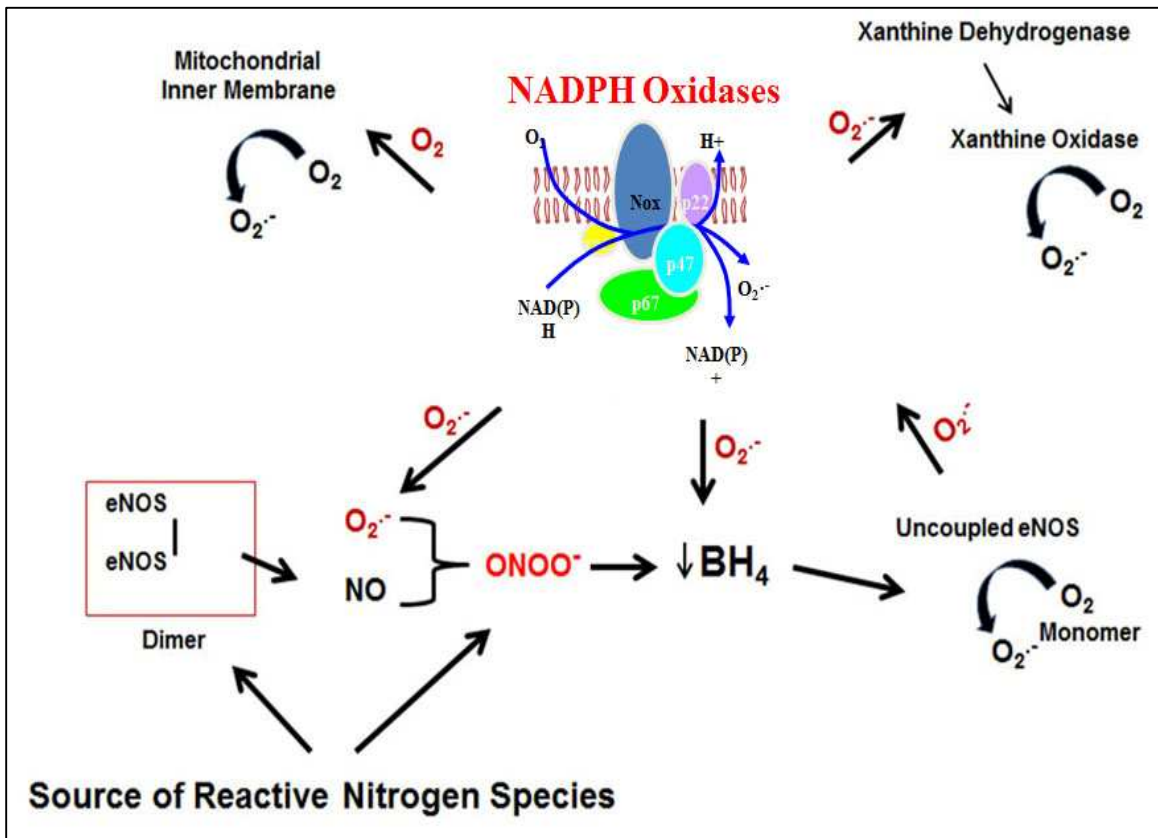
**eNOS as a ROS Source:** Under the atherogenic stimuli, the enzyme eNOS, can 'uncouple' and start to produce superoxide. One of the other mechanism of eNOS uncoupling is oxidation of the cofactor  $BH_4$  (tetrahydrobiopterin) in situations of increased  $O_2^-$  or  $ONOO^-$ , making eNOS a significant source of  $O_2^-$ . Uncoupled eNOS has been shown to be involved in pathophysiology of diabetic vasculopathy, atherosclerosis, hypertension and hypercholesterolemia (Cave et al., 2006; Harrison et al., 2011; Lambeth, 2004).



**Figure 7. Molecular structures of eNOS monomer and dimer. (A) Secondary structure of the eNOS monomer.** The oxygenase (N-terminal) and reductase (C-terminal) domains are separated by calmodulin (CaM) binding site. (B) Detail of the eNOS dimer. The Zn ion is responsible for connecting the monomers at the heme groups, which are resistant to dimerization. The electron dimer shows NADPH-oxidase donating an electron to be ultimately used to convert arginine and oxygen to the reaction products citrulline and nitric oxide. Tetrahydrobiopterin (BH<sub>4</sub>) further stabilized the dimer. Zn, Zinc; Arg, arginine binding site; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide (Gielis et al., 2011).

**NADPH (Nicotine Amide Adenine Dinucleotide phosphate) oxidases (Nox's)** have emerged as major sources of ROS in the vasculature. These enzymes transfer electrons from NADPH to molecular oxygen, thus producing  $O_2^{\cdot-}$ . The enzyme has multiple different isoforms (Nox1, Nox2, Nox4, Nox5) expressed in different cell types. The enzyme complex been shown to be involved in conditions of endothelial dysfunction, hypertension, atherosclerosis, diabetes and coronary artery disease (CAD) (Pagano et al., 1995; Rajagopalan, Kurz, et al., 1996; Rajagopalan, Meng, Ramasamy, Harrison, & Galis, 1996; Rask-Madsen & King, 2007), where there is increased mRNA and protein levels of Nox isoforms and associated subunits. Evidence suggesting their important vascular role comes from pharmacological inhibition or genetic deletion of Nox isoforms and reduction in vascular oxidative stress during disease conditions. (H. Chen et al., 2009; Jackman, Miller, Drummond, & Sobey, 2009; Selemidis, 2008; Selemidis, Sobey, Wingler, Schmidt, & Drummond, 2008). Exposure to pro-inflammatory agents and cardiovascular risk factors also showed up regulation of Nox isoforms (Brandes et al., 2002; Brandes & Schroder, 2008).

Although there are multiple sources of ROS in endothelial cells, NADPH oxidases appear to be central amongst them due to the fact that, a) NADPH oxidases are the only source whose primary function is ROS generation, b) the primary function of the enzyme can be regulated by ROS generated via itself or other ROS sources in a feed-forward cycle, c) the enzyme can amplify ROS production from other sources and induction of eNOS uncoupling (Landmesser et al., 2003) and, d) the enzyme appears to be specifically designed for involvement in localized redox signaling (Figure 8) (Cave et al., 2006; Griending, Sorescu, Lassegue, & Ushio-Fukai, 2000; Lambeth, 2004). In endothelial cells, ROS generation by NADPH oxidase is known to uncouple eNOS, thus amplifying ROS in these cells (Landmesser et al., 2003).



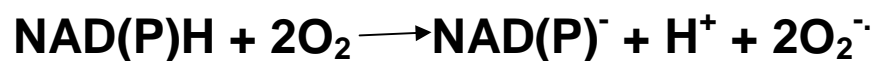
**Figure 8. NADPH oxidase enzyme as the central ROS source.** The enzyme can get activated in response to ROS from other sources; superoxide produced in turn can activate other ROS producing complexes. NADPH oxidase enzyme therefore acts as central ROS generating enzyme in endothelial cells (Adapted from Shah AM et al., 2006).

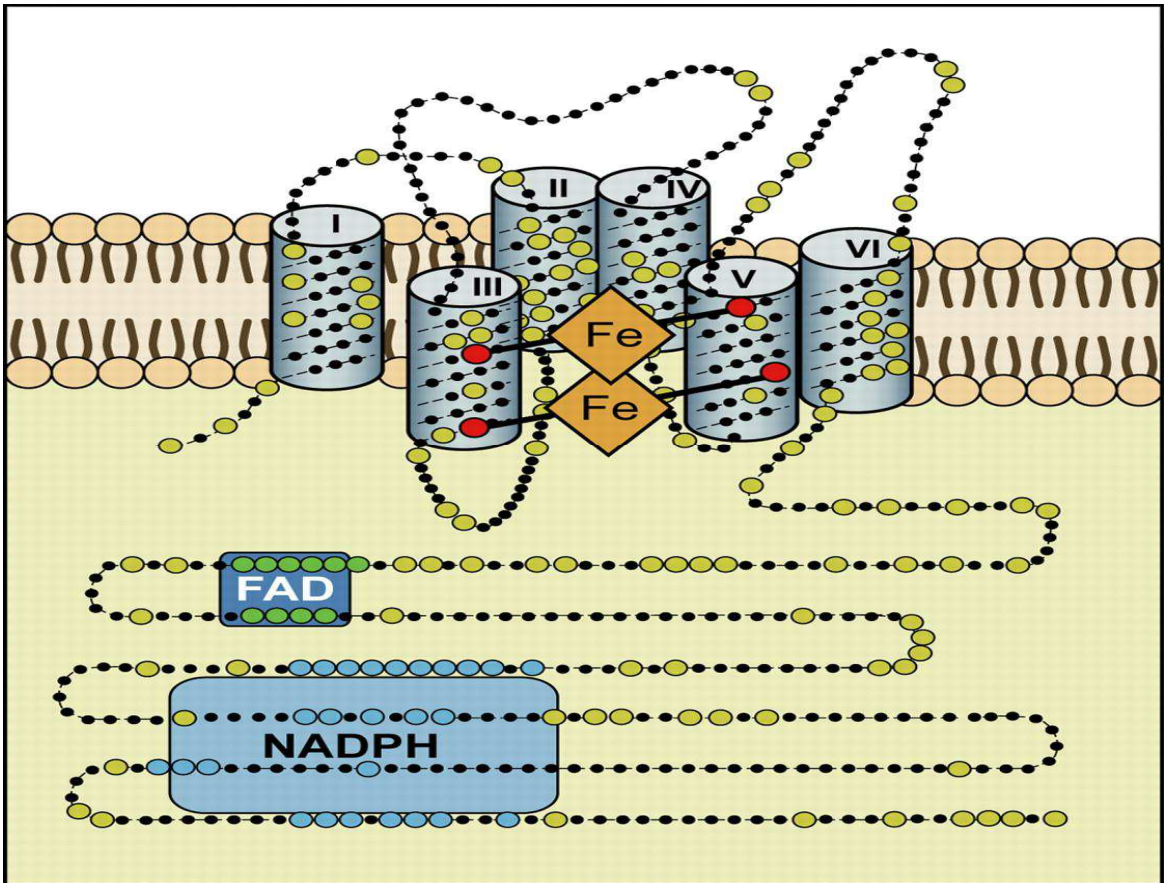
## The NADPH oxidase enzyme complex:

### *Structure and Functions*

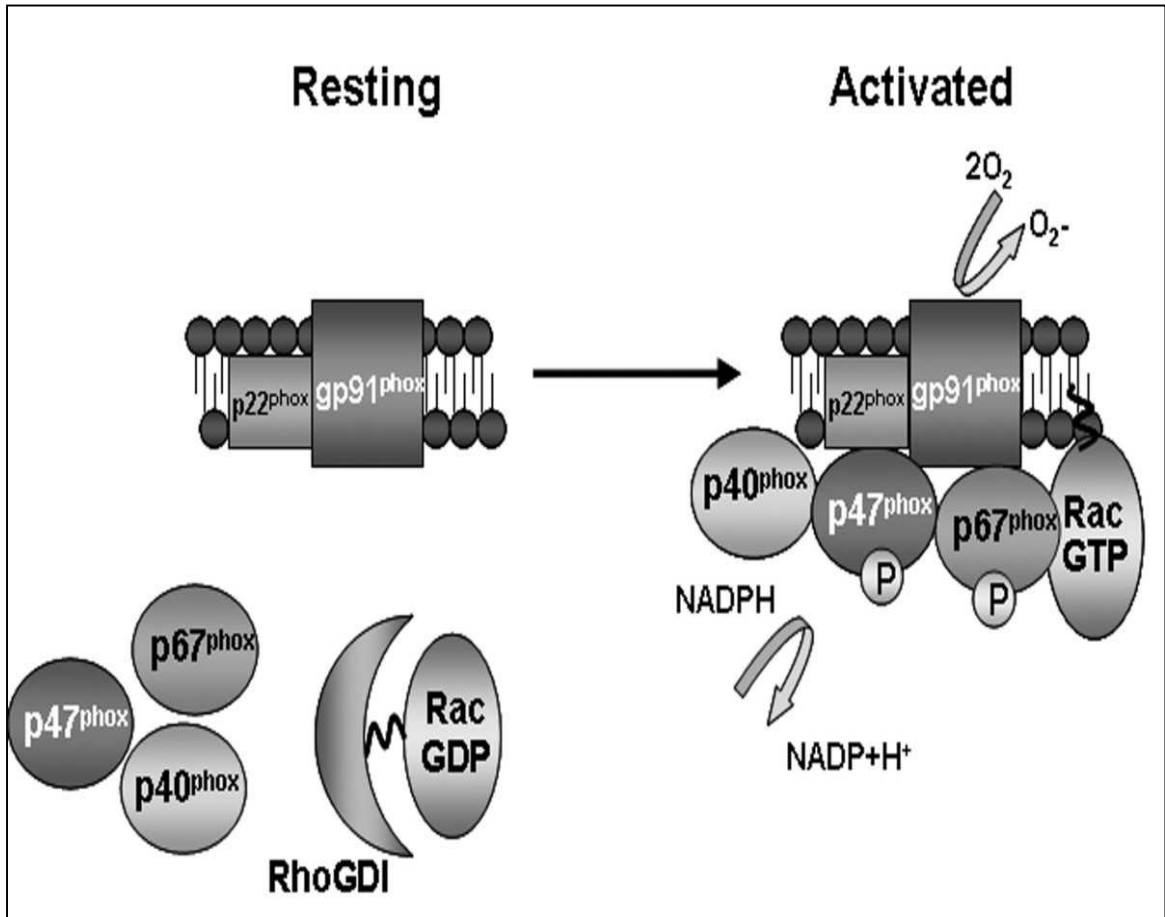
The enzyme NADPH oxidase was first reported in phagocytes, where it is involved in respiratory burst activity to kill invading pathogens (Lambeth JD et al., 2004). The enzyme complex later found in many cell types including fibroblasts, neurons, vascular smooth muscle cells and endothelial cells (Jones SA et al., 1996). It is composed of two membrane integrated catalytic subunit, **Nox (NADPH Oxidase)** or gp91<sup>phox</sup> (**ph**agocytic **ox**idase) and p22<sup>phox</sup> which is directly associated and cytosolic components like p47phox, p67phox, p40phox and other small GTP-binding proteins called Rac1 and Rac2 (Knaus UG et al., 1991).

The central catalytic core of the enzyme complex, gp91 includes, a) a NADPH-binding site at the –COOH terminus, b) a Flavin Adenine Dinucleotide (FAD)-binding region in proximity of the COOH-terminal transmembrane domain, c) six conserved transmembrane domains, d) four highly conserved heme-binding histidines, d) an additional –NH2-terminal transmembrane domain with EF hands and, f) peroxidase homology domain (Figure 9). Activation of NADPH oxidase enzyme involves, recruitment of cytoplasmic subunits (p40, p47, p67) to the membrane subunits (gp91, p22) in endothelial cells (Figure 10).





**Figure 9.** Proposed structure of the core region of NADPH oxidase (NOX) enzyme, gp91. The enzyme contains six highly conserved transmembrane domains III and V, each contain 2 histidines spanning two asymmetrical hemes. The cytoplasmic COOH terminus contains conserved FAD and NADPH binding domains. Enlarged circles represent amino acids that are conserved through human Nox1, Nox2, Nox3, and Nox4 (Adapted from Krause K-H et al., 2007)



**Figure 10. Diagrammatic representation showing activation model of endothelial NADPH Oxidase enzyme complex involving Nox2.** Upon stimulation with either atherogenic stimuli or superoxide produced from other ROS sources, cytoplasmic subunits p40, p47 and p67 translocate to membrane and associate with gp91 and p22 subunits and Rac GTPase, resulting in formation of superoxide radicals (Adapted from Landreth GE et al., 2006).

### *Cellular and sub-cellular distribution*

The gp91phox subunit of NADPH oxidase enzyme assembly exists in multiple isoforms showing different cellular and sub-cellular distribution (Figure 11). These isoforms include, Nox1, Nox2, Nox3, Nox4, and Nox5 which encode predicted proteins around 65kDa and show 21%-59% identity to Nox2, with Nox3 being the most similar and Nox5, the most divergent (Figure 12).

**Nox1** was the first homolog of Nox2 to be described. Nox1 and Nox2 genes appear to be the result of relatively recent gene duplication, as the number and the length of the exons is virtually identical between the two genes. Similarly, at the protein level, there is a high degree of sequence identity (60%) between Nox1 and Nox2. The message for Nox1 is most highly expressed in vascular smooth muscle cells, uterus, placenta, prostate, osteoclasts, and retinal pericytes (Lessegue B et al., 2003; Csanyi G et al., 2009; Zhang G et al., 2007; Hilsenki LL et al., 2004; Miller FJ et al., 2007). There are several studies reporting a subcellular localization of Nox1 in ER and in membrane microdomains called as caveolae of vascular smooth muscle. Small GTPase Rac is required for regulating of Nox1 activity. The novel cytosolic subunits of Nox1 are named NOXO1 (NOX organizer 1 = p47<sup>phox</sup> homolog) and NOXA1 (NOX activator 1 = p67<sup>phox</sup> homolog). In addition to its dependence on cytosolic subunits, NOX1 requires the membrane subunit p22<sup>phox</sup>.

**Nox2** is most commonly found in the endothelial cells along with its associated regulatory proteins under basal conditions (Duerrschmidt, Wippich, Goettsch, Broemme, & Morawietz, 2000; Gorlach, Brandes, Bassus, et al., 2000; Gorlach, Brandes, Nguyen, et al., 2000; Hohler, Holzapfel, & Kummer, 2000; Mollnau et al., 2002; Paravicini, Gulluyan, Dusting, & Drummond, 2002; Peshavariya, Dusting, & Selemidis, 2007;

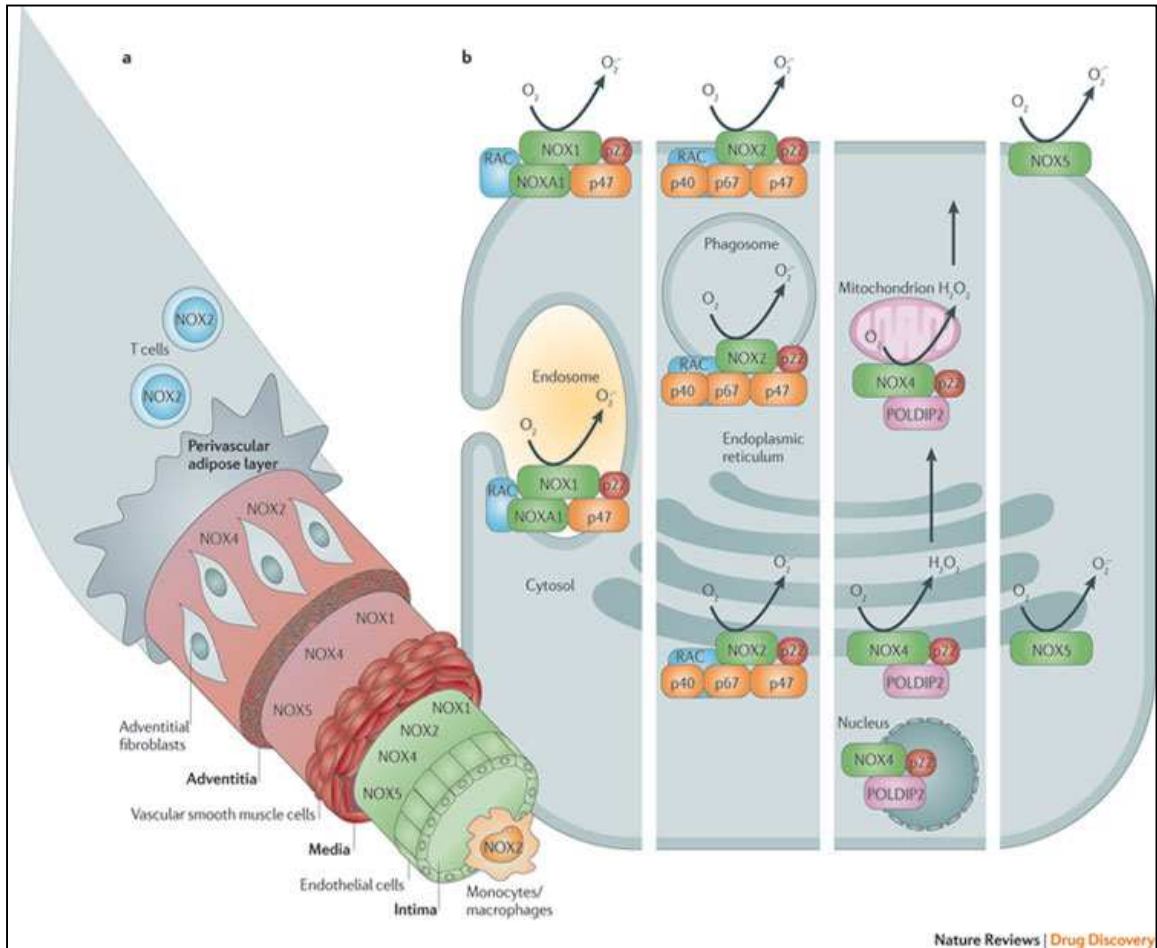
Rueckschloss, Galle, Holtz, Zerkowski, & Morawietz, 2001; Selemidis, 2008; Selemidis et al., 2008; Wagner, Schroeter, & Hecker, 2001). While the exact mechanism of regulation is still undetermined, the present activation model is as follows: Phosphorylation of p47phox, the “organizer subunit” leads to a conformational change allowing its interaction with p22phox, further bringing “activator subunit”, p67phox and p40phox into contact with Nox2. The other cytosolic factor GTPase Rac then interacts with Nox2 and p67phox resulting in superoxide generation by transferring an electron from NADPH in the cytosol to oxygen on the extracellular side (Figure 10). Nox2-oxidase dependent NADPH oxidase activity in endothelial cells is involved in their proliferation during angiogenesis. *In vivo* animal models suggest that under vascular proinflammatory/proatherogenic conditions, such as hypertension, diabetes, hypercholesterolemia, Nox2 levels are upregulated in vessel endothelium (Bengtsson SH et al., 2003). Furthermore, in human arteries from coronary artery disease patients, Nox2 appears to be upregulated in endothelial cells (Guzik TJ et al., 2003). Similarly, AngII infused Wt and Nox2KO mice showed differences in ROS levels and 3-nitrotyrosien staining (Wang HD et al., 2001).

**Nox3** has sequence similarity to other NOX isoforms. Nox3 shares □56% amino acid identity with Nox2. The overall structure of Nox3 is highly similar to that of Nox1 and Nox2, in terms of transmembrane domains, the length of the extracellular loops, NADPH- and FAD-binding sites, and the localization of the heme-coordinating histidines. Nox3 has been shown in different tissues, including fetal spleen, fetal kidney, skull bone, brain and that of inner ear. Nothing is known about the subcellular localization of Nox3. Nox3 is a p22<sup>phox</sup>-dependent enzyme; its expression stabilizes the p22<sup>phox</sup> protein and its translocation to the plasma membrane. In functional studies, p22<sup>phox</sup> is required for Nox3

activation and truncated p22<sup>phox</sup> inhibits ROS generation by Nox3. Nox3 lacks cytosolic subunits like other Nox's. Heterologously expressed Nox3 is found either to be inactive, weakly active or substantially active, whereas activation of Nox3 can be induced by heterologous expression p47<sup>phox</sup> and p67<sup>phox</sup> of other Nox's.

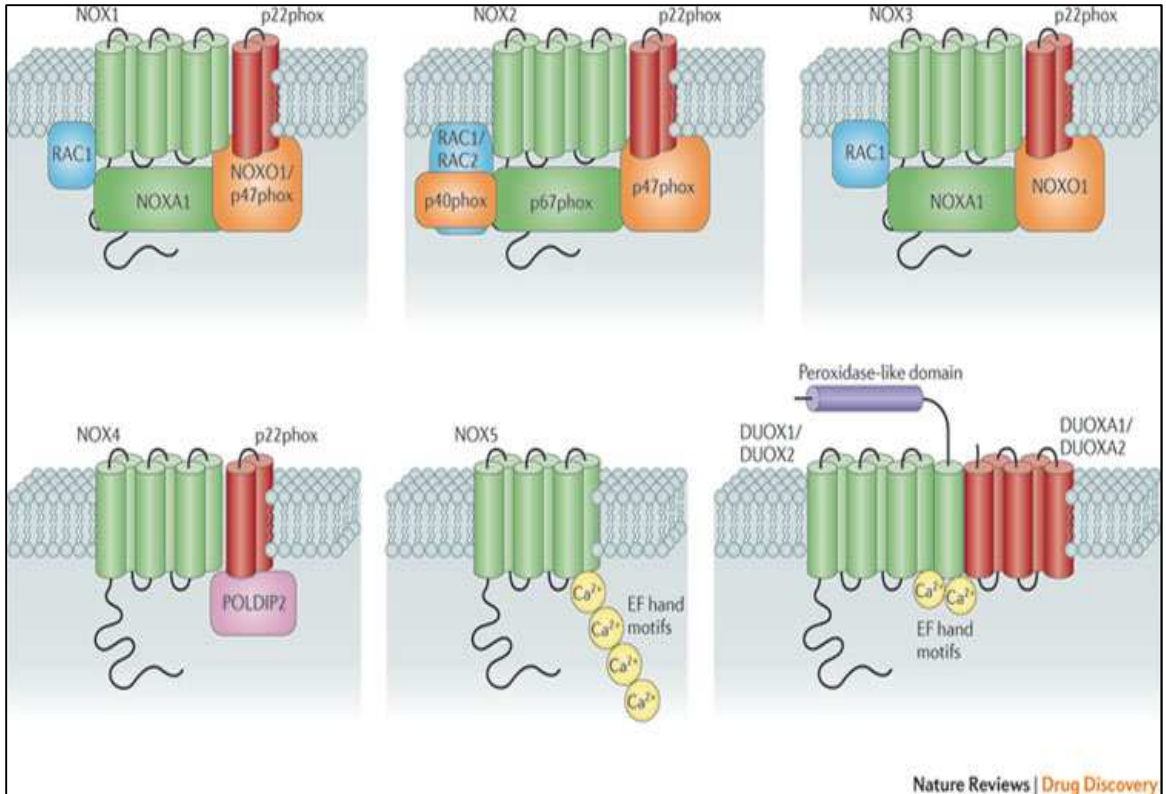
**Nox4** is highly expressed in endothelial cells and is unique amongst the Nox2 isoforms due to the fact that it only requires membrane p22<sup>phox</sup> subunit and not cytosolic subunits for its activity. It is therefore constitutively active isoform. The other cells showing expression of NOX4 include, VSMCs, osteoclasts, fibroblasts and hematopoietic stem cells. Nox4 sub-cellular localization includes focal adhesions in VSMCs and most surprisingly has also been found in nucleus of endothelial cells. Under conditions of endoplasmic reticulum stress, shear stress, carotid artery injury, hypoxia, and in pathologies showing increased transforming growth factor (TGF- $\beta$ ) and tumor necrosis factor (TNF- $\alpha$ ), Nox4 shows increased expression in VSMCs and endothelial cells.

**Nox5** is the most divergent in sequence homology to Nox2 and is also shown to function in a cell-free system without requirements of any cytosolic organizer or activator subunits. Nox5 has a Ca<sup>+2</sup>-binding domain, which undergoes conformation changes and interacts with its own COOH-terminus in response to Ca<sup>+2</sup> changes resulting in its activation. Nox5 is also expressed in VSMCs, bone marrow, testis, ovary, and stomach tissues (BelAiba RS et al., 2007).



**Figure 11. Showing cellular and subcellular distribution of NADPH oxidase isoforms in blood vessel wall.** a) Show distribution of Nox's in vessel cells. Endothelial cells express Nox1, Nox2, Nox4, Nox5. b) Schematic of a hypothetical cell in which all of the vascular NADPH oxidase isoforms (starting with NOX1 oxidase in the left hand column and finishing with Nox5 oxidase in the right hand column) are expressed in each of their possible subcellular locations (Adapted from Drummond GR et al., 2011).

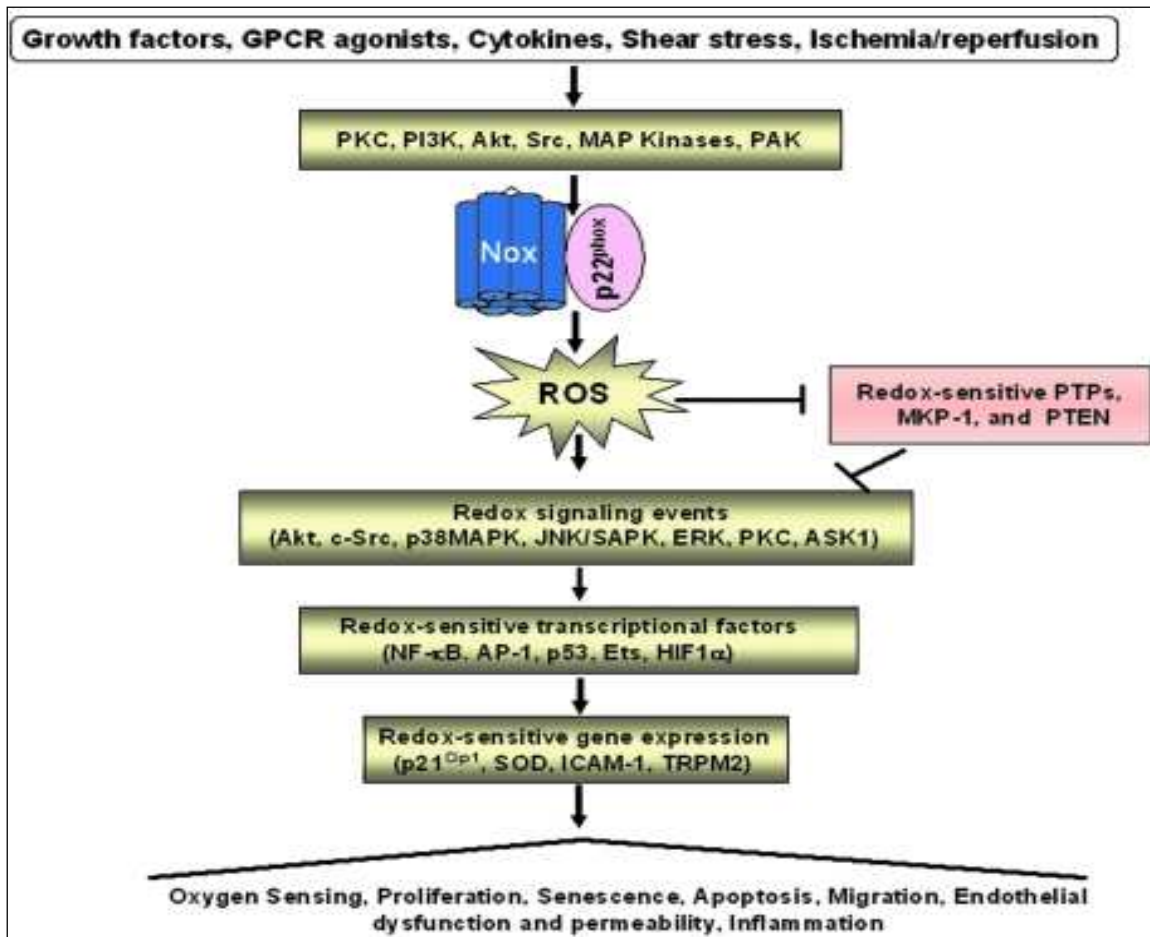
**Dual Oxidases (DUOX1 and DUOX2)** are very large Nox isoforms (earlier called thyroid oxidases, based on the organs they were discovered in). These proteins have an extra trans-membrane peroxidase domain along with the six commonly found in all Nox isoforms (Lambeth, 2004). It had been known for a long time that thyroid epithelial cells produce  $H_2O_2$  at the apical plasma membrane in a  $Ca^{2+}$ - and NADPH-dependent manner. In addition to a Nox1–4-homology domain and an EF-hand region, DUOX proteins have a seventh transmembrane domain at the  $NH_2$  terminus with an ecto-facing peroxidase like domain. Within the Nox backbone, DUOX isoforms share ~50% identity with Nox2. Both DUOX enzymes are N-glycosylated. Both DUOX1 and DUOX2 are highly expressed in the thyroid, airway epithelia, in prostate. In thyrocytes, DUOX enzymes localize to the apical membrane and in the ER. When heterologously expressed, DUOX enzymes tend to be retained in the ER, and superoxide generation can be measured only in broken cell preparations. DUOX enzymes do not require activator or organizer subunits; however, the  $p22^{phox}$  requirement is still a matter of debate. DUOX enzymes coimmunoprecipitate with  $p22^{phox}$ , but there is no evidence for enhanced DUOX function upon co-expression of  $p22^{phox}$ .



**Figure 12 NADPH oxidase enzyme isoforms and activation mechanisms.** Nox1 requires p22phox, p47, p67, small GTPase Rac. Nox2 requires p22phox, p47phox, p67phox, and Rac1, p47 phosphorylation is essential for Nox2 activation. Nox3 requires p22phox, and NOXO1, and requirement for Rac is debated. Nox4 requires p22phox, but it is constitutively active without the requirement for other subunits. Nox5, DUOX1 and DUOX2 are activated by  $Ca^{+2}$  and do not appear to require subunits (Adapted from Drummond GR et al., 2011).

### *Regulation of the endothelial NADPH oxidase and redox signaling*

It has become more important to study the endothelial NADPH oxidase due to its regulation by wide range of patho-physiologically relevant factors like, 1) agonists of G-protein coupled receptors such as Angiotensin-II, 2) growth factors such as thrombin and VEGF, 3) cytokines such as TNF $\alpha$ , 3) metabolic factors such as increased glucose, insulin, non-esterified fatty acids (NEFA) or advanced glycation end products (AGEs), 4) oxidized lipids, 5) oscillatory shear stress, and 6) hypoxia/reoxygenation and nutrient deprivation. The mechanisms underlying an increase in oxidase activity are either an acute increase in oxidase complex formation secondary to post-translation modification of regulatory subunits (p47<sup>phox</sup> and Rac1) or a chronic increase in the expression and abundance of component subunits. A major mechanism that is involved is PKC dependent phosphorylation of the p47<sup>phox</sup> regulatory subunit and its translocation to the Nox2/p22<sup>phox</sup> hetero-dimer to form more fully assembled complexes (Figure 13). It has been shown that p47<sup>phox</sup> phosphorylation is induced via acute activation by angiotensin-II, phorbol esters, TNF $\alpha$ , VEGF and oscillatory shear stress (J. Li et al., 2008). The role of p47 phosphorylation is an important event in the TNF $\alpha$  induced activation of the enzyme. These Coronary microvascular endothelial cells (CMECs) derived from p47KO mice showed decreased MAPK activation and cell surface adhesion, ICAM-1, suggesting important roles in redox signaling and endothelial activation and dysfunction.



**Figure 13.** Schematic showing role of cell signaling pathways in NADPH oxidase activation to produce ROS and its role in inducing redox signaling mediated endothelial activation and dysfunction. Under the influence of atherogenic stimuli, signaling mediators activate to regulate NADPH oxidase enzyme activity in producing ROS, which further activates specific redox signaling events. The signaling pathways converge to regulate expression of antioxidant enzymes and inflammatory adhesion molecules such as ICAM-1, all-important in cellular pathophysiology (Frey SF et al., 2009).

### *Endothelial NADPH Oxidase system*

Endothelial cells express mRNA and protein for Nox2 and its associated regulatory proteins, p22phox, p47phox, p67phox, p40phox, and Rac1 under basal conditions and high levels of both Nox4 and Nox5. In contrast, they express almost undetectable (Petry, Weitnauer, & Gorch, 2010) or lower levels of Nox1. Although the expression levels of Nox4 appear to be markedly higher (upto ~1000-fold) than Nox1, Nox2 or Nox5, Nox2 still appears to be a major source of superoxide in endothelial cells. All the Nox's in endothelial cells predominantly show peri-nuclear localization on the membranes of the endoplasmic reticulum and nucleus (J. Li et al., 2008). It has recently been shown by various groups that Nox2 is localized on the plasma membrane micro-domains, namely lipid-rafts/caveolae. The receptors for most of the pro-atherogenic ligands like Angiotensin-I (AT<sub>1</sub>R), Platelet derived growth factor (PDGF) and Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ R1) are also located in lipid-raft and caveolae microdomains (Stehr et al., 2003; Zuo et al., 2005) and are shown to increase endothelial superoxide production in a Nox2 or its regulatory partner expression dependent manner. *It is highly possible that these micro-domains could serve as major signaling platforms for patho-physiological ROS generation.*

### Therapies to reduce ROS/RNS induced endothelial dysfunction

Various therapeutic strategies have been tried to reduce pathological effects of increased oxidative stress in patients with cardiovascular diseases along with enhancing endogenous anti-oxidant systems. These include: a) **Vitamin C**: The efficacy of vitamin C in preventing or reverting endothelial dysfunction is controversial. In cultured endothelial cells, the antioxidants increase eNOS activity via regeneration of BH<sub>4</sub> (Heller

L et al., 2001). Long-term *in vivo* vitamin C treatment restored endothelial function and eNOS activity in the aortae of apoE<sup>-/-</sup> mice. In addition, in patients with coronary artery disease, acute infusions of high-dose vitamin C have been found to improve endothelial function. Some studies of patients with coronary artery disease have also demonstrated improved endothelial function and endothelial NO production and reduced levels of ROS in the vascular wall after longer term, oral treatment with vitamin C. The exact mechanism of action of L-ascorbate in improving endothelial dysfunction is not clear, but the most important mechanism might prove to be the enhanced regeneration or stabilization of BH<sub>4</sub> and eNOS recoupling, rather than the scavenging of superoxide. Disappointingly, however, the results of long-term epidemiological trials with oral vitamin C have been ambiguous at best and do not support vitamin C supplementation to reduce the risk of coronary disease or other types of cardiovascular morbidity or mortality, b)

**Vitamin E:** The Vit E was initially believed to have a role in preventing diseases associated with oxidative stress because of its anti-oxidative properties. Unfortunately, recent large-scale randomized clinical trials undertaken to prove this hypothesis failed to verify a consistent benefit in terms of prevention of coronary heart disease and death, c)

**Angiotensin-converting-enzyme inhibitors and angiotensin II receptor antagonists:**

Angiotensin II activates NADPH oxidases via AT<sub>1</sub> receptor stimulation. In addition, expression of the AT<sub>1</sub> receptor is upregulated *in vitro* by LDL cholesterol. Angiotensin-converting-enzyme (ACE) inhibitors and AT<sub>1</sub> receptor antagonists could, therefore, have indirect antioxidant effects by preventing the activation of NADPH oxidase (Klingbeil et al., 2003) and increasing the activity of SOD3. Indeed, clinical evidence indicates that inhibition of the renin–angiotensin system has a beneficial effect; ACE inhibitors and AT<sub>1</sub> receptor antagonists improve endothelial function in patients with coronary artery disease and in hypertensive patients. Furthermore, the Heart Outcome Prevention

Evaluation (HOPE) trial showed that treatment with Ramipril greatly reduced the incidence of death, myocardial infarction and stroke in high-risk patients without heart failure (Yusuf, Dagenais, Pogue, Bosch, & Sleight, 2000). The European Trial on Reduction of Cardiac Events with Perindopril in Stable Coronary Artery Disease (EUROPA) also demonstrated a 20% reduction in relative risk for cardiovascular end points with perindopril in a patient population with stable coronary heart disease (Fox KM et al., 2003), d) **Statins:** Statins are shown to have pleiotropic beneficial effects on vascular oxidative stress (Liao, 2002a, 2002b). Statins can inhibit endothelial superoxide formation by preventing the isoprenylation of p21 Rac, which is critical for the assembly of NADPH oxidase after activation of protein kinase C (Wagner et al., 2001). In addition, SOD3 activity more than doubled and the number of functionally active endothelial progenitor cells increased in individuals treated with simvastatin. In addition, statins can increase the expression of eNOS by inhibition of isoprenylation of the small GTPase RhoA (Landmesser et al., 2003) and statins directly activate eNOS via post-translational mechanisms involving activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway. Taken together, statin treatment improves endothelial function at least in part by reducing oxidant stress and improving eNOS function.

#### Possible reasons for the failure and solutions

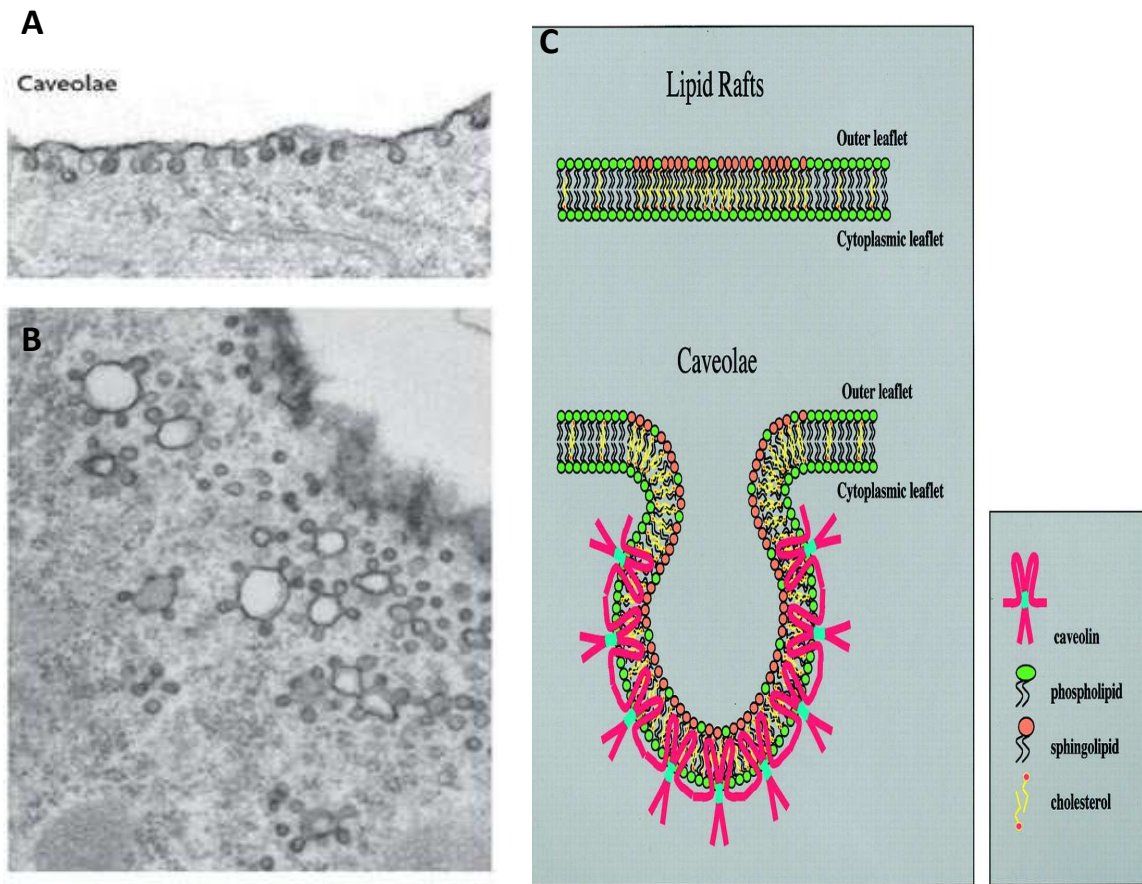
Therapeutically, despite promising initial observations, clinical trials investigating supplementation with vitamins C or E failed to show improved cardiovascular outcome. There are several potential reasons for this lack of effect. The anti-oxidants used could have been inappropriate or dosing may have been insufficient. The rate constants for reactions between vitamins C or E and  $O_2^{\cdot -}$  are five to six orders of magnitude less than the rate constant for the reactions of  $O_2^{\cdot -}$  with NO. Furthermore, radical-scavenging vitamins can become radicals themselves with pro-oxidant activity (vitamin E—the

tocopheroxyl radical; vitamin C—the ascorbyl radical). Antioxidant vitamins C and E do not scavenge hydrogen peroxide or hypochlorous acid, oxidants that may be involved in the induction of vascular damage. Moreover, vitamin E is concentrated in lipid membranes and lipoproteins. Oxidative events occur in the cytoplasm and in extracellular space and would not be affected by lipid-soluble antioxidants. Finally, the signaling effects of ROS could mean that any long-term intervention into the system provokes counter-regulations leading to rebound ROS production. It is also possible that orally administered anti-oxidants may be inaccessible to the sources of free radicals, particularly if ROS are generated in intracellular compartment and organelles. Furthermore, antioxidant vitamins do not scavenge  $H_2O_2$  or HOCl, which may be more important than  $O_2^-$  in hypertensive vascular damage. Another factor, which should be considered, is that antioxidants do not inhibit ROS production. Theoretically, agents that reduce oxidant formation should be more efficacious than scavengers in ameliorating oxidative stress. This is based on experimental evidence where it has been shown unambiguously that inhibition of ROS source, NADPH oxidase leads to regression of vascular remodeling, improved endothelial dysfunction, and lowering of blood pressure. The experimental evidence supporting a role for oxidative stress in vascular injury and hypertension is convincing, whereas what remains to be known is, a) mechanism by which ROS regulate specific cellular signaling related to cardiovascular system diseases, b) what tips the balance to pro-oxidant state in hypertension, and c) whether scavenging free radicals is indeed the best way to decrease ROS bioavailability. The failure in anti-oxidant and other therapeutic studies to reduce oxidative/nitrative stress suggests our lack of complete understanding of mechanism of ROS/RNS production and its regulation during physiological and pathological conditions. The missing pieces in puzzle of radical stress mediated cardiovascular diseases include, 1) elucidating

mechanisms whereby free radicals modify signaling proteins in cardiovascular system, 2) exploring, why ROS/RNS generating systems are up regulated and anti-oxidant systems are down regulated during pathologies? 3) determining, whether it is preferable to prevent or limit formation of free radicals by targeting the sources rather than by scavenging them once they have been generated, and 4) Since enzymes responsible for oxidative and nitrative stress in cardiovascular diseases are localized in membrane microdomains, lipid-rafts and caveolae, elucidating regulatory mechanisms of compartmentalized spatio-temporal radical generation and associated nitroxidative signaling can prove to be very beneficial in developing effective therapies.

#### Lipid-rafts and caveolae

For many years the plasma membrane was believed to be a bilayer of phospholipids with free-floating cholesterol, lipid and other proteins. This has been replaced by the concept that plasma membrane is a phospholipid bilayer with unevenly distributed lipids, cholesterol and proteins organized into discrete domains called as membrane microdomains or lipid-rafts/caveolae (Palade, 1953; Yamada, 1955) (Figure 14). The lipid-rafts microdomains are found to be rich in cholesterol, sphongolipids, sphingomyelin, saturated fatty acids and most importantly signaling proteins (Anderson, Kamen, Rothberg, & Lacey, 1992) and are therefore also known to serve as “signaling platforms”.



**Figure 14. Micrographs and cartoon showing structure of Caveolae.** A) Electron micrographs showing endothelial cell caveolae, and B) showing budding off of caveolae structures from plasma membrane to form cytoplasmic “caveosomes”. C) Schematic showing molecular components of lipid rafts and caveolae and their structural differences (Adapted from Simons K et al., 2007).

### *Caveolae Associated Proteins:*

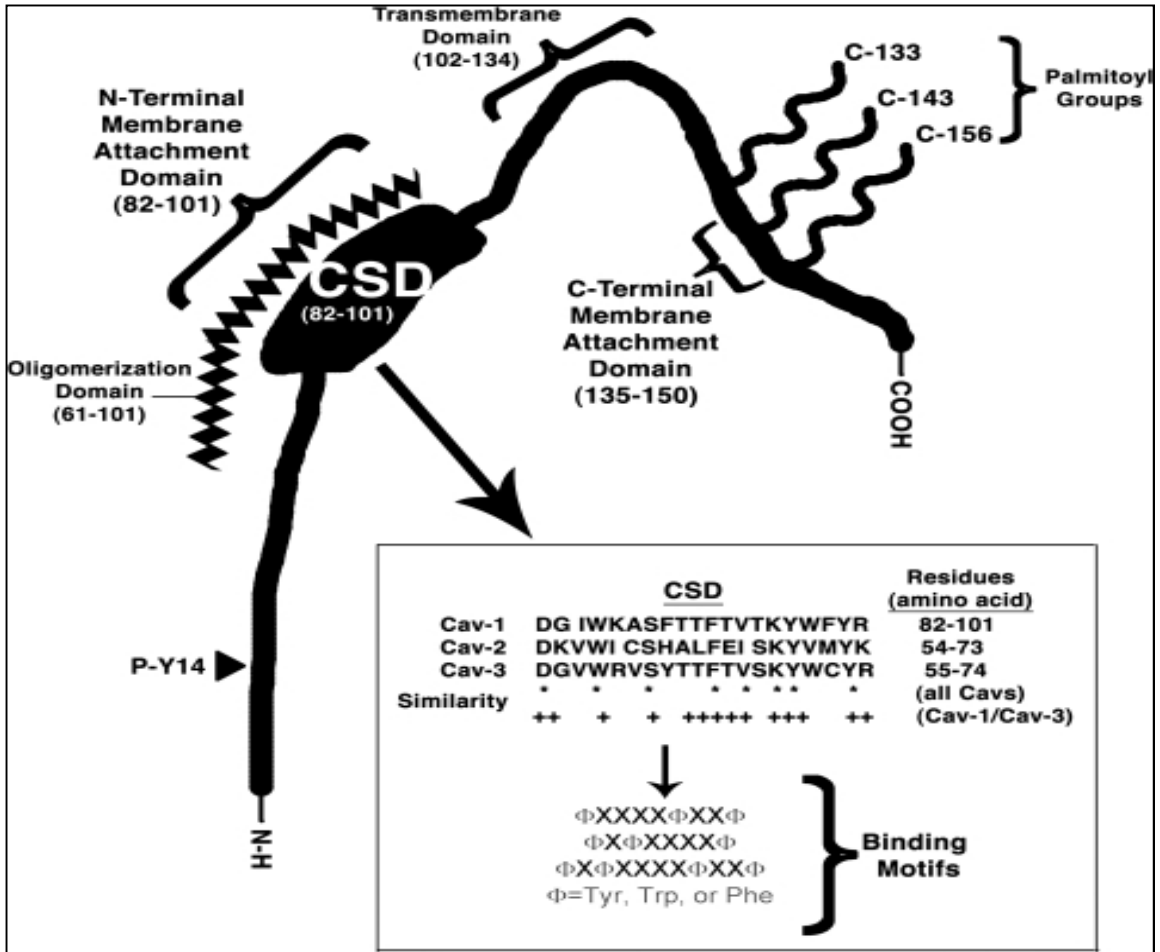
Caveolae microdomains are small (50-100nm) flask shaped invaginations of lipid-rafts associated with caveolin family of proteins (Yamada, 1955). They are abundantly found in adipocytes, endothelial cells, type I pneumocytes, epithelial cells, smooth muscle and striated muscle cells. Caveolae structures are dynamic structures, which constantly recycle between the plasma membrane, endosomes, and the *trans*-Golgi network. Caveolin proteins directly bind cholesterol and represent the major structural components of caveolae. The caveolin gene family consists of caveolin-1, -2, and -3. Caveolins -1 and -2 are co-expressed and form a hetero-oligomeric complex in many cell types like endothelial cells and adipocytes, whereas expression of caveolin-3 is muscle-specific. These caveolin homo- and hetero-oligomers directly interact with cholesterol and represent the functional assembly units of caveolae.

### *Caveolin-1 - Structure and Functions:*

Caveolin-1 is a 22-kDa protein of 178 amino acids with intracellular -NH<sub>2</sub> and -COOH termini and an intervening hydrophobic domain inserted into the membrane. The -COOH terminus of caveolin-1 is palmitoylated and the -NH<sub>2</sub> terminus is tyrosine phosphorylated. Caveolin-1 contains an oligomerization domain mapping residues 61-101, which mediates the homo-oligomerization via -COOH terminal interactions forming large network of caveolin-1 providing scaffold for caveolae organelle. The residues between 82-101 forms Caveolin-1 scaffolding domain (CSD, a region capable of mediating protein-protein interactions). Using a GST-fusion protein containing the caveolin-1 scaffolding domain as a receptor to select peptide ligands from a bacteriophage display library, two related but distinct caveolin binding motifs (CBM) were identified in most

proteins shown to interact with caveolin-1 (CBM;  $\Phi$ XXXX $\Phi$ XX $\Phi$  and  $\Phi$ X $\Phi$ XXXX $\Phi$ , where  $\Phi$  represents an aromatic amino acid and X denotes any amino acid) (Couet, Li, Okamoto, Ikezu, & Lisanti, 1997) (Figure 15). The scaffolding domain serve dual roles of acting as, a) an anchor holding various proteins within caveolae (Src-like kinases, H-Ras, eNOS, and heterotrimeric G-proteins) (Anderson et al., 1992), and b) a regulatory element capable of either inhibiting or enhancing signaling activity of anchored protein.

Based on functional studies, caveolae are known to primarily function in transcytosis of macromolecules through plasma membrane, endocytosis of ligand-receptor complexes, lipid and cholesterol homeostasis and signaling. Transcytosis (transcellular transport) of macromolecules from the luminal side of capillary endothelial cells to the interstitial space is mediated via caveolae. The introduction of novel caveolae-specific tracer molecules and development of caveolin-1 knockout mice (Cav1KO) showed the transcytosis role of caveolae (Komarova & Malik, 2010).



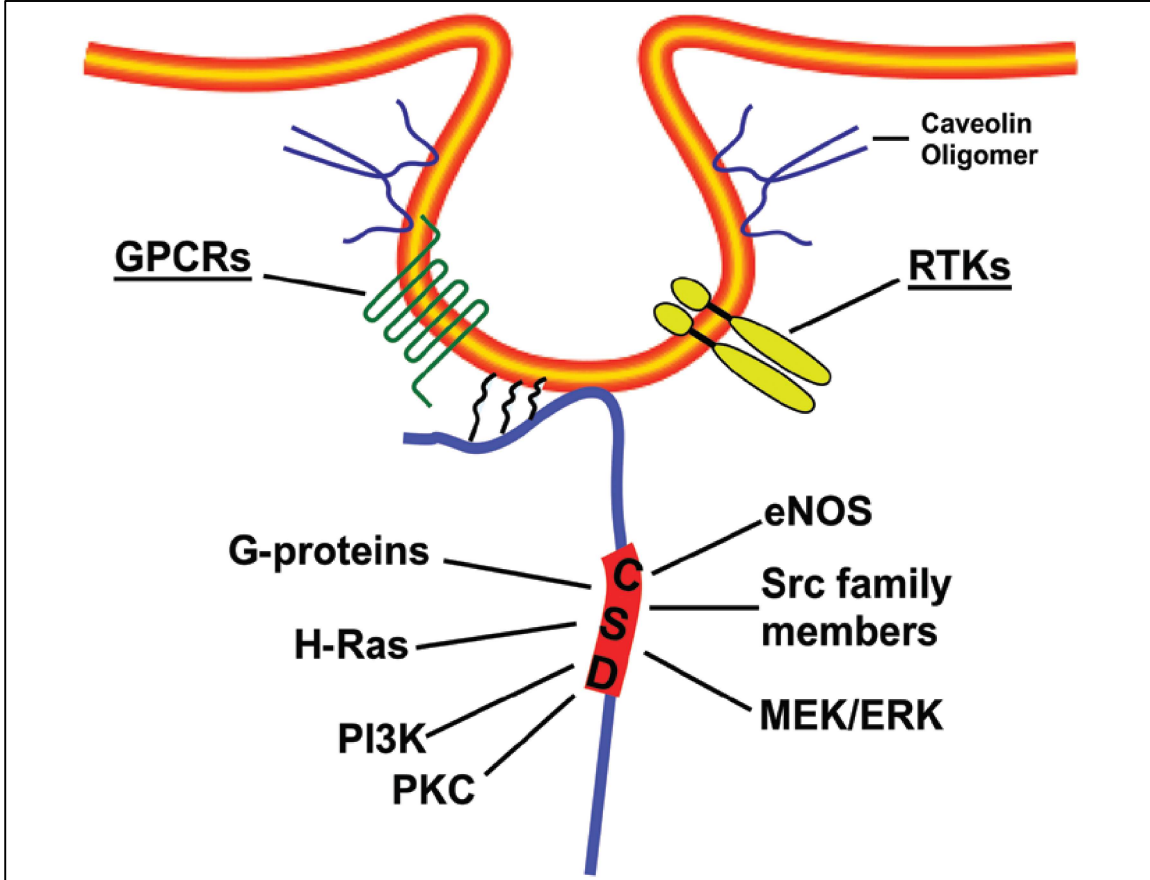
**Figure 15. Schematic showing structure of Caveolin-1.** Cav1 contains hair-pin loop like structure with scaffolding domain (CSD), N-terminal phosphorylation prone tyrosine residue, C-terminal membrane attachment domain with palmitoylation Cysteine residues. Inset shows differences in CSD of Cav1, 2 and 3 with conserved aromatic residues essential for peptide binding (Adapted from Patel HH et al., 2004).

#### Lipid and cholesterol homeostasis:

Caveolae are shown to regulate trafficking of fatty acids and their accumulation into the cells. The support for this role comes from the exogenous expression of Cav1 in cells that normally have no endogenous caveolin. The expression was shown to facilitate the uptake of fatty acids into cells as well as to increase the levels of cholesterol and of cholesterol export (Fielding & Fielding, 2004). Cav1KO mice are also shown to be resistant to diet induced obesity and show decreased adiposity as well as decreased levels of free cholesterol in adipocytes (Ley & Zarbock, 2006; Razani et al., 2002). The close association between Cav1 and plasma membrane fatty acid can corroborate this fact. Cav1-null mice show dramatic reduction in the formation of lipid droplets during liver regeneration and decreased survival after hepatectomy. *In vitro* studies in HEK293 cells expressing Cav1 and Cav3, showed increasing lipid storage in lipid-droplets.

#### *Signaling platforms:*

Lipid-raft/caveolae structures harbor variety of signaling molecules and therefore are also known to act as signaling platforms for activation of cellular signaling. Cav1 regulates many G protein receptors, G $\alpha$  subunits, tyrosine kinases and receptor tyrosine kinases (RTKs), GTPases, components of the MAPK pathway (Anderson et al., 1992). Cav-1 has also been shown to sequester p42/44 MAPK cascade members including EGFR, raf, MAP-1, and ERK-2, inhibiting the activity of this pathway (Engelman et al., 1998) (Figure 16). These signaling molecules bound to caveolin-1 can sometimes keep the signaling molecules inactive, whereas in others their activation is positively regulated. Caveolae also serve important roles in integrin signaling, a key component of mechanotransduction and the inflammatory response.



**Figure 16. Diagram showing caveolae roles as signaling platforms.** Caveolae harbor GPCRs like  $\beta$ -adrenergic receptors, receptor tyrosine kinases (RTKs), enzyme eNOS and diverse signaling mediators such as H-Ras, PI3K, PKC, Src family members and MEK/ERK, which bind to cavolin-1 via CSD, thus regulating specific cell signaling cascades (Panneerselvam M et al., 2012).

## Caveolae Dependent Endocytosis

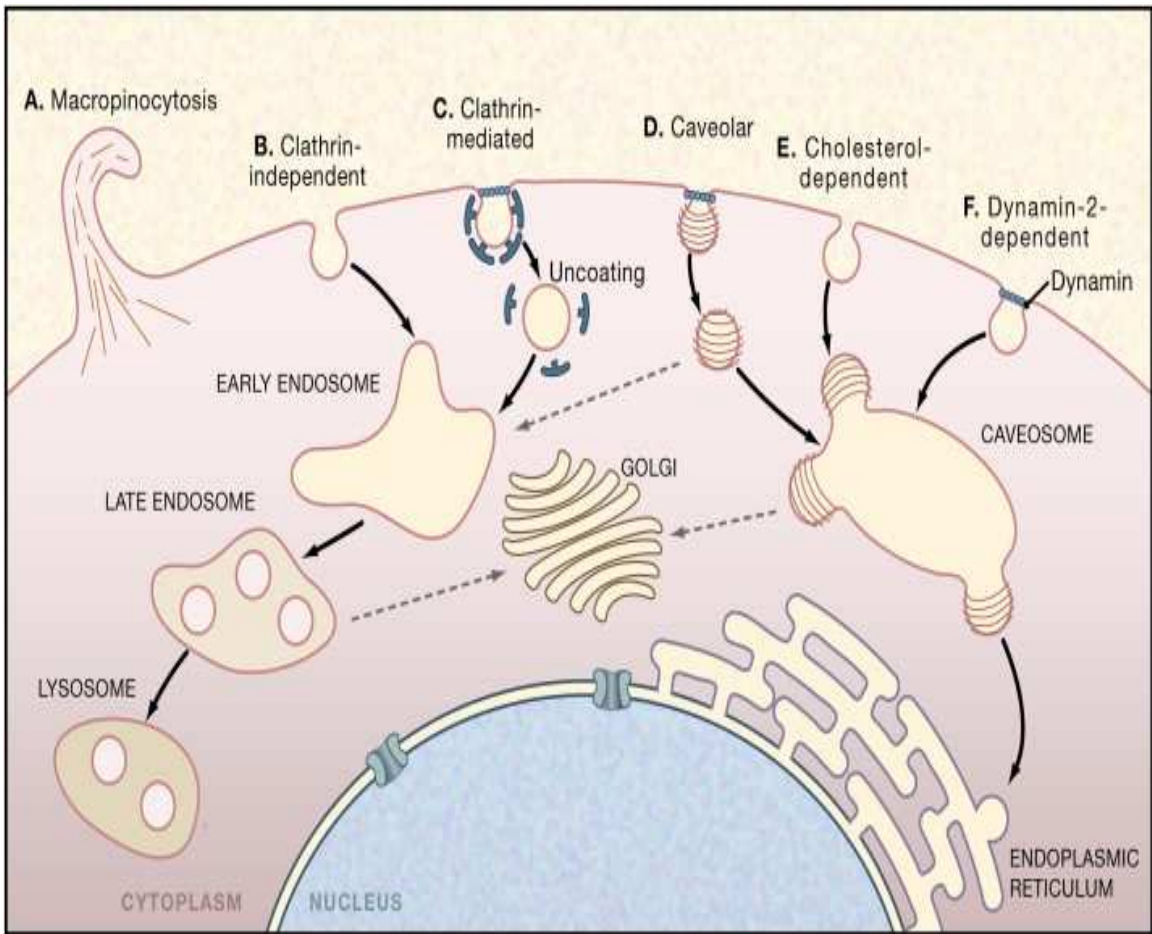
In the absence of any stimulus, caveolae are either present on the membrane as stationary structures or undergo continuous kiss-and-run dynamic cycles of fission and fusion between plasma membrane and short distances within cytoplasm. Whereas upon stimulation with ligands like, folic acid, albumin, alkaline phosphatases, lactosyl ceramide, pro-inflammatory cytokines (like  $\text{TNF}\alpha$  and IL-1) and pathogens like cholera toxin, SV40 virus, these membrane domains can undergo Clathrin-independent internalization (Anderson et al., 1992; Parton, Joggerst, & Simons, 1994; Puri et al., 2001). These ligands stimulate caveolae internalization from short-distance to long-distance dynamics via binding to caveolae/caveolin protein, cross-linking of caveolae components, accumulation of cognate receptors in caveolae or activation of downstream signaling events.

Caveolae endocytosis is dependent on cholesterol and caveolin-1 protein levels. Studies involving depletion of total cholesterol via cholesterol-modulating agents, including methyl- $\beta$ -cyclodextrin, Nystatin, Filipin and overexpression of dominant-negative caveolin-1 showed loss of caveolae formation and its internalization. Caveolin-1 is directly involved in vesicle formation and internalization via caveolin-1 phosphorylation (tyrosine-14). It has been suggested as a negative regulator of raft-dependent endocytosis (Lajoie & Nabi, 2007; Nabi & Le, 2003). Caveolin-1 phosphorylation status is regulated by different kinases and phosphatases. Inhibition of caveolin-1 (Y-14) tyrosine phosphorylation via tyrosine kinase inhibitors blocks caveolae endocytosis, while treatment with phosphatase inhibitor okadaic acid, triggers endocytosis (Botos et al., 2008; Botos et al., 2007; Kiss, Botos, Turi, & Mullner, 2004; Parton et al., 1994).

The cytoskeleton also plays an important role in distribution of caveolae (Mundy, Machleidt, Ying, Anderson, & Bloom, 2002). The, the internalization of caveolae also depends on the integrity and/or organization of the cytoskeleton (Parton et al., 1994).

The other important players involved in internalization of caveolae include GTP-binding protein Dynamin, originally known to be involved in pinching off of Clathrin coated vesicles (CCVs). Dynamin protein exists in 3 different isoforms, dynamin 1, 2 and 3. Dynamin-2 is the most abundantly expressed isoform in endothelial cells. In endothelial cells Dynamin-2 is found at the neck of caveolae and is known to mediate their budding by constriction of plasma membrane (Figure 17) (Oh, McIntosh, & Schnitzer, 1998). Over expression of dominant negative dynamin-2 or mutants unable to hydrolyze GTP inhibits endocytosis by both Clathrin dependent (CCPs) as well as Clathrin-independent/raft-caveole dependent pathways (Oh et al., 1998). Caveolae-mediated endocytosis and transendothelial albumin transport via transcytosis is dependent on dynamin-2 phosphorylation.

Src kinase dependent phosphorylation of Dynamin-2 at tyrosine 231 has been shown as an important modification regulating caveolae endocytosis. The mechanism of Dynamin-2 phosphorylation in caveole internalization has been suggested via its interaction with phosphorylated Caveolin-1. The phosphorylated Dynamin2-Cav1 interaction drives caveole endocytosis via an unknown mechanism. Microvascular endothelial cells transfected with mutant form of Dyn2, a non-Src phosphorylatable Dynamin2 (Y231F/Y597F), lacked caveolin-rich membrane fraction translocation to the cytosol. These cells showed reduced trans-endothelial albumin transport, suggesting important roles of Dyn2 phosphorylation in uptake of macromolecules via caveolae endocytosis.



**Figure 17. Dynamin2 mediated internalization of caveolae.** Caveolae unlike clathrin-coated pits (CCPs), undergo internalization without clathrin coat. The internalization of caveolae structures is dependent on the cholesterol levels, caveolae phosphorylation and like CCPs on phosphorylation state of Dynamin2 (Marsh M et al., 2006).

## Caveolin-1 deficient mice, pathologies and Atherosclerosis

Caveolin-1 protein knockout mice, Cav1<sup>-/-</sup> (Cav1KO) has provided excellent models to study the relevance of physiological functions assigned to caveolae. Cav1KO mice generated are viable and fertile. These mice show a remarkable lack of caveolae in all non-muscle tissues confirming the necessity of this protein in caveolae biogenesis. Since Cav1 and Cav2 are known to form hetero-oligomeric complexes, Cav1 knockout mice showed drastic reduction of caveolin-2 expression, without any noticeable change at the transcription level. Aortic rings derived from Cav1 KO mice show drastic reduction in radiolabeled albumin uptake compared to wild-type mice (Schubert et al., 2002), suggesting vesicular trafficking alterations, implicated in vascular diseases.

The Cav-1KO mice show increased activation of eNOS and NO production in vasculature, cardiomyopathy, impaired vascular reactivity, mechanotransduction, cell proliferation and severe cardiovascular and pulmonary phenotypes. When these Cav-1KO mice were specifically reconstituted with Cav-1 in endothelium, it rescued the cardiovascular and pulmonary phenotypes by correcting vascular leakage, abnormal vasomotion. This demonstrates that diverse cardiovascular phenotypes in these mice are attributable to caveolae in the endothelium and not to other cell types that express Cav-1.

Role of Caveolae in Atherosclerosis: Cav1KO mice plasma lipoprotein analysis shows a shift towards an atherogenic profile, characteristic for a hypertriglyceridemia (elevated VLDL/chylomicrons) (Razani et al., 2002), but have normal plasma cholesterol levels. The knockout mice display a marked intolerance in the clearance of an oral fat load independent of lipoprotein and hepatic lipase activities. Whereas, when Cav1<sup>-/-</sup> mice

were crossed with atherosclerosis-prone apoE<sup>-/-</sup> mice (double-knockout-DKO), the progeny displayed a significant decrease (70% decrease) in atherosclerotic lesion formation in aortas in spite of the elevations in plasma cholesterol and triglycerides compared to apoE<sup>-/-</sup> mice (Frank et al., 2004; Frank & Lisanti, 2004). The protective effects can be explained by the defects in expression of pro-atherogenic molecules like scavenger receptor for oxidized and modified LDL, CD36 and the vascular cell adhesion molecule-1 (VCAM-1), found to be markedly reduced in aortic extracts from DKO mice.

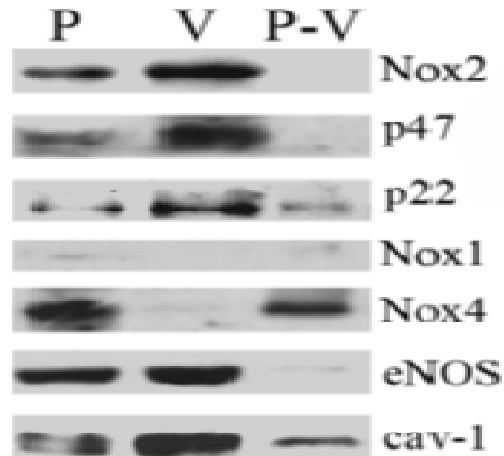
#### Lipid-raft/caveolae Localization of eNOS and NADPH oxidase Complex

##### *eNOS localization in Caveolae:*

The direct interaction of eNOS and Caveolae has been studied very well and it appears to be primary regulatory mechanism for the activation of eNOS (Garcia-Cardena et al., 1997; Shaul et al., 1996). The Cav-1/eNOS interaction tonically inhibits eNOS activity resulting in sequestration of eNOS in caveolae and reduced NO production. Caveolin-1 has been found to be a potential molecular chaperone, which directly inactivates eNOS via a reversible process, modulated by Ca<sup>+2</sup>-calmodulin. The interaction of eNOS and Cav1, with enrichment in the micro-domain associated with reduced enzyme activity, led to the “caveolar paradox,” whereby enrichment does not lead to enhanced activity (Feron & Kelly, 2001). The paradox was resolved by the evidence that eNOS is dually regulated: direct interaction with caveolin under basal conditions maintaining enzyme activity in an inactive state but enrichment of eNOS in caveolae providing a means for rapid, high-fidelity response upon stimulation (Sbaa, Frerart, & Feron, 2005). These studies suggest that the functional consequence of eNOS targeting to caveolae are most likely temporally and spatially distinct events in regulating NO production in endothelial cells.

*Compartmentalization of NADPH oxidase enzyme in Lipid-rafts and Caveolae:*

Studies in neutrophils showed that Nox2 is constitutively present in lipid-rafts and that other NADPH oxidase subunits- p47<sup>phox</sup>, p40<sup>phox</sup>, and p67<sup>phox</sup> are recruited in response to an activating stimulus (Shao, Segal, & Dekker, 2003). This was further demonstrated by depleting membranes of lipid-rafts by exposing cells to the cholesterol-sequestering agent Methyl- $\beta$ -Cyclodextrin (M $\beta$ CD), inhibiting Nox2 activation. Nox1 and Nox2 have been shown to localize in lipid-rafts/caveolae of non-phagocytic cells also, like VSMC's and endothelial cells. Lipid-raft localized Nox1 in VSMCs are suggested to play Ang-II induced redox-signaling roles. Other direct evidence for the presence of Nox1 in lipid rafts comes from the observation that the TNF $\alpha$  receptor (TNFR1) and the other components of the TNF $\alpha$  signaling pathway are recruited to lipid rafts following ligand stimulation (Legler, Micheau, Doucey, Tschopp, & Bron, 2003). In endothelial cells NADPH oxidase complex previously shown to be primarily associated with cytoskeletal elements. Previously, it has been shown that NADPH oxidase enzyme isoform, Nox2, is localized and active in endothelial lipid-raft/caveolae compartments (Yang & Rizzo, 2007). Additionally, previous studies in laboratory utilizing specific methodology to isolate caveolae structures showed lack of Nox4 isoform in caveolae domains and almost negligible expression of Nox1 (Figure 18). The TNF $\alpha$ , PDGF, EGF, and Ang-II pathways have all been associated with lipid rafts (Stehr et al., 2003; Zuo et al., 2005). Each of these receptors also appears to utilize Nox1 and/or Nox2 during signal transduction (Legler et al., 2003). NADPH oxidase localization in caveolae plays an important role in activation of specific redox signaling events. Taken together, the constitutive presence of Nox2 in caveolae micro-domains appears to be a common starting point for compartmentalized ROS production and associated redox signaling.



**Figure 18. NADPH oxidase isoform, Nox2, is enriched in caveolae compartments.**

Previous studies from our group using silica coated bovine aortic endothelial cell plasma membrane isolation and sub-fractionation to purify their subtending caveolae. P-Plasma membrane showed presence of Nox2, p47 and p22 subunit, Nox4, eNOS and negligible levels of Nox1. V-caveolae fractions showed enrichment of Nox2, eNOS, P47 and p22, whereas no Nox4 or Nox2.

Caveolae as centers for dual activation of eNOS and NADPH oxidase enzymes in  
endothelial cells:

Bovine Aortic Endothelial Cells (BAECs) when stimulated with pro-inflammatory cytokine TNF $\alpha$  shows enrichment of p47<sup>phox</sup> subunit of NADPH oxidase and enzyme eNOS in caveolae domains to produce ROS and NO respectively. Therefore, under the influence of atherogenic stimuli, caveolae appears to be the sites for activation of both NADPH oxidase and eNOS. Based on the cross-reactive nature of superoxide and NO, these radicals are required to be produced in close proximity to each other for ONOO generation, caveolae can act as compartments for spatial and temporal regulation for this reaction. The ONOO produced can subsequently induce protein tyrosine nitration of proximally located signaling mediators. Furthermore, death receptors such as Fas and TNF receptor 1 (TNFR1) also localize in lipid-rafts/caveolae and are known to stimulate raft clustering and formation of redox signaling platforms (A. Y. Zhang, Yi, Zhang, Gulbins, & Li, 2006; D. X. Zhang, Zou, & Li, 2003).

As discussed above, caveolae organelles undergo endocytosis via phosphorylation of Dynamin-2 (Botos et al., 2008; Botos et al., 2007; Parton et al., 1994). The activation mechanism of enzyme eNOS involves dynamin-2-dependent internalization (Sanchez, Kim, Duran, Meininger, & Duran, 2008; Sanchez et al., 2009). Similarly, there is some evidence relating to internalization of NADPH oxidase enzyme as an activation mechanism for ROS generation, whereas role of caveole in these processes is not very clear. Based on these observations it is imperative to believe that caveolae compartments function as essential ROS/RNS generation centers in activating localized cellular signaling during endothelial dysfunction related vascular pathologies such as atherosclerosis.

## Overall Hypothesis

The fact that enzymes, eNOS as well as NADPH oxidase are localized in endothelial caveolae compartments and active under similar patho-physiological stimuli suggest that caveole domains serve as a compartment for ROS/RNS generation and associated nitroxidative (oxidative and nitrative) signaling. Therefore, we hypothesize that Caveolae membrane domains act as micro-compartments for ONOO generation and protein tyrosine nitration (PTN), thereby serving as platforms allowing activation of specific nitroxidative signaling pathways essential for inducing endothelial cell activation and dysfunction.

We plan to test our hypothesis using aims discussed below:

Specific Aim 1: Caveolae as spatio-temporal compartments for ROS generation and sites for localized protein-tyrosine nitration

**Aim 1 Hypothesis:** We hypothesize that proximal localization of enzymes, NADPH oxidase, eNOS in caveolae micro domains (spatial) and simultaneous activation (temporal) of both enzymes in response to inflammatory stimuli,  $TNF\alpha$ , will result in compartmentalized ROS/RNS generation and PTN of local proteins. Testing this aim will involve,

- a)  $TNF\alpha$  induced ROS generation and tyrosine nitration of total proteins *in vitro* and *in vivo*.
- b) Role of Caveolae roles as spatio-temporal compartments will be tested using pharmacological agents to disrupt rafts and siRNA mediated caveolin-1 knockdown *in vitro* and measuring ROS/RNS generation. *In vivo* correlates will be tested using Caveolin-1KO mice.

c) Determining functional consequences of caveolae compartmentalized ROS/RNS generation in regulating endothelial phenotype.

Specific Aim 2: Caveolae as nitroxidative signaling platforms in regulating endothelial cell functioning

**Aim 2 Hypothesis:** We hypothesize that caveole compartments act a platforms in regulating proinflammatory nitroxidative signaling by activating proximally localized signaling mediators, resulting in endothelial activation and dysfunction. Testing of this aim will involve:

- a) Characterizing ROS/RNS induced nitroxidative-signaling pathways in response to  $TNF\alpha$  *in vitro*.
- b) Role of caveolae compartments as signaling platforms by disrupting caveole domains (Cav1 siRNA) and measuring SFK and NFkB nitroxidative signaling
- c) Identification of signaling mediators undergoing PTN and its functional roles in adhesion molecule expression *in vitro*.

Specific Aim 3: Caveolae internalization as an essential step in mechanism for NADPH oxidase enzyme activation and redox signaling

**Aim 3 Hypothesis:** Based on the previous studies showing dynamin-2-mediated internalization of NADPH oxidase enzyme functions in serving as redoxosomes, we hypothesize that internalization of caveolae structures is an essential step for compartmentalized ROS generation, NFkB redox signaling in inducing endothelial dysfunction. Testing of this aim will involve:

- a) Characterization of dynamin-2-dependent caveolae endocytosis roles in ROS generation by use of Dyn2 siRNA and pharmacological agents to block Dynamin-2 GTPase activity.

- b) Characterization of dynamin-2-dependent ROS producing caveolae structures in regulating NFkB redox signaling and adhesion molecule expression *in vitro*.

Specific Aim 4: Inhibition of Caveolae compartmentalized ROS generation as therapeutic targets in limiting vascular oxidative stress

**Aim 4 Hypothesis:** Based on the data here showing caveolae localized Nox2 as one of the major source of endothelial ROS, we hypothesize that development of caveolae targeting Nox2 inhibitor can be developed as therapeutic target in reducing pathological ROS. Testing of this aim will involve:

- a) Designing membrane permeable, caveolin-1 scaffolding domain (caveolae) targeting Nox2 inhibitory (therapeutic) peptide.
- b) Characterizing peptide targeting to caveolae domains.
- c) Testing therapeutic effects of peptide in limiting TNF $\alpha$ -induced ROS generation.

#### Significance

The successful *in vitro* and as *in vivo* testing of these aims will help us in understanding mechanistic roles of caveolae in regulation of ROS/RNS generation, importance in modulating nitration events and characterizing signaling cascades responsible for initiation of inflammatory vascular diseases involving endothelial cell dysfunction.

## Materials and Methods

**Cell culture:** Bovine aortic endothelial cells (BAECs) (VEC Technologies, Inc, Rensselaer, NY) were grown in MCDB-131 cell culture medium (SIGMA-ALDRICH Chemical Inc, St. Louis, MO) supplemented with 15% FBS (Atlanta Biol, Flowery Branch, GA) and 0.04mg/ml gentamycin sulfate (Cambrex Bio Science, East Rutherford, NJ) and maintained at 37°C, 97% humidity, and 5% CO<sub>2</sub>. To stimulate oxidant production, cells were incubated with 100 U/ml TNF $\alpha$  (SIGMA-ALDRICH) for indicated time points. In some experiments, cells were pretreated with Uric acid (SIGMA-ALDRICH) or gp91ds-tat peptide (Biosynthesis Inc, Lewisville, TX).

**Inflammatory Cytokine, TNF $\alpha$ , treatment:** Confluent BAECs were serum starved overnight in low-serum MCDB-131 media (0.1% FBS) or on the day of experiment for 3 hours in serum-free media (0% FBS). Cells were challenged with TNF $\alpha$  100U/ml (SIGMA-ALDRICH) at 37°C for the desired length of time.

**Treatment with Inhibitors of ROS, NO and ONOO-:** Confluent cells were serum starved for 3 hours in serum-free media (0% FBS) and pre-treated with inhibitors for NADPH oxidase enzyme, gp91-ds-tat (25  $\mu$ M), eNOS enzyme, L-NAME (100  $\mu$ M), peroxynitrite scavenger, Uric acid (100  $\mu$ M) for 1 hour each prepared in MCDB-131 media. The pharmacological inhibitors containing media was removed and cells were rinsed in serum-free MCDB-131 warm media (37°C) twice and new media was added containing TNF $\alpha$  for desired time points.

**Detection of intracellular superoxide production (DHE Assay):** The in vitro studies utilized cells pretreated with indicated compounds for 60 minutes washed several times

in 1XPBS (with  $\text{Ca}^{+2}$ ,  $\text{Mg}^{+2}$ ) and loaded with Dihydroethidium (DHE, 10  $\mu\text{M}$  for 30 mins). Cells were extensively washed to remove free probe and then incubated with  $\text{TNF}\alpha$ . Cells were then washed twice with ice-cold Tricine buffer, gently scraped into 0.5ml of the same buffer and centrifuged at 1,400 g for 10 mins at 4°C. The cell pellets were resuspended in 0.5 ml of ice-cold PBS and sonicated on ice for 10 secs. One hundred microliters of material were then assayed for ethidium fluorescence in a FLUOstar galaxy (Polar Star) plate reader at excitation and emission wavelengths of 390/490nm, respectively. Fluorescence values were normalized to sample protein concentration and results presented as fold difference in fluorescence values of experimental/control.

**Oxidative Fluorescent Microtopography for in vivo measurement of ROS:** To evaluate superoxide production in mouse aortas *in situ*, oxidative fluorescent microtopography method was used as previously described (Nakane, Miller, Faraci, Toyoda, & Heistad, 2000). The aortic tissue was removed following in vivo exposure to  $\text{TNF}\alpha$  (1-3hrs). Aortas were cleaned of adventitia, embedded in OCT (source) and frozen at -80°C. Vessels were then serial cryosecti oned (30 $\mu\text{m}$ ) using the Cryocut 1800 microtome (Reichert Jung). Sections were placed on microscope slides, and equilibrated in Krebs-HEPES buffer for 30 mins at 37°C. Fresh buffer containing DHE (2 $\mu\text{m}/\text{l}$ ) was topically applied to each tissue section and incubated for 30 minutes in a light-protected, humidifier chamber at 37°C. Oxidized DHE was detected by fluorescence microscopy using a Nikon Eclipse 80i with excitation and emission wavelengths set at 488 and 610nm, respectively.

**Western blotting:** Protein sample concentrations were determined with the Advanced Protein assay reagent kit (Cytoskeleton, Inc, Denver, CO). Samples were mixed with

modified Laemmli buffer (BIO-RAD, Hercules, CA) denatured at 100°C for 10 mins, separated by SDS-PAGE on 5-20% gradient gels, and electrotransferred to nitrocellulose filters (BIO-RAD). Immunoblotting included incubation with following primary antibodies anti-caveolin-1 (pAb) (BD Transduction labs, San Jose, CA), anti-p-p65 (pAb), anti-p65 (pAb), anti-pSrc (Tyr416) (pAb), and total Src family kinase (pAb) (Cell Signaling Inc, Boston, MA), anti-VCAM-1 (pAb) and anti-ICAM-1 (pAb) (Santa Cruz Biotech, Inc, Santa Cruz, CA), anti-nitrotyrosine (mAb) (Abcam, Cambridge, MA; anti- $\beta$ -actin) and anti- $\beta$ -actin (mAb) (SIGMA-ALDRICH). Species-matched secondary antibodies conjugated with horseradish peroxidase (HRP) (Amersham Biosciences Corp, Waltham, MA) were used. Proteins were detected with enhanced chemiluminescence substrate (Pierce, Rockford, IL). Densitometry quantification of immunoblots with Image J software allowed for direct comparisons between experimental sets.

***In vivo studies:*** The Temple University Institutional Animal Care and Use Committee reviewed and approved the protocols for animal use in these studies. Wild type and Cav1<sup>-/-</sup> (Cav1KO) male mice (Jackson Labs, Barr Harbor, ME) between 9-10 weeks of age and weighing approximately 20g were used in these studies. To induce ROS/RNS within the vascular compartment, mice received a bolus of either saline (control vehicle) or TNF $\alpha$  (2 $\mu$ g/25gm) via tail vein injections. Animals were sacrificed 3-hour post-TNF $\alpha$  exposure.

**Disruption of Lipid-Raft and Caveolae:** Both Lipid-rafts and caveolae compartments were disrupted via treatment of cell monolayers with M $\beta$ CD (10mM for 30 mins) (SIGMA-ALDRICH), a cholesterol-sequestering agent, which binds to membrane cholesterol and

disturb the organization of cholesterol-rich raft domains. Media containing M $\beta$ CD was applied for 30 minutes at 37°C, followed by brief rinse with serum-free media prior to any additional treatments or cell lysis.

**Depletion of caveolin-1 in BAECs:** Expression of Cav1 was inhibited with a SMARTpool Cav1siRNA antisense sequence, 5'-AACUGUGUGUCCUUCUGG-dT-Dt-3' (containing 4 mismatches for all known gene sequences) and scrambled control siRNA (Dharmacon, Inc, Pittsburg, PA), as described in our past work (Yang & Rizzo, 2007). Briefly, BAECs at 80% confluence were transfected with 100nM siRNA USING DharmaFECT-1. Similar to previous results, cells were used 48 hours after transfection when expression levels of caveolin-1 were reduced by >90%. The expression level of caveolin-1 was determined by Western blot analysis.

**Immunoprecipitation of tyrosine nitrated proteins:** A mAb against 3-nitrotyrosine (3-NT) (Abcam, Cambridge, MA) conjugated to sheep anti-mouse-coated paramagnetic Dynabeads (Invitrogen, Grand Island, NY) according to manufacturer's protocol. Cell lysates were isolated from TNF $\alpha$  treated and untreated BAECs, with or without siRNA pre-treatments. Equal amounts of protein (200  $\mu$ g) from each sample was adjusted to a final volume of 1.2 ml with PBS containing 60mM octyl- $\beta$ -D-glucopyranoside (SIGMA-ALDRICH) and incubated with the antibody-bead conjugates overnight at 4°C. The nitrotyrosine immunoprecipitates were collected and washed three times with cold PBS. Samples incubated with beads lacking nitrotyrosine antibody served to control for nonspecific protein binding.

**Disruption of caveolae endocytosis via Dynamin-2 siRNA and Dynasore:**

Expression of Dynamin-2 was inhibited with a SMARTpool Dynamin-2 antisense sequence, 5'-UUGUUCUCCAGGACAUCCCUU-dT-Dt-3' (containing 4 mismatches for all known gene sequences) and scrambled control siRNA (Dharmacon, Inc, Pittsburg, PA). Briefly, BAECs at 80% confluence were transfected with 100nM siRNA using DharmaFECT-1. Similar to previous results, cells were used 48 hours after transfection when expression levels of Dynamin-2 were reduced by >90%. The expression level of caveolin-1 was determined by Western blot analysis. Dynasore was brought from commercial available sources (Tocris, Biosci.). Confluent BAECs were pretreated with 60 $\mu$ M concentration of Dynasore for 60 mins in serum free media. Excess Dynasore was removed by rinsing cells with serum free media and stimulated with TNF $\alpha$  for indicated time points.

**Immunocytochemistry:** BAECs were grown to 85% confluence on glass coverslips coated with 0.2% gelatin made in 1XPBS with calcium and magnesium, for 30 mins. Gelatin was aspirated off and coverslips were air-dried. The confluent cells were washed twice with PBS then fixed in 4% paraformaldehyde (PFA) for 10 mins. Labeling with p65 (NF $\kappa$ B), Caveolin-1 and FITC primary antibodies was performed on ice prior to permeabilization with 0.1% Triton100 made in 1XPBS. All the following steps were carried out at room temperature in a humidified chamber. Coverslips were blocked in 10% normal goat serum for 1 hour before application of primary antibodies followed by desired Alexa-conjugated secondary antibodies (Invitrogen, Inc). Cells were viewed under Nikon (model) fluorescence microscope. Individual red, green and blue (DAPI) images were of the same field were overlaid and processed using Adobe Photoshop

software. In red and green merged images, areas of dual co-localization were confirmed by presence of yellow.

**Immunofluorescence:** Adhesion molecules, VCAM-1 and ICAM-1, expression were measured *in vivo* in aortic tissue derived from both WT and Cav1KO mice. Aortic vessels were collected in OCT and 5 $\mu$  sections were cut using cryostat (Cryocut 1800, Reichert Jung). The tissue sections were washed in DDH<sub>2</sub>O for 5 mins and rinsed in 1XPBS. Tissue sections were fixed in 4% paraformaldehyde solution for 10 mins and non-specific binding was blocked using 10% Normal Rat Serum (NRS) (Jackson Immunoresearch) for 2 hours. After washing with PBS the tissue sections were incubated with primary mAb VCAM-1 (1:100; BD Bioscie) overnight at 4°C. The sections were washed 3 times in 1XPBS-Tween (0.1%) for 5 mins each and incubated with secondary antibody (Goat-anti-Rat Alexa594) (Invitrogen Molecular probes A11007) for 2 hours. The tissue sections were washed again for 3 times in 1XPBS-Tween (0.1%) for 10 mins each and mounted WITH DAPI containing mounting media (Vectashield Laboratories, Burlingame CA) before imaging using Nikon microscope.

**Immunohistochemistry:** For detection of protein tyrosine nitration in whole vessels, aortic tissue was obtained from WT and Cav1KO mice following administration of TNF $\alpha$  (SIGMA-ALDRICH, St. Louis, MO). Aortas were processed for embedding in paraffin wax. Tissue blocks were cut into 5 $\mu$ m sections and placed on slides. Tissue sections were deparaffinized, rehydrated and soaked in antigen retrieval solution (Vector Labs, Burlingame, CA) with citrate buffer. Sections were then blocked in 5% normal goat serum (NGS) (Jackson Immunoresearch, West Grove, PA) for 2 hours and incubated with primary anti-nitrotyrosine antibody (1:100) (Abcam) in PBS (Fisher, Pittsburg, PA)

containing 1% BSA (SIGMA-ALDRICH) and 0.1% Tween20 overnight. Primary antibody was labeled with a biotinylated secondary antibody followed by an avidin-biotin peroxidase complex (Pierce, Rockford, IL). Non-specific, isotype-matched antibodies were used as negative controls. Sections were then incubated with diaminobenzidine (Vector Labs) and counterstained with haematoxylin (SIGMA-ALDRICH).

**Lipid-raft/Caveolae Isolation:** Following experimental challenge, BAECs were immediately placed on ice and scraped in detergent-free Tricine buffer (250Mm sucrose, 1Mm EDTA, 20Mm Tricine, pH 7.4). The cellular material was homogenized and centrifuged at low speed (1,400xg for 5 minutes at 4°C) to precipitate nuclear material. The resulting supernatant was collected, mixed with 30% Percoll in Tricine buffer and subjected to ultracentrifugation for 25 minutes (Beckmann MLS50 rotor, 77,000xg, at 4°C). Plasma membranes were collected and sonicated (3x3-second bursts). The sonicated material was mixed with 60% sucrose (to a final concentration of 40%), overlaid with a 35-5% step sucrose gradient and subjected to overnight ultracentrifugation (Beckman MLS50 rotor, 87,400xg at 4°C). Fractions were collected every 400uL from the top sucrose layer and proteins were precipitated using 0.25 volume TCA-deoxycholic acid [100% (wt/vol)] TCA in double distilled water, 0.1% (wt.vol) deoxycholic acid] to precipitate proteins.

**NADP/NADPH Detection Assay:** BAECs at 100% confluence were pretreated with M $\beta$ CD followed by TNF $\alpha$  stimulations for additional 2 hours in serum free media. Cell lysates were collected in NADP/NADPH Extraction buffer (NADP<sup>+</sup>/NADPH Quantification Kit-MBL Intern, Corp) and processed for suggested protocol to detect NADP/NADPH

ratio. The final collected samples were plated on a 96 well plate and developed color after reaction was detected using plate reader (FluoStar Galaxy) at OD of 450 nM.

**Statistical analysis:** Differences between groups were determined by two-way ANOVA followed by Bonferroni post-tests using Graph Pad PRISM statistical analysis software. Differences between control and experimental groups were significant at  $p < 0.05$ , with all n-values at least equal to three separate experiments.

## CHAPTER 2 LIPID-RAFTS AND CAVEOLAE AS COMPARTMENTS FOR ROS GENERATION AND LOCALIZED PROTEIN TYROSINE NITRATION

### Introduction

Abnormalities in endothelial cell function underlie vascular pathologies that contribute to cardiovascular disease. There is mounting support from basic science (Lassegue & Griendling) and preclinical studies (Ye & Song, 2008) for increased production of reactive oxygen (ROS) and nitrogen (RNS) species as a significant contributor towards endothelial dysfunction.

The family of NADPH oxidase enzymes (Nox1, Nox2, Nox4 and Nox5), are primary ROS generating sources in vasculature. Several studies have demonstrated that vascular disease inducing factors such as proinflammatory cytokines (X. L. Chen, Varner, et al., 2003; Frey, Rahman, Kefer, Minshall, & Malik, 2002; J. M. Li, Fan, Christie, & Shah, 2005; J. M. Li & Shah, 2002), Ang-II (Landmesser et al., 2002) (J. M. Li et al., 2005; J. M. Li & Shah, 2002) (H. Zhang et al., 1999), and oscillatory shear stress can enhance the expression and activity of both Nox1 and Nox2, endothelial Nox isoforms. In addition, roles for Nox1 and/or Nox2 as sources of excessive vascular ROS production in experimental models of vascular disease is supported by studies using transgenic and knockout mice (Rivera, Sobey, Walduck, & Drummond, 2010).

Reactive nitrogen species, peroxynitrite, is produced under near-diffusion controlled conditions upon cross-reaction between superoxide (ROS), and Nitric oxide (NO, another RNS), inducing nitration of tyrosine residues in proteins, commonly referred to as, protein-tyrosine nitration (PTN). The PTN events are known to be important mediators of pathophysiological processes in tissues. The presence of nitrated proteins is established in a myriad of cardiovascular diseases and is considered as marker of oxidant imbalance

(Peluffo & Radi, 2007). Human sclerotic tissue derived from atherosclerotic vessels has shown increased tyrosine nitration of total proteins (Buttery et al., 1996).

Recent clinical trials have shown that plasma levels of nitrotyrosine are correlated with severity of coronary artery disease (CAD) and that PTN levels diminish after statin treatment which strongly suggest dependence on nitroxidative stress, since statins are known to act via modulation of antioxidant system (Shishehbor et al., 2003). Thus, limiting nitroxidative (oxidative and nitrative) stress is a viable approach for maintaining redox status and abating endothelial/vascular dysfunction.

In order to counteract increased ROS and RNS in vasculature, several interventional trials found little benefit from “traditional” antioxidants (Vitamins C and E, folic acid, Q10 and polyphenols) in limiting cardiovascular disease (Ye & Song, 2008). A key factor, which may explain the discrepancy between the mechanistic/preclinical studies and clinical trials, is the indiscriminate scavenging of oxidants by these compounds. Such lack of specificity may interfere with some of the protective functions attributed to oxidants such as ischemic preconditioning and angiogenesis. Thus, there is a need to understand the mechanisms that precisely control ROS/RNS production in a pathogenic environment so that more targeted therapeutic compounds can be developed. Features that may determine the specific biological consequences of ROS/RNS production include the enzymatic source, moieties and amounts of oxidant produced. Our group (Yang, Oo, & Rizzo, 2006; Yang & Rizzo, 2007) and others (K. Chen, Craige, & Keaney, 2009; Ushio-Fukai, 2009) have postulated that the subcellular location where oxidants are produced can impact both the generation and downstream effects of ROS/RNS. In a past study, we reported that components of NADPH oxidase enzyme complex, particularly Nox2, are enriched and functional in endothelial cell caveolae (Yang & Rizzo,

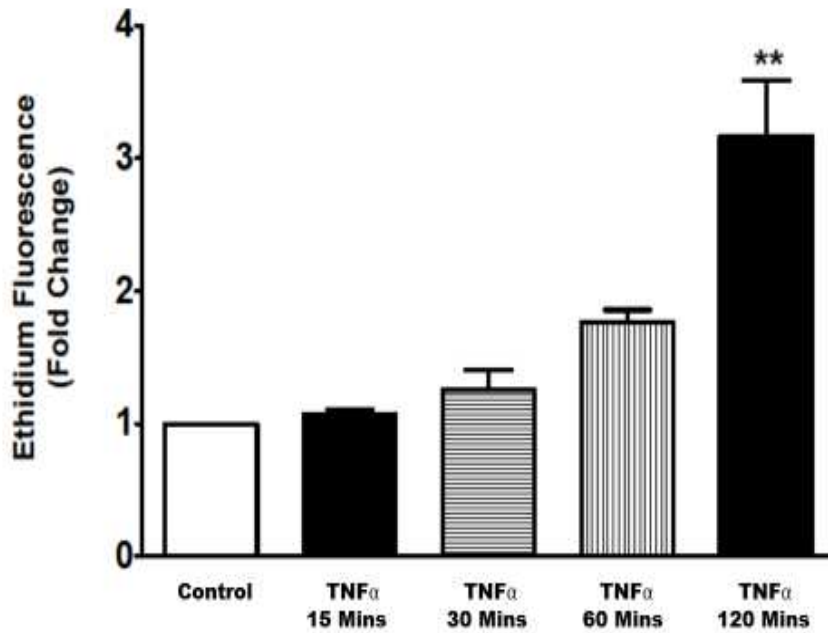
2007). In addition, cytokine stimulation also induced acute production of nitric oxide (NO) through activation of eNOS, which is present in the same caveolae membrane compartment. The dual activation of superoxide and nitric oxide generating systems in caveolae provides a spatially favorable environment for nitration of tyrosine containing proteins localized to these membrane domains. Based on these findings, we hypothesize that the subcellular positioning of oxidant producing enzyme provides a mechanism for the spatial regulation of ROS production as well as compartmentalized RNS generation and allows for nitroxidative signaling specificity through access to redox sensitive local targets. The data presented here provides new experimental evidence that supports this concept.

Here, we show that under proinflammatory conditions, caveolae domains act as subcellular organelles required for compartmentalized activation of NADPH oxidase enzyme in generating ROS and its cross-reaction with locally produced NO, to induce protein-tyrosine nitration of proteins. We propose that the functional result of this nitroxidative stress (ROS/RNS) has significance in inducing overexpression of adhesion molecules, ICAM-1/VCAM-1, a hallmark feature of dysfunctional endothelium during vascular diseases.

## Results

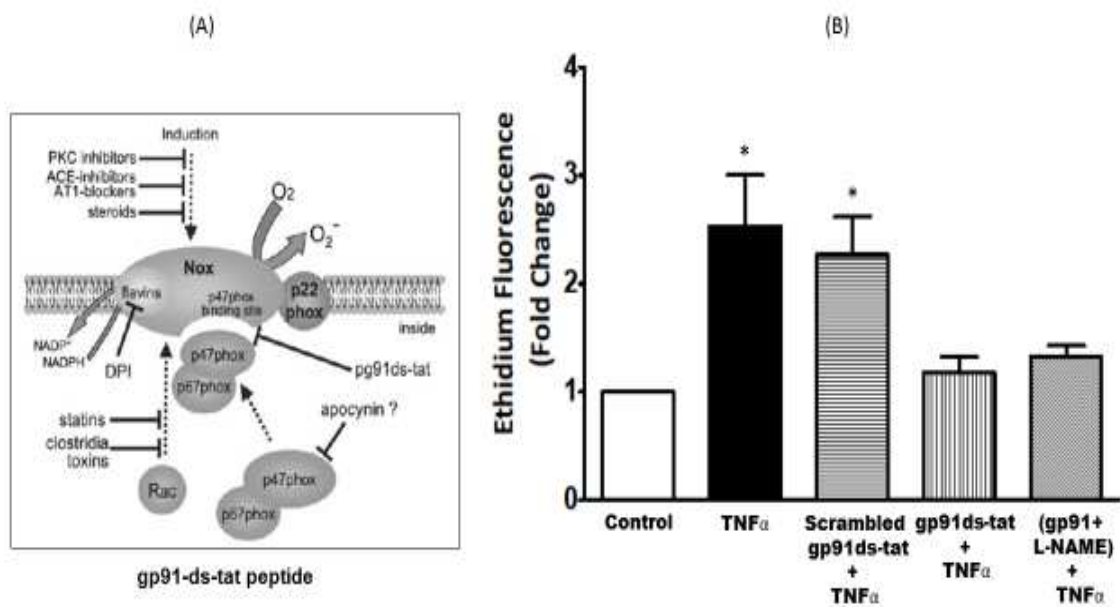
*Inflammatory cytokine, TNF $\alpha$  induces time-dependent increases in ROS production in primary endothelial cells:*

Inflammatory cytokines such as TNF $\alpha$  stimulate ROS production in endothelial cells (Poerber & Min, 2006). These ROS are associated with the redox signaling mechanisms that lead to impaired endothelial function. Bovine aortic endothelial cells (BAECs) stimulated with TNF $\alpha$  (100U/ml) showed steady and progressive increases in ROS production as measured by DHE fluorescence at indicated time points (Figure 19). Stimulation with TNF $\alpha$  at 2-hour time point showed significant increases (3.5 fold change from controls) in ROS generation. Therefore, this time point was used for all further studies.

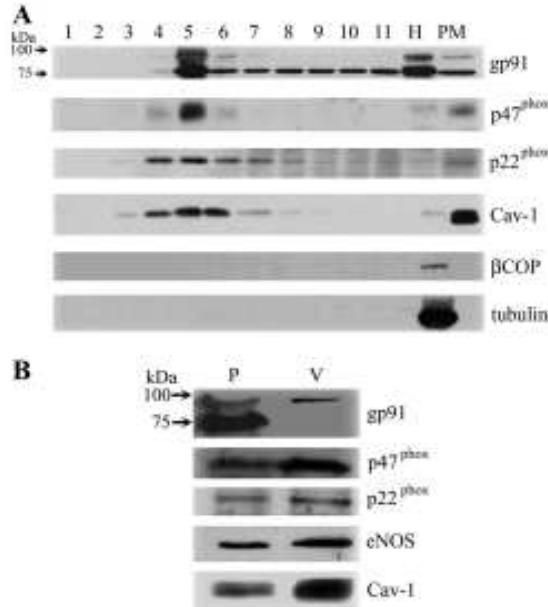


**Figure 19. Proinflammatory cytokine, TNF $\alpha$  induces time dependent increases in superoxide generation in endothelial cells.** BAECs were labeled with dihydroethidium (10 $\mu$ M for 25 min) and washed to remove free probe. The cells were then stimulated with TNF $\alpha$  at 100U/ml for 15, 30, 60 and 120 mins, and superoxide production was detected by ethidium fluorescence. TNF $\alpha$  stimulation showed slight increases in superoxide generation at time points, 15 and 30 mins, whereas most significant ROS generation was seen at 120 min. Fluorescence values were by sample protein concentration and presented as fold difference in Experimental/control. The 120 min time point for TNF $\alpha$  stimulation were used for all further *in vitro* experiments \*\* $P < 0.01$ .

While the NADPH oxidases are considered to be the primary sources of cytokine induced ROS, pharmacological compounds used to inhibit NADPH oxidases (alopyrinole and DPI) have reported off target effects. Here, we used a cell permeable peptide, gp91ds-tat, which specifically binds to and sequesters p47phox, a subunit necessary for Nox1 and Nox2 activation (Rey, Cifuentes, Kiarash, Quinn, & Pagano, 2001) (Figure 20A). In response to TNF $\alpha$ , BAEC's showed rapid production and accumulation of ROS. Endothelial cells pretreated with the gp91ds-tat peptide failed to produce ROS after several hours of exposure to TNF $\alpha$  (Figure 20B). These data confirm that NADPH oxidase is the source of acute ROS production in endothelial cells exposed to inflammatory cytokines. Based on this data we deduce that Nox2 is one of the major source of ROS, since Nox1 is expressed at very low levels in this endothelial cells (Wendt et al., 2005) and Nox4 does not localize to membrane raft/caveole compartments (Figure 21). Uncoupling of NO producing enzyme eNOS has been shown to produce superoxide generation under pathological conditions, therefore we pre-treated BAECs with eNOS inhibitor, L-NAME, and Nox2 inhibiting, gp91ds-tat peptide in response to TNF $\alpha$ , and ROS generation was measured. Blockade of both enzymes attenuated total ROS generated in response to TNF $\alpha$  (Figure 20B).



**Figure 20. TNF $\alpha$  induced superoxide generation is dependent on endothelial NADPH oxidase enzyme.** (A) Schematic shows mechanism of action of NADPH oxidase enzyme inhibiting, gp91 docking-site-tat peptide. Other than the membrane permeabilizing HIV-Tat, peptide is composed of sequence resembling the cytoplasmic subunit, p47 of NADPH oxidase enzyme required for docking to membrane subunit, gp91, thus preventing activation of enzyme, inhibiting ROS production (Rey et al. 2001). (B) BAECs were labeled with dihydroethidium (10 $\mu$ M for 25 min) and pretreated with scrambled gp91ds-tat, gp91ds-tat peptide (25 $\mu$ M for 30 mins) or gp91ds-tat peptide and eNOS inhibitor, L-NAME (10 $\mu$ M for 60 mins), prior to TNF $\alpha$  treatment. The superoxide production was detected by ethidium fluorescence. Fluorescence values were by sample protein concentration and presented as fold difference in Experimental/control \* $P < 0.05$



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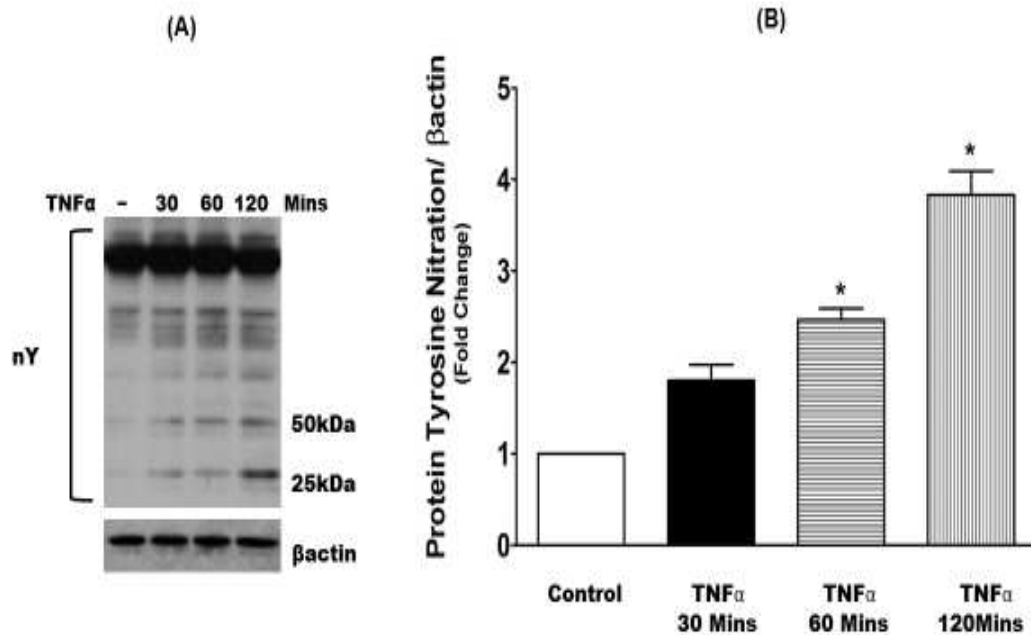
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**Figure 21. NADPH oxidase components are present in caveolae.** (A) BAECs were processed to purify plasma membranes with the use of Percoll gradient centrifugation. The plasma membranes were sonicated and subsequently subfractionated by sucrose-gradient centrifugation to isolate light buoyant density membranes from bulk membranes. Whole cell homogenate (H), plasma membrane (PM), and plasma membrane fractions (1–11) were resolved by SDS-PAGE, and Western blotted with indicated primary antibodies. (B) silica-coated apical endothelial cell plasma membranes were isolated and then subfractionated to purify their subtending caveolae. Proteins from the indicated fractions (P, plasma membranes; V, caveolae) were probed using indicated antibody. Cav-1, caveolin-1; eNOS, endothelial nitric oxide synthase; βCOP, Golgi membrane coat protein (Yang & Rizzo, 2007).

TNF $\alpha$  induces ROS generation and protein-tyrosine nitration events:

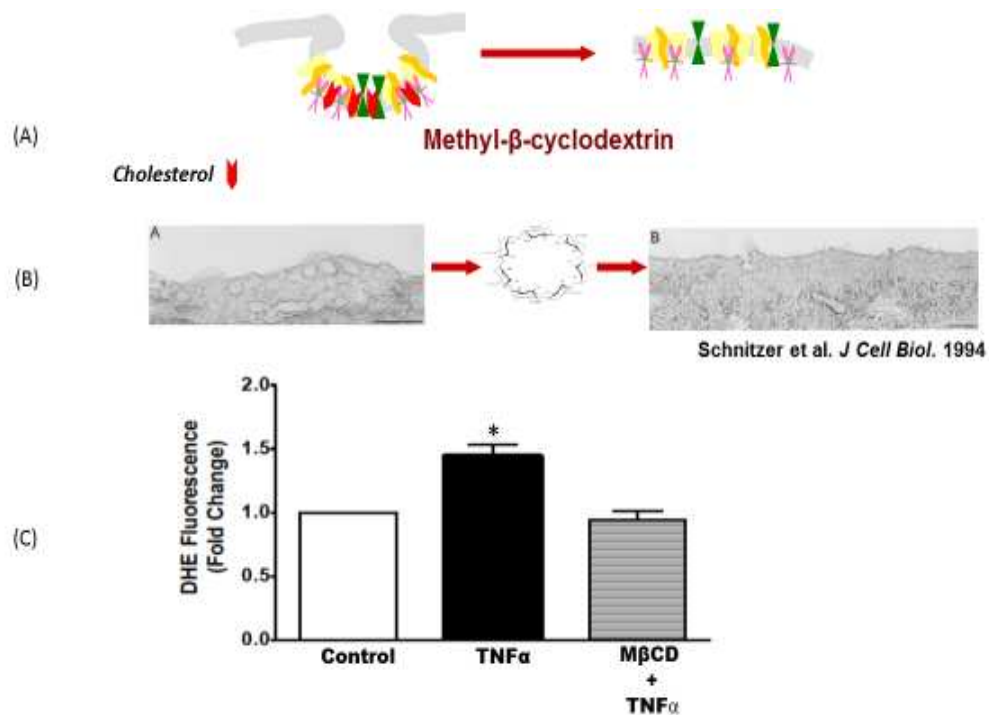
Simultaneous generation of superoxide and NO from enzymes Nox2 and eNOS, respectively can cross-react under diffusion-controlled conditions to generate formation of reactive nitrogen species (RNS), peroxynitrite. Peroxynitrite is a potent oxidizing as well as nitrating agent, implicated in inflammatory vascular diseases such as atherosclerosis (Upmancis, 2008). We measured generation of peroxynitrite via an indirect “foot-print assay” where the nitrogen radical induced protein-tyrosine nitration can be measured. We found that TNF $\alpha$  treatment induced a steady and progressive increase in the tyrosine nitration of several endothelial proteins (**Figure 22**). To address whether peroxynitrite served as the source for these nitration events, BAECs were pretreated with a, competitive inhibitor of NADPH oxidase, gp91ds-tat peptide, or a peroxynitrite scavenger, Uric acid, and challenged with TNF $\alpha$ . In each case, TNF $\alpha$ -induced tyrosine nitration of cellular proteins was significantly reduced compared to cells receiving no pretreatment compound (Figure 26A, B).



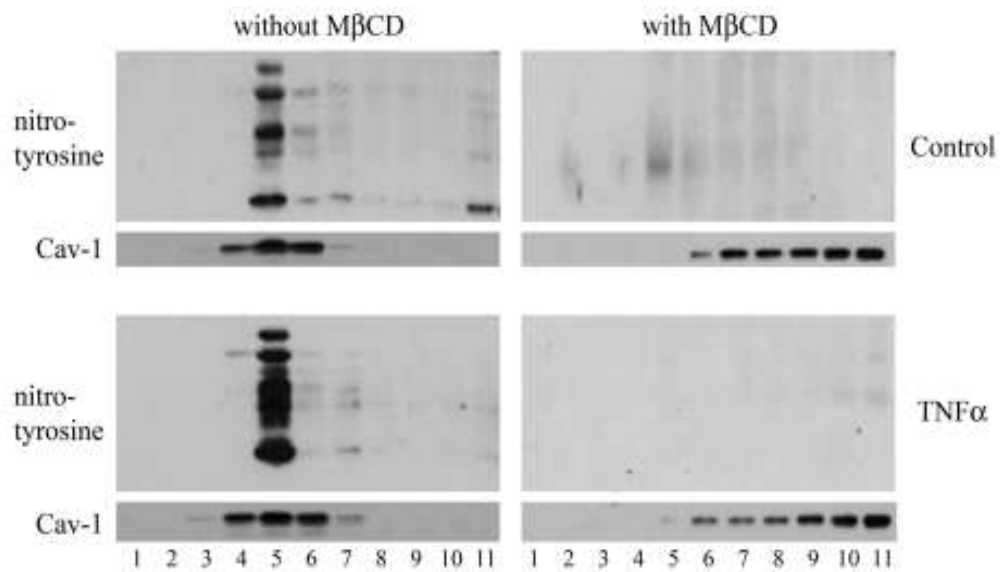
**Figure 22 TNF $\alpha$ -induced protein-tyrosine nitration** (A) BAECs were treated with TNF $\alpha$  at 100U/ml for indicated time points. Cell lysates were collected and Western blotted analysis for detection of protein tyrosine nitration using an antibody directed against 3-nitrotyrosine (nY).  $\beta$ -actin served to verify equal protein loading between samples. (B) Densitometric analysis of the collective band intensities measured in each sample (n=3) were averaged and expressed as fold change relative to  $\beta$ -actin \* $P$ < 0.05.

*Disruption of lipid-rafts and caveolae compartments result in loss of ROS and localized nitration of proteins In vitro:*

Previously we (Yang & Rizzo, 2007) and others (Feron et al., 1996; Garcia-Cardena, Fan, Stern, Liu, & Sessa, 1996; Garcia-Cardena et al., 1997) have shown that both enzymes Nox2 and eNOS are enriched and functional in lipid-raft and caveolae membrane microdomains (Figure 21). Based on these, we tested whether generation of both ROS and tyrosine nitration of proteins (RNS) is dependent on lipid-raft and caveolae domains in endothelial cells. Disruption of lipid-rafts/caveolae using cholesterol sequestering agent, M $\beta$ CD, showed decreases in ROS generation (Figure 23). Previously, we showed that disruption membrane rafts (M $\beta$ CD) resulted in loss of PTN in Cav-1 enriched fractions of the plasma membrane in response to TNF $\alpha$  (Figure 6). In this study, we tested effects on PTN upon depletion of Cav1. BAECs were pretreated with Cav1siRNA and challenged with TNF $\alpha$ . We found that depletion of caveolin-1/caveolae markedly reduced both ROS generation (Figure 25) as well as nitration of tyrosine containing proteins (Figure 26C).



**Figure 23. Compartmentalized activation of NADPH oxidase enzyme and generation of superoxide in lipid-raft and caveolae organelles.** (A) Schematic showing depletion of membrane cholesterol content upon MβCD pretreatment, resulting in flattening of invaginated caveolae domains. (B) Electron-micrographs obtained from cells showing disappearance of membrane caveolae structures post-treatment with MβCD. (C) BAECs were pretreated with MβCD (10mM) before loading with DHE and were challenged with TNFα. Sequestration of cholesterol, disrupting both lipid-raft and caveolae organelles showed significant attenuations in superoxide production. Fluorescence values were by sample protein concentration and presented as fold difference in Experimental/control \* $P < 0.05$ .



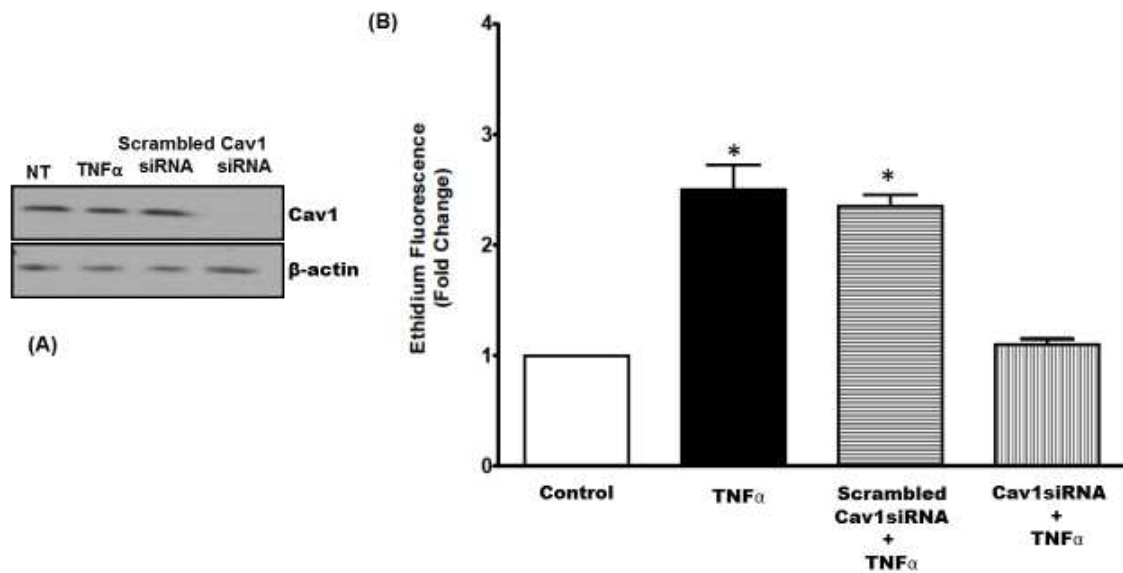
Yang B , and Rizzo V Am J Physiol Heart Circ Physiol  
2007;292:H954-H962

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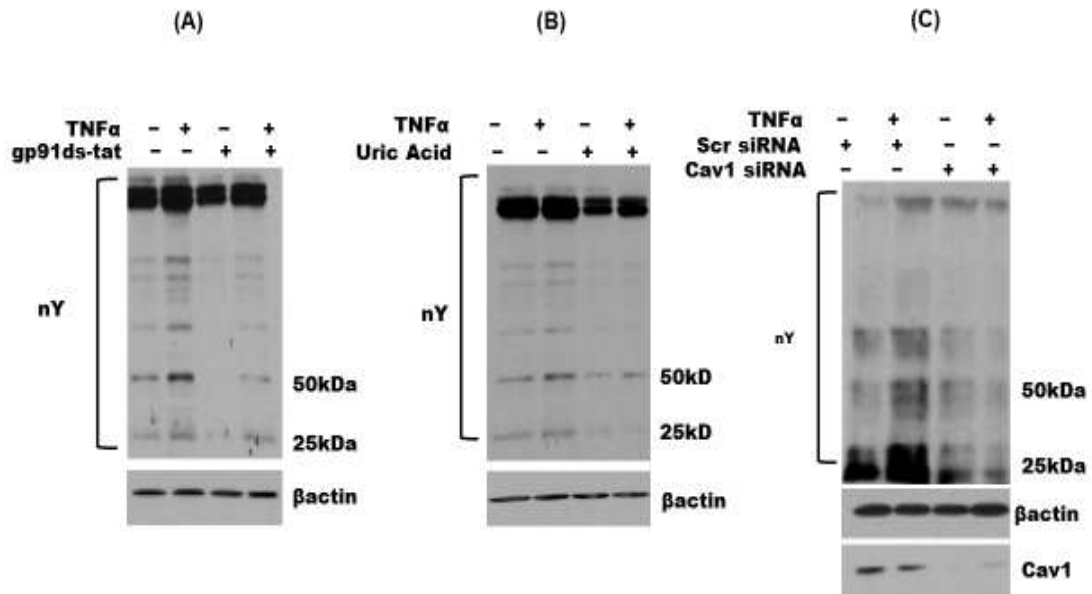
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**Figure 24. Cholesterol depletion blocks TNF- $\alpha$ -induced protein-tyrosine nitration.**

BAECs were pretreated with or without 10 mM M $\beta$ CD for 1 h, washed, and treated with or without TNF- $\alpha$  at 100 U/ml for an additional 1 h. The plasma membranes were subfractionated as described (materials and methods) and blotted for protein-tyrosine nitration as an indicator of peroxynitrite production. Densitometry quantification showed that TNF- $\alpha$ -induced protein-tyrosine nitration in raft fractions of the plasma membrane was essentially abolished after lipid raft ablation with M $\beta$ CD.



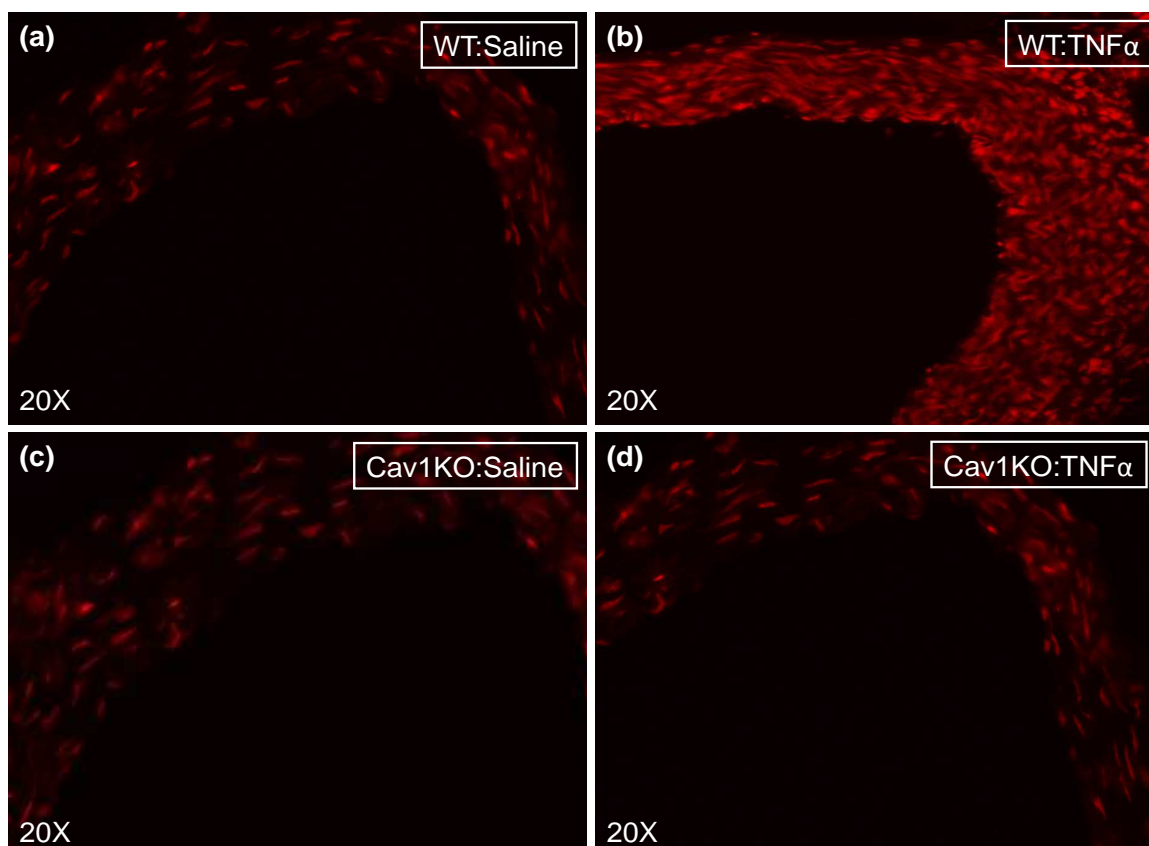
**Figure 25. Compartmentalized activation of NADPH oxidase enzyme and generation of superoxide in caveolae organelles.** (A) BAECs were pretreated with scrambled siRNA or Cav1 siRNA before loading with DHE and were challenged with TNF $\alpha$ . Pretreatments with scrambled siRNA showed significant (greater than 2 fold) increases in superoxide generation similar to that of TNF $\alpha$ , whereas disruption of caveolae organelles showed attenuations in superoxide production. Fluorescence values were by sample protein concentration and presented as fold difference in Experimental/control. \* $P < 0.05$ . (B) Western blot illustrating expression levels of Cav1 in BAECs pretreated with cav1siRNA or scrambled siRNA related to non-treated (NT) cells. B-actin served to verify equal loading between samples.



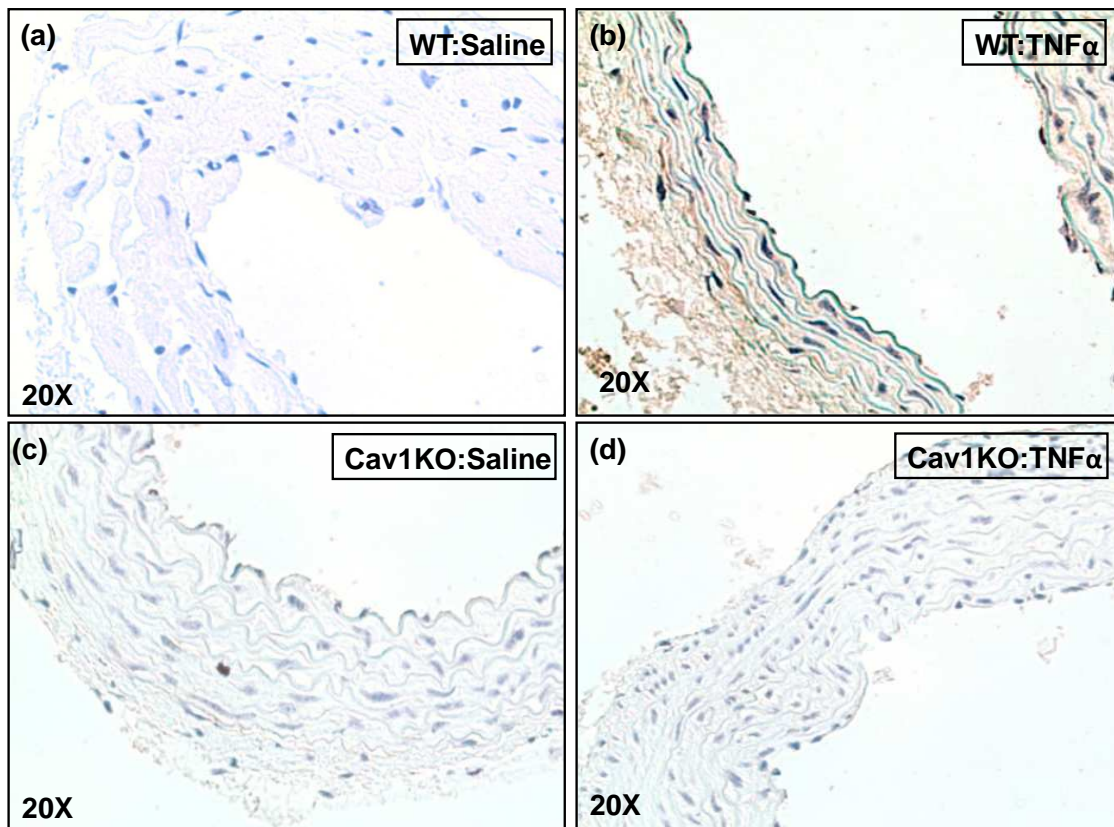
**Figure 26. Inhibition of NADPH oxidase, peroxynitrite scavenging and depletion of Caveolin-1 blocks TNF $\alpha$  induced protein tyrosine nitration.** BAECs were pre-treated with oxidant inhibitors or siRNA as indicated, washed and incubated with or without TNF $\alpha$  at 100U/ml for 2 hours. Cell lysates were collected and processed for Western blot analysis of protein-tyrosine nitration (nY), an indicator of peroxynitrite formation. While TNF $\alpha$  enhanced tyrosine nitration on several proteins, a) inhibition of NADPH oxidase (gp91ds-tat peptide), B) scavenging peroxynitrite (Uric acid) and C) depletion of caveolin-1 (Cav1siRNA), showed marked attenuation in protein tyrosine nitration. Equal protein loading and reduction in Cav1 protein expression was confirmed by detection of  $\beta$ -actin and Cav1, respectively. All the blots are representative of at least 3 independent experiments

*Depletion of Caveolin-1/Caveolae attenuates ROS production and Protein-tyrosine nitration events In vivo:*

To evaluate the role of Cav1/caveolae in cytokine-stimulated ROS production and protein tyrosine nitration in an *in vivo* setting, Wt and Cav1KO were injected with bolus of either saline or TNF $\alpha$ . After 3hrs, animals were sacrificed and aortas removed for histological staining and analysis. Consistent with the culture findings, circulating TNF $\alpha$  increased ROS generation and nitration of PTN within cells residing in each layer of the vessel wall, including the endothelium in Wt, whereas Cav1KO mice aortic tissue showed no increases in ROS or PTN events (Figure 27 and 28).



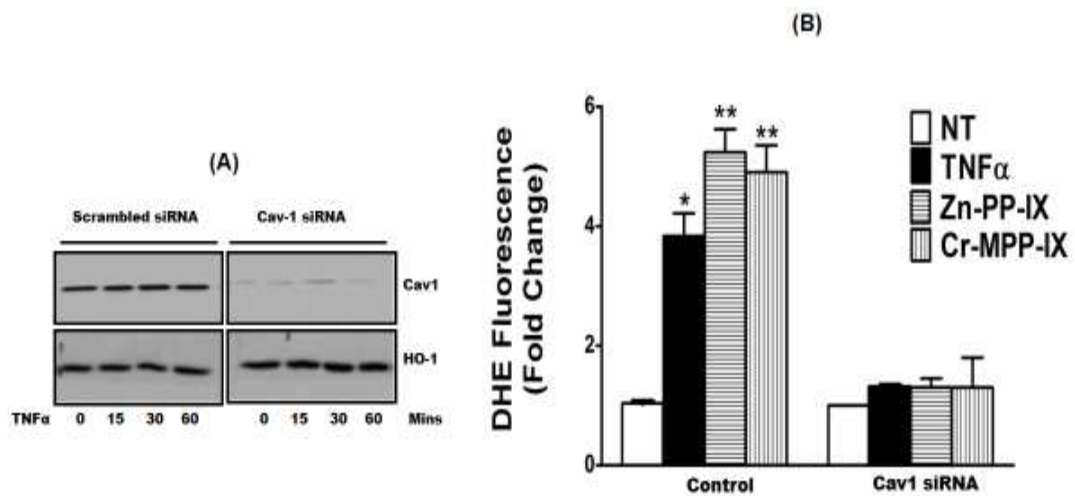
**Figure 27. TNF $\alpha$  induced ROS generation in mouse aortic tissue is dependent on Caveolin-1.** *In situ* detection of superoxide in mouse aortic tissue. Wild-type C57Blk6 mice were injected (I.V) with saline or a bolus of TNF $\alpha$  (3 $\mu$ g/25gm of animal) for 3 hours. Similarly, Cav1KO mice were also injected with saline (C) or TNF $\alpha$  (D) to induce ROS generation. Animals were sacrificed, and aortic tissue was dissected out, embedded in OCT and cryosectioned. DHE was topically applied to the tissue sections on slides and viewed under fluorescence microscope to visualize amount of ROS generation. At identical laser and microscope capture settings, fluorescence in Wt mouse aortas derived from TNF $\alpha$  (A) injected animals was markedly increased compared to their saline injected (B) animals. However, Cav1KO mice derived aortas showed no increases in ROS in TNF $\alpha$  injected (D) animals. Fluorescent photomicrographs of sections are representative of multiple sections from three different mice.



**Figure 28. Protein-tyrosine nitration events *In vivo*.** Saline or TNF $\alpha$  (2 $\mu$ g/25gm animal) was introduced into the circulation of Wt C57Blk6 and Cav1KO mice via tail vein injections. After 3hrs of exposure, animals were sacrificed and the vasculature was perfused with paraformaldehyde (4%). Aortas were embedding in parraffin wax. To detect protein tyrosine nitration, sections were incubated with an anti-nitrotyrosine antibody followed by secondary antibody coupled to an avidin-biotin peroxidase complex and developed with DAB substrate. (A) Vessels from saline injected Wt mice showed no evidence of protein tyrosine nitration whereas (B) Wt animals injected with TNF $\alpha$  showed strong reaction product throughout the vessel wall. Aortas from Cav1KO mice showed little to no protein-tyrosine nitration in response to (C) saline or (D) TNF $\alpha$ . Tyrosine nitration IHC micrographs shown here are representative of 3 different sets of Wt and Cav1KO injected animals. L denotes vessel lumen.

*Antioxidant enzyme, Hemeoxygenase-1 (HO-1) expression levels as well as activity remain unchanged in Caveolin-1/Caveolae depleted cells:*

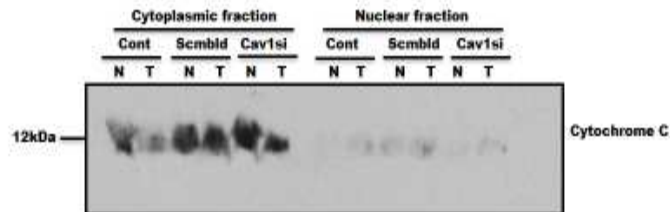
Hemeoxygenase-1 (HO-1), an antioxidant enzyme, directly interacts with caveolae protein, caveolin-1 and is localized in caveolae compartments. Levels of protein caveolin-1 negatively regulate the enzyme activity. Therefore, depletion of caveolin-1 in endothelial cells may increase antioxidant system in these cells resulting in neutralization of superoxide. In order to test if caveolin-1 depletion alters either expression of HO-1 protein or enzyme activity, we measured HO-1 protein levels and ROS generation in cells depleted with caveolin-1 and then pre-treated with HO-1 enzyme inhibitors, Zn-Protoporphyrin-IX-Cl (Zn-PP-IX) and Chromium-Mesoporphyrin-IX-Cl (Cr-MPP-IX). Our results showed no changes in HO-1 levels in cells pre-treated with Cav1siRNA prior to TNF $\alpha$  stimulation. Also, the results showed no increases in ROS generation in Cav1 depleted cells when pre-treated with HO-1 inhibitors (Figure 29).



**Figure 29. Loss of ROS production upon Cav1 depletion is not dependent on Hemeoxygenase-1 (HO-1) expression or activity levels.** (A) BAECs were pretreated with scrambled or Cav1 siRNA followed by TNF $\alpha$  treatment for indicated time points. Cell lysates were collected and processed for Western blot analysis to detect HO-1 and Cav1. Neither TNF $\alpha$  stimulation nor depletion of Caveolin-1 altered changes in HO-1 expression. The blots here are representative of 3 independent experiments. (B) BAECs depleted of Cav1 (Cav1 siRNA) were labeled with dihydroethidium and pretreated with HO-1 enzyme inhibitor, Zn-Protoporphyrin-IX-Cl (Zn-PP-IX) and Chromium-Mesoporphyrin-IX-Cl (Cr-MPP-IX) prior to TNF $\alpha$  treatment for additional 2 hours. The superoxide production was detected by ethidium fluorescence. Fluorescence values were by sample protein concentration and presented as fold difference in Experimental/control \*\* p< 0.01.

*Mitochondrial functioning stays intact in Caveolin-1 depleted cells in response to TNF $\alpha$ :*

Mitochondria is another major source of ROS generation in endothelial cells and its dysfunction under pathological conditions can induce significant increases in oxidative stress. Previously (Bosch et al., 2011) it has been shown that depletion of caveolin-1 in fibroblasts result in mitochondrial dysfunction and increases in ROS generation. Based on these we tested mitochondrial functioning in our endothelial cell system lacking caveolin-1 protein by measuring release of cytochrome c into nucleus, an indicator of mitochondrial dysfunction. Unlike fibroblasts derived from Cav1KO mice, Cav1 depleted endothelial cells showed no increases in Cytochrome-c release into the nucleus prior to TNF $\alpha$  stimulation at early as well as late time points (Figure 30).

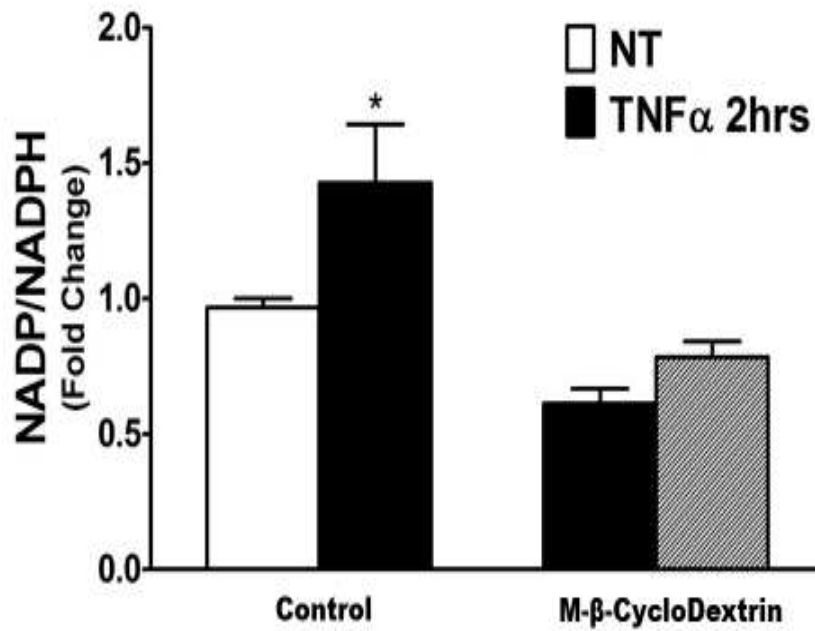


**Figure 30. Caveolin-1 depletion does not alter mitochondrial stability.** BAECs were pretreated with scrambled or Cav1 siRNA followed by  $TNF\alpha$  treatment for indicated time points and processed for isolation of nuclear and cytoplasmic extracts using Nucleo-cytoplasmic extraction kit. Collected fractions were processed for western blotting to detect nuclear localization of cytochrome-c (Cyt-c) as an indicator of mitochondrial dysfunction. Nucleo-cytoplasmic.

*NADP/NADPH ratio, determinant of NADPH oxidase enzyme activity, remains unaltered in endothelial cells depleted of membrane cholesterol or caveolin-1/caveolae knockdown:*

NADPH oxidase enzyme requires NADPH molecule as a substrate to oxidize it to NADP and as a result reduce molecular oxygen into free radical, superoxide. Therefore, ratio of NADPH/NADP is an important determinant of enzyme activity. Disruption of lipid-rafts using M $\beta$ CD requires depletion of membrane cholesterol and therefore cells may initiate biochemical process of cholesterol biosynthesis.. Cholesterol biosynthesis involves generation of NADP, presence of which will keep enzyme non-functional due to non-availability to substrate.

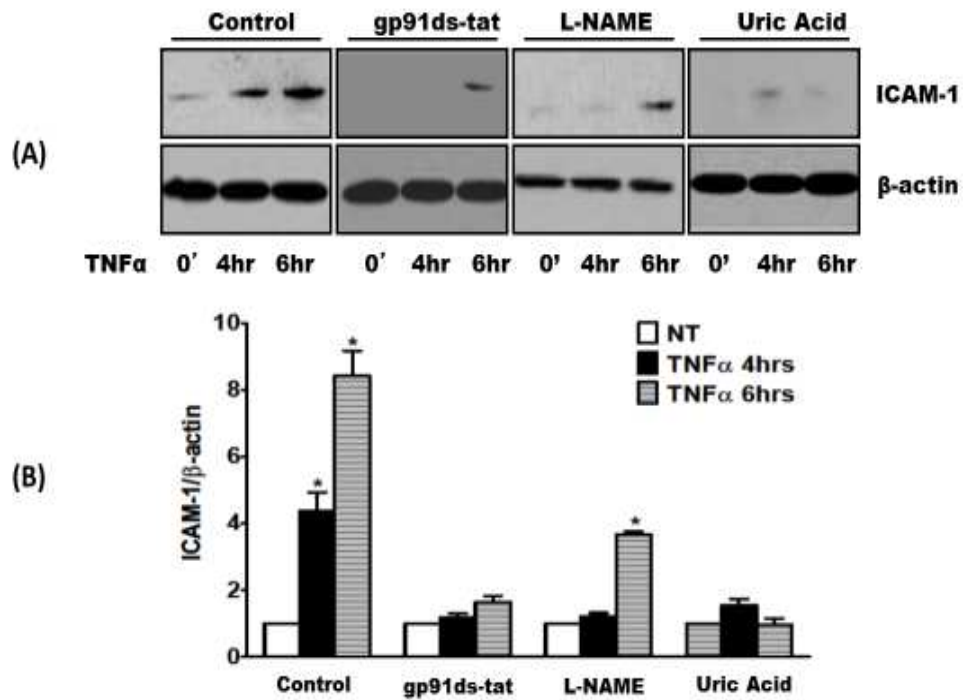
Based on this, we measured if NADPH/NADP ratio is altered in cells depleted of cholesterol. Surprisingly, NADPH/NADP ratios in cholesterol-depleted cells remain unchanged (Figure 31).



**Figure 31. NADP/NADPH ratios in cells remain unaffected after disruption of lipid-rafts and Caveolae.** BAECs were pretreated with M $\beta$ CD for 30 mins followed by TNF $\alpha$  treatments for additional 2 hours. Cells were lysed in buffer from NADP/NADPH detection kit; samples were collected and processed for NADP and NADPH detections under plate reader. Fluorescence values were by sample protein concentration and presented as fold difference in Experimental/control \* $P < 0.05$ .

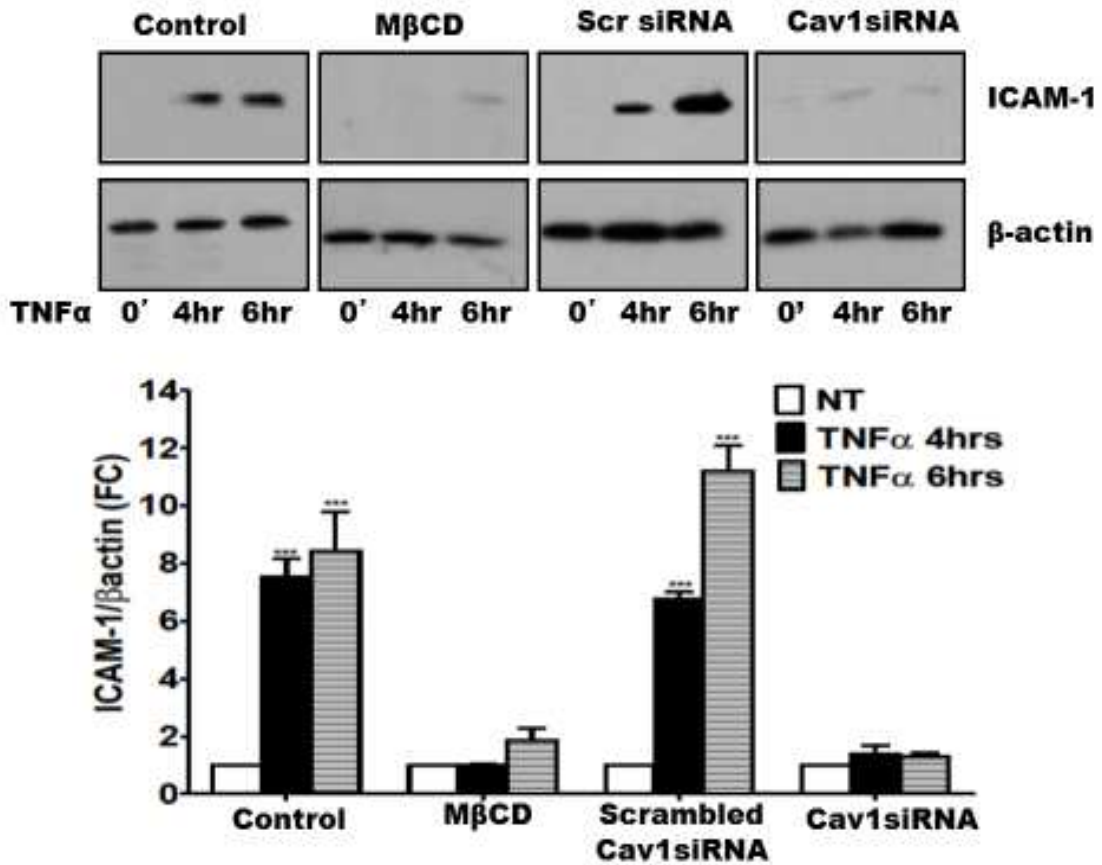
*Excessive ROS/RNS generation induces endothelial cell dysfunction In vitro:*

A hallmark feature of vascular pathophysiology is a shifting of the endothelial phenotype from quiescent to proinflammatory, where adhesion molecules such as ICAM-1 and VCAM-1, are upregulated and expressed on the cell surface (X. L. Chen, Zhang, et al., 2003; Giannotti & Landmesser, 2007). While ROS have been linked to redox signaling pathways that regulate adhesion molecule expression (Dworakowski, Alom-Ruiz, & Shah, 2008), the role for RNS is less clear. Consistent with previous reports (Wertheimer, Myers, Wallace, & Parks, 1992), TNF $\alpha$  stimulated increased expression of ICAM-1 protein at 4 and 6 hours post-treatment (4 and 8 fold increase compared to non-treated controls, respectively). However, in the presence of Nox2 inhibitor, gp91ds-tat, eNOS inhibitor, L-NAME, or peroxynitrite scavenger, Uric acid, ICAM-1 up regulation was attenuated and expression remained similar to baseline controls at both time points of exposure to TNF $\alpha$  (Figure 32).



**Figure 32. TNF $\alpha$  induces peroxynitrite dependent adhesion molecule expression.**

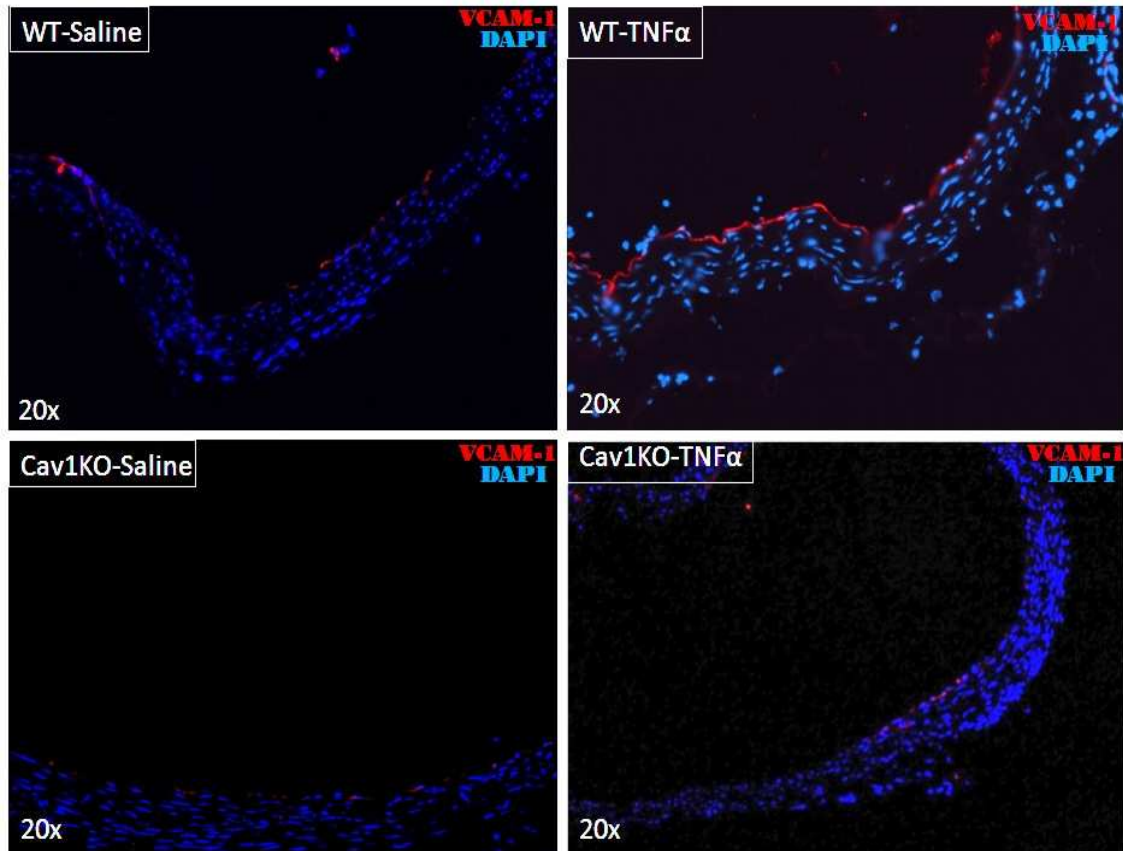
(A) BAECs were pre-treated with gp91ds-tat peptide or/and L-NAME, Uric acid prior to challenge with TNF $\alpha$  for 4 to 6 hours. Cell lysates were processed for Western-blot analysis for detection of ICAM-1 protein expression. The blots are representative of 3 independent experiments. (B) Densitometric analysis of each experimental set was performed and expressed as fold change with respect to levels of total  $\beta$ -actin. \* $P < 0.05$



**Figure 33: Lipid-raft/caveolae mediate TNF $\alpha$ -induced adhesion molecule expression.** (A) BAECs were pre-treated with MbCD, Cav1 siRNA or scrambled siRNA prior to challenge with TNF $\alpha$  for 4 to 6 hours. Cell lysates were collected and processed for Western-blot analysis for detection of ICAM-1 protein expression. The blots are representative of 3 independent experiments. (B) Densitometric analysis of each experimental set was performed and expressed as fold change with respect to levels of total  $\beta$ -actin. \* $P$ <0.05, \*\*\* $P$ <0.01

*TNF $\alpha$ -induced endothelial adhesion molecule expression is dependent on lipid-raft/caveolae compartmentalized ROS and RNS generation, In vitro as well as In vivo:*

Given the fact that disruption of caveolae compartments result in loss of protein tyrosine nitration (figure 33), we examined adhesion molecule expression upon either disruption of lipid-raft/caveolae compartments (M $\beta$ CD) or specifically depleting cells of caveolin-1/caveolae using Cav1siRNA. BAECs disrupted of lipid-raft/caveolae compartments failed to upregulate ICAM-1 in response to TNF $\alpha$  (Figure 34). In order to corroborate our in vitro findings in primary endothelial cells *in vivo*, we injected Wt as well as Cav1KO mice with a bolus of TNF $\alpha$  intravenously for 3 hours and measured adhesion molecule (VCAM-1) expression on aortic tissue derived from Wt and Cav1KO mice. TNF $\alpha$  injected Wt animals showed significant increase in expression of luminal VCAM-1 expression (Figure 34B) as compared to saline injected control animals (Figure 34A). However, Cav1KO mice derived aortic tissue showed blunting of VCAM-1 expression upon TNF injections (Figure 34D), similar their saline injected controls (Figure 34C).



**Figure 34. Inflammatory cytokine induced adhesion molecule expression is dependent on caveolin-1/caveolae.** *In vivo* Wt C57Blk6 and Cav1KO were injected (*i.v*) with saline or TNF $\alpha$  (2 $\mu$ g/25gm animal). After 3hrs, aortic tissue was removed, snap-frozen in OCT and cryosectioned (5 $\mu$ m). Sections were stained with anti-VCAM-1 antibody followed by goat-anti-rat-AlexaFluor594. Red fluorescence signal for VCAM-1 was measured using laser wavelength (590/617) and microscope settings. Cell nuclei was detected using blue DAPI. (A) Basal level of VCAM-1 protein was observed in the endothelium of saline injected Wt mice. (B) In contrast, TNF $\alpha$  increased VCAM-1 expression in the endothelium of the vessel wall. Neither (C) saline nor (D) TNF $\alpha$  induced upregulation of VCAM-1 expression in the endothelium of Cav1KO mice.

## Discussion

The main findings of our study here include, a) Nox2 isoform of NADPH oxidase enzyme as primary source of ROS in endothelial cells undergoing inflammation; b) caveolae organelles as localized compartments for regulating Nox2 mediated ROS generation in response to TNF $\alpha$  in endothelial cells, *in vitro* as well as *in vivo*; c) simultaneous generation of ROS and RNS induces protein tyrosine nitration events in cultured endothelial cells as well as aortic tissue. Our findings therefore are suggestive of a compartmentalized protein-tyrosine nitration as a novel mechanism for endothelial activation and dysfunction by inducing expression of adhesion molecules such as ICAM-1 and VCAM-1.

The activation of NADPH oxidase enzyme involves recruitment of cytoplasmic subunits (p47, p40, p67) to the membrane (gp91, p22) subunits. Based on our previous results (Yang & Rizzo, 2007), we stimulated bovine aortic endothelial cells with proinflammatory cytokine, TNF $\alpha$  at different time points and observed time dependent increases in ROS generation (Figure 19). Various pharmacological compounds such as DPI have been used in the past to inhibit NADPH oxidase enzymes, however most have been non-specific due to inhibition of not only enzyme NADPH oxidase but also other ROS sources, resulting in abolishment of both pathological as well as physiological ROS. Here, we used specific NADPH oxidase enzyme blocker, gp91ds-tat peptide, which prevents binding of cytoplasmic p47 subunit of NADPH oxidase enzyme to membrane subunits (gp91 and p22), preventing ROS generation. Although, Nox1 and Nox2 are primary enzyme isoforms implicated in endothelial ROS generation, we believe peptide blocks Nox2 induced ROS generation based on the evidence that Nox1 is expressed at very low levels in endothelial cells (Figure 21). Nox inhibiting peptide, gp91ds-tat showed

attenuations in TNF $\alpha$  induced ROS generations in endothelial cells compared to the scrambled control peptide (scrambled-gp91ds-tat). The primary function of enzyme eNOS is for NO production, whereas under chronic inflammatory conditions, the cofactor for enzyme eNOS, tetrahydrobiopterin (BH<sub>4</sub>), can undergo oxidation, resulting in uncoupling of enzyme to produce superoxide. We here used an acute TNF $\alpha$  treatment, which doesn't seem to uncouple eNOS at these early time points. Consistently, results with use of eNOS blocker, L-NAME, did not show any further loss in ROS generation compared to when only gp91ds-tat peptide was used, ruling out possibility of eNOS uncoupling (Figure 20).

There are previous reports suggesting that membrane rafts function in regulating ROS production through subcellular targeting of ROS-producing enzyme systems (Hilenski, Clempus, Quinn, Lambeth, & Griending, 2004; Shao et al., 2003). Endothelial cells contain all components of the classical phagocyte-type NADPH oxidase enzyme (Bayraktutan, Blayney, & Shah, 2000; Gorlach, Brandes, Bassus, et al., 2000; Gorlach, Brandes, Nguyen, et al., 2000). Another group (J. M. Li & Shah, 2002; Oakley, Smith, & Engelhardt, 2009) reported that NADPH activity and superoxide generation were found predominately within a Triton X-100-insoluble cell fraction. The authors concluded that functional NADPH oxidase enzyme complexes are associated with the cytoskeleton in resting endothelial cells. Although a detergent-insoluble cell fraction is most often considered to be composed of cytoskeletal elements, Triton-X 100 insolubility is also a primary characteristic of plasma membrane lipid rafts and caveolae. Our previous data showed that several major NADPH oxidase subunits are present at the plasma membrane and significantly enriched within membrane raft microdomains relative to the plasma membrane proper (Figure 21) (Yang & Rizzo, 2007). It was therefore proposed

that the activation of endothelial NADPH oxidase enzyme (Nox2) requires close association of membrane subunits for efficient recruitment of cytoplasmic subunits. Based on these studies, our group in the past showed that NADPH oxidase enzyme subunits assembled in lipid-raft compartments are required for enzyme activation, ROS generation in endothelial cells (Yang & Rizzo, 2007). Also, a different mechanism has been proposed suggesting, enzyme activation via ceramide enriched Nox2 positive membrane rafts following FasL stimulations in human coronary endothelial cells (A. Y. Zhang et al., 2006). Several other groups have also shown localization of gp91 in raft microdomains in neutrophils (Shao et al., 2003; Vilhardt & van Deurs, 2004), vascular smooth muscle cells (Hilenski et al., 2004) for efficient activation of NADPH oxidase enzyme. The findings discussed above were restricted to roles of lipid-raft domains in NADPH oxidase activation, whereas the specific roles of caveolae compartments as spatial centers for enzyme activation and ROS generation are not clear. There is evidence under conditions of flow-induced shear stress, where caveolae function as sensors to induce Nox2 dependent ROS generation in pulmonary microvascular endothelial cells (PMVEC) (Milovanova et al., 2008) or in response to environmental pollutants such as PCB153 causing caveolae induced ROS generation and endothelial dysfunction (Han, Eum, Toborek, Smart, & Hennig, 2010). Here, we tested caveolae roles (Cav1 siRNA) in enzyme activation in response to TNF $\alpha$ , which showed significant attenuations in ROS generation, suggesting roles of caveolae domains in the enzyme activation and compartmentalized ROS generation (Figure 25).

During vascular disease, increased oxidative stress (ROS) is accompanied by decreases in bioavailability of protective RNS, NO. The reasons for decreasing NO include, either uncoupling of enzyme eNOS due to oxidation of substrate, BH<sub>4</sub> or sequestering of NO by superoxide to generate harmful RNS, peroxynitrite. Generation of

peroxynitrite involves near-diffusion-controlled reaction between superoxide and NO. Peroxynitrite generation is of pathophysiological consequences due to its oxidizing as well as nitrating potential (Beckman et al., 1990; Radi, Peluffo, Alvarez, Naviliat, & Cayota, 2001). When produced in high quantities these radicals exert cytotoxic effects in the cell through oxidative damage to proteins, lipids and DNA (Zou, Cohen, & Ullrich, 2004). Although less well characterized, peroxynitrite also appears to modulate protein functioning by inducing conformational changes. Peroxynitrite and its metabolite,  $\text{NO}_2^-$ , can nitrate tyrosine residues in proteins, forming 3-nitrotyrosine (Radi, 2004; Radi et al., 2001). Due to the very short half-life and lack of direct assays for measuring peroxynitrite, an indirect “footprint assay” measuring protein-tyrosine nitration (PTN) is standard. The relevance of heightened protein-tyrosine nitration to human disease is evidenced by an observed increase in tyrosine nitration in atherosclerotic lesions and abdominal aortic aneurysms in both patients (Buttery et al., 1996) and mouse models of these diseases (Parastatidis et al., 2007; Takayanagi et al., 2013). Furthermore, plasma nitrotyrosine levels correlates strongly with severity of coronary artery disease (Shishehbor et al., 2003). Thus, an increase in PTN may serve as a biomarker for oxidant imbalance and an indirect indicator of cardiovascular disease (Peluffo & Radi, 2007). However, important details regarding the precise mechanism by which RNS are formed, the identity of cellular components that are targets of nitration and the consequence of PTN in endothelial and vessel function are poorly understood. In these studies, we determined the extent of PTN under inflammatory conditions. We found that  $\text{TNF}\alpha$  induced rapid and sustained PTN (Figure 22), consistent with past *in vitro* studies (Neumann et al., 2006; Yang & Rizzo, 2007). Moreover, we observed enhanced protein-tyrosine nitration in the vessel wall of mice injected with  $\text{TNF}\alpha$  into the systemic circulation (Figure 28). The *in vitro* data here in BAECs pre-treated with NADPH oxidase

inhibitor, gp91ds-tat (Figure 26A) or use of peroxynitrite scavenger, Uric acid (Figure 26B), resulted in attenuation in PTN events.

As discussed above, peroxynitrite is formed by near-diffusion-controlled reaction between superoxide and NO at balanced rates under hydrophobic environment. Therefore, both the radical sources are required to be present in spatially confined compartments to favor peroxynitrite generation and tyrosine nitration of proteins. The first evidence for this concept came from studies in eosinophil and neutrophils where both the NO producing enzyme, iNOS, and superoxide generating machinery co-localized in specific subcellular organelles such as secretory granules, mitochondria and peroxisomes (Heijnen et al., 2006). Consistent with this, our previous data showed that localization of both Nox2 and eNOS in spatially confined compartments of plasma membrane (lipid-rafts) provides favorable environment for interaction between these two reactive radicals and tyrosine nitration of localized proteins. In this study we take this concept further by showing roles of caveolae domains in compartmentalized cross-reaction of superoxide and NO radicals, inducing PTN of local protein molecules and their roles in endothelial dysfunction. Our current findings support this concept, where disruption of caveolae compartments using Cav1siRNA prevented PTN events (Figure 26C). *In vivo* results showed abolishment of PTN events in aortic tissue derived from Cav1KO mice under inflammatory conditions (Figure 28) are in line with *in vitro* results observed in endothelial cells. Taken together, all these findings are consistent with proposed roles of caveolae as functional centers mediating localized PTN.

Our data so far shows that disruption of caveolae domains result in loss in activity of enzyme NADPH oxidase mediated attenuations in ROS production, whereas alternations in activity of other ROS producing and neutralizing enzymes also need to be

taken in to consideration. These limitations may interfere with interpretation of data obtained from our studies. There is evidence that loss of caveolin-1 can either exacerbate oxidative stress through alteration of mitochondrial function (Bosch et al., 2011) or limit oxidative stress by enhancement of antioxidant systems such hemoxygenase-1 (HO-1) (Kim, Wang, Galbiati, Ryter, & Choi, 2004). To evaluate these possibilities as contributing factors in our findings, we measured expression of HO-1 and mitochondrial integrity (Cytochrome-c release). In both cases, we found no evidence of change in these elements between cell expressing normal or reduced levels of caveolin-1 (Figure 29 and 30). Sequestration of membrane cholesterol can also initiate cellular cholesterol biosynthesis, contributing to increases in NADPH molecule, a substrate for NADPH oxidase activity to reduce molecular oxygen to superoxide. However, M $\beta$ CD treatments showed no changes in basal or TNF $\alpha$ -induced NADP/NADPH ratios (Figure 31).

A well-characterized consequence of increased ROS (oxidative stress) in endothelial cells include over expression of adhesion molecules such as ICAM-1/VCAM-1, which is an important early event in progression of vascular diseases such as atherosclerosis (Galkina & Ley, 2007a, 2007b) by recruiting monocytes into the artery wall. Consistent with these findings, it has been shown that Cav1KO mice with background of apoE<sup>-/-</sup> mice show attenuations in expression of VCAM-1 following high fat diet (Frank et al., 2004; Frank & Lisanti, 2004). Another evidence supporting role of Cav1/caveole progression of atherosclerosis comes from studies involving endothelial-specific over expression of caveolin-1 in apoE<sup>-/-</sup> mice, showing increased expression of VCAM-1 (Fernandez-Hernando, Yu, Davalos, Prendergast, & Sessa, 2010). However, the connections between Caveolin-1/caveolae, increased ROS, PTN and adhesion molecules levels under inflammatory conditions remain unexplored. While the caveolae-

associated mechanisms that governed adhesion molecule expression were not completely identified in those studies, our findings suggest that caveolae-dependent protein tyrosine nitration may play a role in regulation of ICAM-1/VCAM-1 expression. Here we show roles of excessive ROS and RNS in inducing TNF $\alpha$  induced expression of ICAM-1 in cultured endothelial cells. TNF $\alpha$  induced expression of ICAM-1, whereas blockage of ROS (gp91ds-tat peptide) or RNS (L-NAME or Uric acid) attenuated adhesion molecule expression (Figure 32). Similarly, disruption of raft and caveolae compartments or depletion of caveolin-1 showed attenuations in adhesion molecule expression (Figure 33). Our *in vivo* results in aortic tissue derived from Cav1KO mice showing lack of VCAM-1 expression (Figure 34) is consistent with loss of ROS and PTN in these mice (Cav1KO) in response to TNF $\alpha$ , confirming caveolae compartment roles in localized ROS generation and PTN induced endothelial dysfunction.

Further work focused on functional significance of caveolae in generating compartmentalized ROS generation and tyrosine nitration of localized proteins. Various signaling pathways emanate in response to TNF $\alpha$  induced ROS and RNS generation called as nitroxidative signaling pathways. Therefore, caveolae may also serve as nitroxidative signaling platforms to confer directionality to these cascades. Based on the clinical evidence, peroxynitrite-induced PTN events play a role in disease progression, whereas exact role of these post-translational modifications in regulating protein functioning in endothelial cells, are not clear. Also, the knowledge that if any of the signaling molecules discussed above are targets of PTN is lacking.

## CHAPTER 3 CAVEOLAE AS NITROXIDATIVE SIGNALING PLATFORMS IN REGULATING ENDOTHELIAL CELL FUNCTIONING

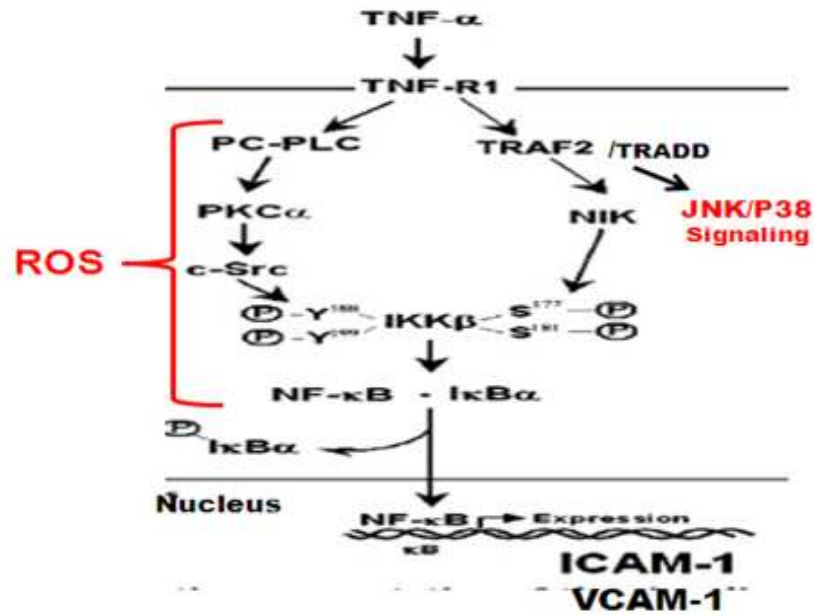
### Introduction

The direct effects of excessive ROS production in the endothelium including protein and lipid oxidation and sequestration of NO are well established. Given the short half-life and limited diffusion distance of primary superoxide and peroxynitrite, central questions emerge as to what signaling cascades these radicals activate and how these can confer directionality to signaling. Caveolae functions as redox-signaling platforms by providing close proximity of oxidants to local enzyme targets are well documented (Oakley, Abbott, Li, & Engelhardt, 2009; Oakley, Smith, et al., 2009). However, their functional significance in relaying TNF $\alpha$  induced protein-tyrosine nitration associated nitroxidative signaling and identification of molecules undergoing tyrosine nitration in endothelial cells, is not clear. Our previous data demonstrated that Caveolae localization of Nox2, eNOS and dual activation of these enzymes is important for protein-tyrosine nitration events (Chapter 1). Here, we extended these initial observations and provide new evidence that the functional significance of ROS/RNS compartmentation is to regulate specific nitroxidative sensitive signaling pathways resulting in endothelial activation and dysfunction.

## Results

### *TNF $\alpha$ -induced oxidative and nitroxidative stress signaling involves I $\kappa$ B/NF $\kappa$ B and Src-Family Kinase (SFK) pathways:*

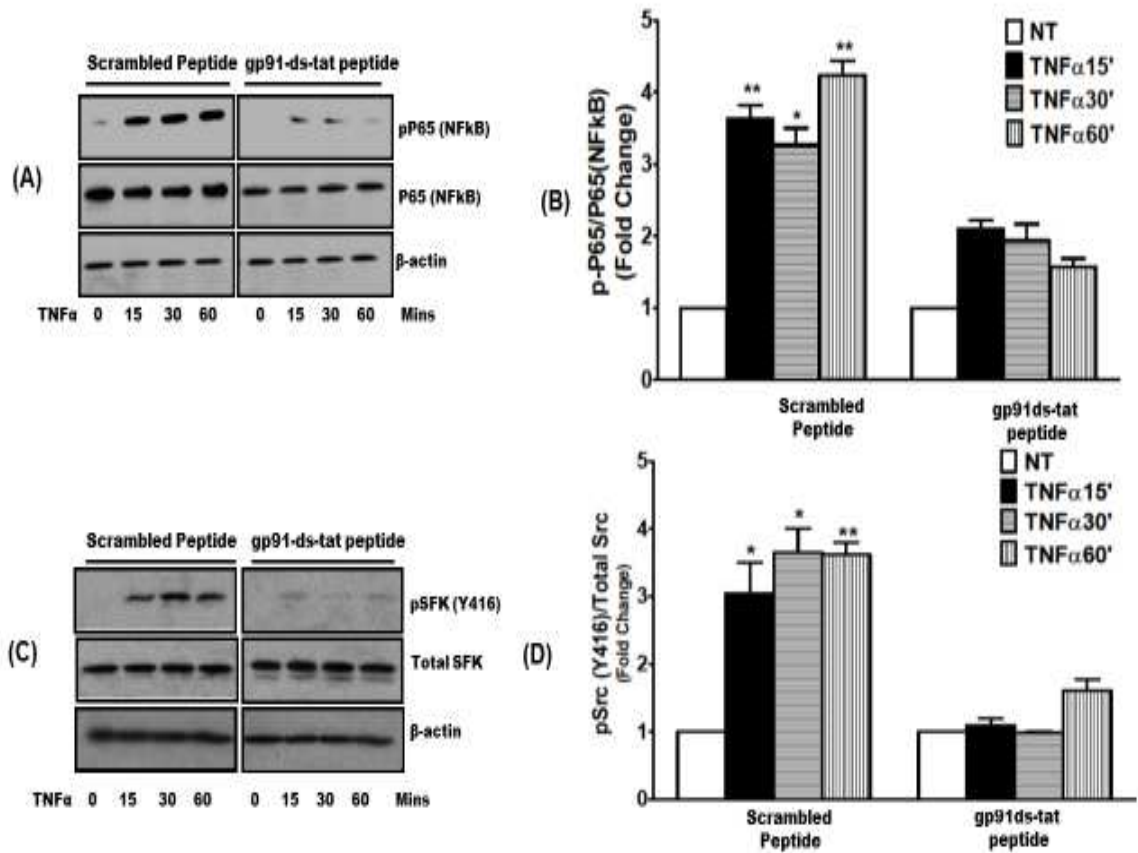
There are several pathways stemming from TNF $\alpha$  receptors to activation of NF $\kappa$ B and up regulation of the adhesion molecules described in the literature (Madge & Pober, 2001) including mechanisms that function through Src-family kinases (SFK) (Huang, Chen, & Chen, 2003). (Figure 35). Similarly, previous studies have shown roles of excessive ROS in activation of NF $\kappa$ B redox signaling to induce adhesion molecule expression (True, Rahman, & Malik, 2000). In order to test specific roles of Nox2 enzyme mediated ROS in activating redox signaling pathways, endothelial cells were pre-treated with gp91ds-tat peptide prior to TNF $\alpha$  stimulation for indicated time points. TNF $\alpha$  stimulation showed significant (4 fold change at 60 mins) increases in phosphorylation of both p65 subunit of NF $\kappa$ B (Figure 36A,C) compared to cells pre-treated with scrambled sequence of gp91ds-tat peptide. Out of the many signaling pathways upstream of NF $\kappa$ B, important one implicated in TNF $\alpha$  induced inflammatory signaling and adhesion molecule expression involves, Src-family kinases (SFKs) (Huang, Chen, & Chen, 2003; Huang, Chen, Inoue, & Chen, 2003). Therefore, we evaluated TNF $\alpha$  induced ROS roles in SFK activation. TNF $\alpha$  induced tyrosine-416 phosphorylation of total SFKs was studied. Similar to p65 phosphorylation, TNF $\alpha$  induced SFK phosphorylation (Y-416) at different time points and inhibition of ROS (gp91ds-tat peptide), blocked phosphorylation (Figure 36B,D).



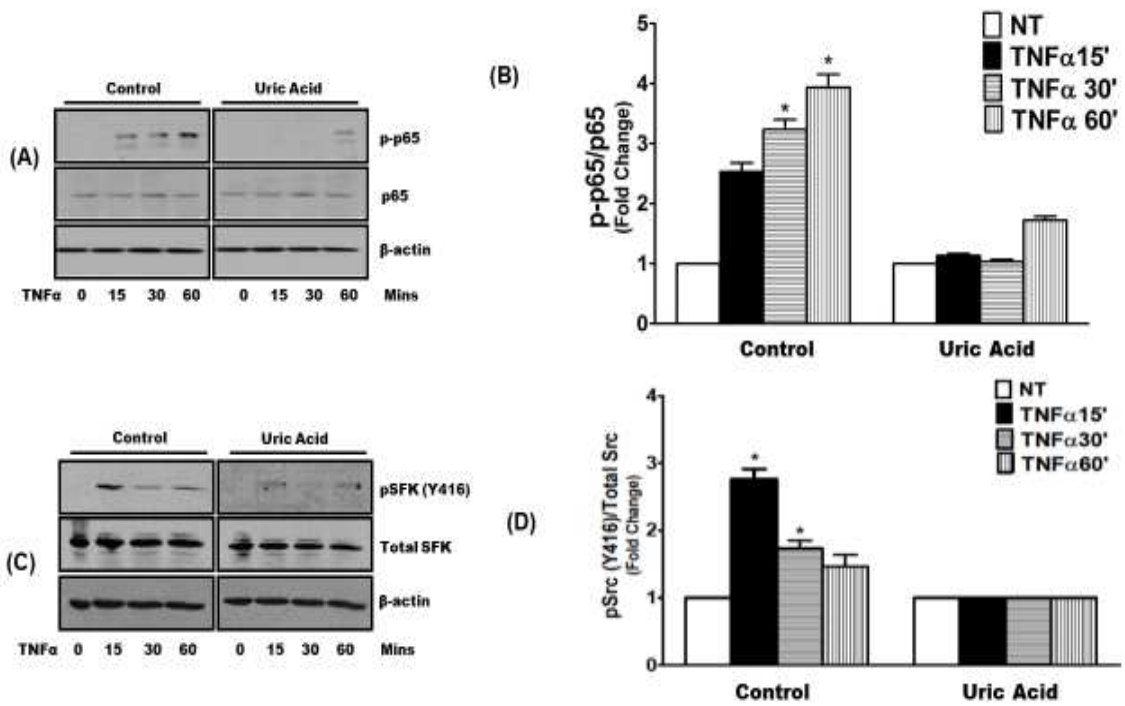
Huang W-C et al., 2003 Journal of Biol Chem

**Figure 35: TNF $\alpha$  induced inflammatory signaling:** Schematic shows TNF $\alpha$  induced activation of Src-family kinases (SFKs), I $\kappa$ B/NF $\kappa$ B and stress activated kinase, JNK signaling resulting in overexpression of adhesion molecules, ICAM-1/VCAM-1 in endothelial cells (Huang, Chen, Inoue, et al., 2003). The signaling scheme also shows that SFK activation is upstream of I $\kappa$ B/NF $\kappa$ B pathway in mediating adhesion molecule expression. The other signaling emanating from TNF $\alpha$  involves TNF $\alpha$ Receptor associated, TRADD/TRAF signaling resulting in JNK pathway activation. This signaling is also a function of TNF $\alpha$ Receptor functioning.

ROS induced oxidative signaling, NFκB, and SFK have been previously shown, and whereas peroxynitrite induced nitroxidative (oxidative and nitrative) signaling mediators are not known. Based on these, we tested roles of peroxynitrite generation in activation of both NFκB as well as SFK signaling. Scavenging of peroxynitrite using Uric acid, attenuated TNF- induced phosphorylation of p65 subunit of NFκB (Figure 37A,C) as well as SFK at tyrosine-416 residues (Figure 37B,D).



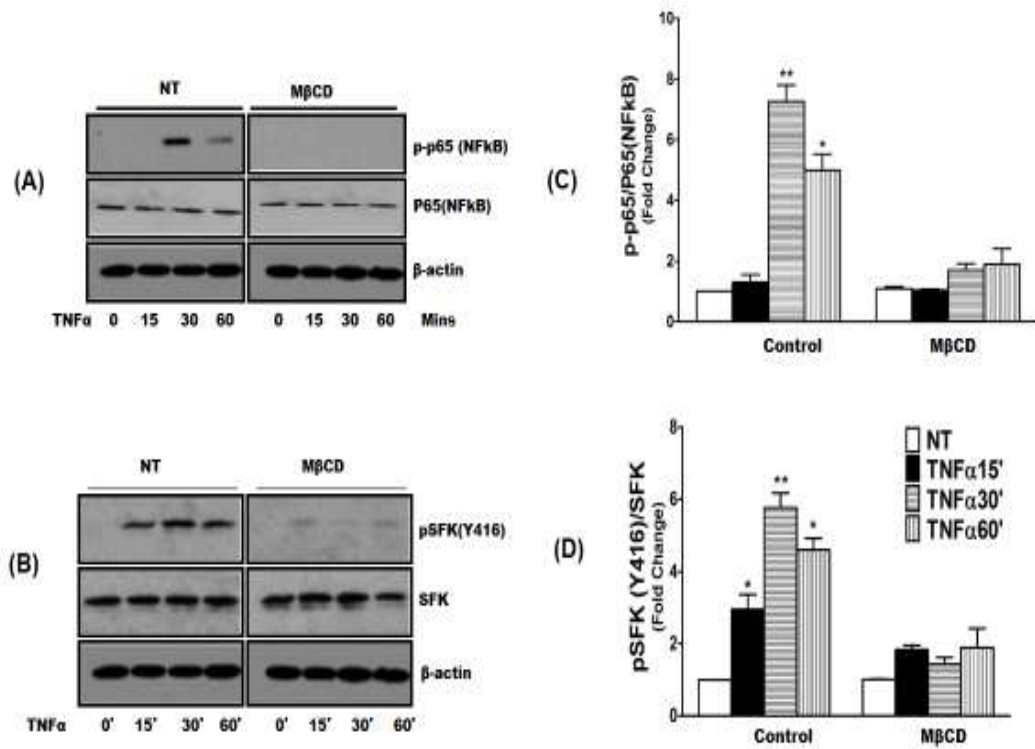
**Figure 36. ROS/RNS induced signaling involves activation of both NFκB and SFK pathway.** (A) BAECs were pretreated with gp91ds-tat peptide or Uric acid prior to challenge with TNFα. Cell lysates were collected, processed for Western blot and probed for phosphorylation of the p65 subunit of NFκB, total p65, pSFK, total SFK and β-actin. TNFα induced phosphorylation of p65 at all-time points, whereas pretreatments with (A) gp91ds-tat peptide or (B) Uric acid, attenuated this phosphorylation event. The blots here are representative of 3 independent experiments. (B), (D) Blots from 3 different experiments were quantified by densitometry and expressed as fold change compared to total p65 \*P<0.05.



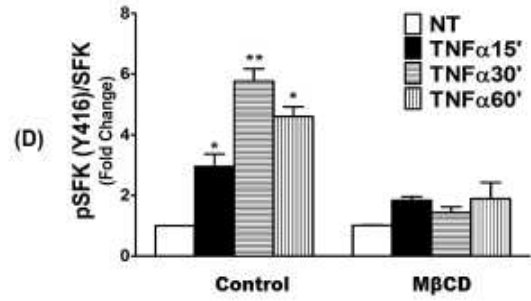
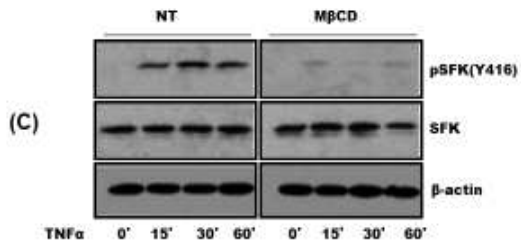
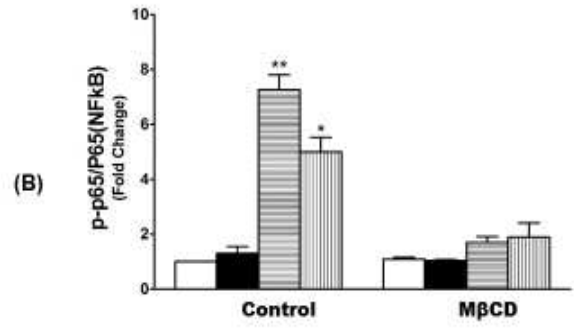
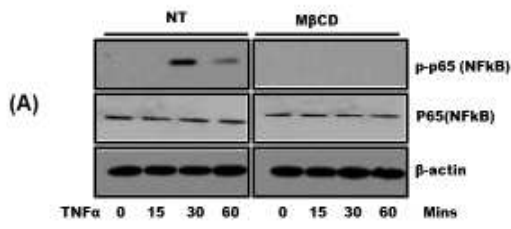
**Figure 37. Peroxynitrite induced nitroxidative signaling involves activation of both NFκB and SFK pathway**(A) BAECs were pretreated with gp91ds-tat peptide or Uric acid prior to challenge with TNFα. Cell lysates were processed for Western blot and probed for pSFK, total SFK and β-actin. TNFα induced phosphorylation of p65 at all-time points, whereas pretreatments with (A) gp91ds-tat peptide or (B) Uric acid, attenuated this phosphorylation event. The blots here are representative of 3 independent experiments. (B), (D) Blots from 3 different experiments were quantified by densitometry and expressed as fold change compared to total p65 \*P<0.05.

*TNF $\alpha$  induced activation of nitroxidative signaling is compartmentalized in lipid-raft/caveolae domains:*

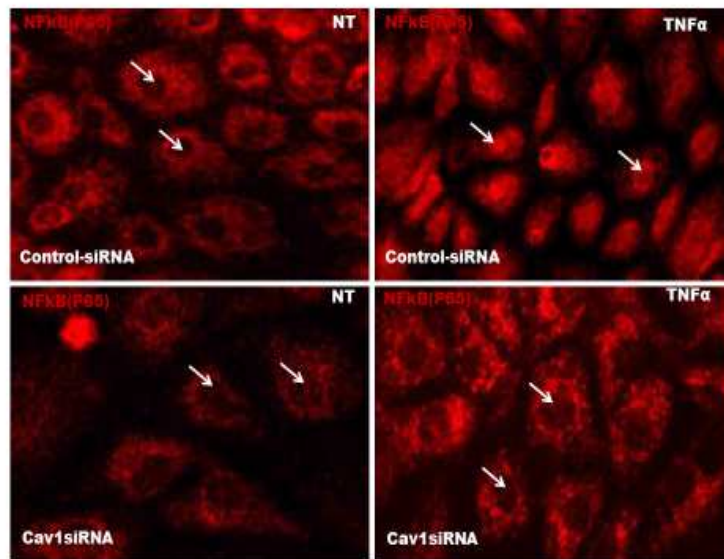
The functional significance of caveole compartmentalized ROS and RNS generation was tested by activation of NF $\kappa$ B and SFK nitroxidative signaling mediators upon disruption of raft and caveolae domains using M $\beta$ CD (Figure 38). Based on the previous data showing SFKs regulation by ROS/RNS and evidence showing enrichment of SFKs in caveolae (Lisanti, Scherer, Tang, & Sargiacomo, 1994; Liu, Oh, Horner, Rogers, & Schnitzer, 1997), we evaluated the relationship of this proximal signaling molecule to caveolae-based RNS production and endothelial activation induced by TNF $\alpha$ . Consistent with the patterns of activation for adhesion molecule expression, TNF $\alpha$  rapidly induced phosphorylation of SFKs on the tyrosine<sup>416</sup> residue, an event that strongly correlated with kinase activation. Similar to peroxynitrite scavenging compound, Uric acid, pretreatment of endothelial cells with cholesterol sequestering M $\beta$ CD prior to TNF $\alpha$  stimulation resulted in significant attenuations in activation of both nitroxidative-signaling mediators, NF $\kappa$ B and SFKs (Figure 38) compared to control cells. Similarly, depletion of caveolin-1 protein also resulted in significant decreases in activation of NF $\kappa$ B and SFK pathways (Figure 39).



**Figure 38. Peroxynitrite induced NFκB and SFK nitrooxidative signaling is compartmentalized in lipid-rafts and caveolae**(A) BAECs were pretreated with MβCD prior to challenge with TNFα. Cell lysates were collected, processed for Western blot and probed for phosphorylation of the p65 subunit of NFκB, total p65, pSFK, total SFK and β-actin. TNFα induced phosphorylation of p65 as well as SFK at all-time points, whereas pretreatments with MβCD, attenuated these phosphorylation events. The blots here are representative of 3 independent experiments. (B), (D) Blots from 3 different experiments were quantified by densitometry and expressed as fold change compared to total p65 and total SFK \* $P < 0.05$



(E)



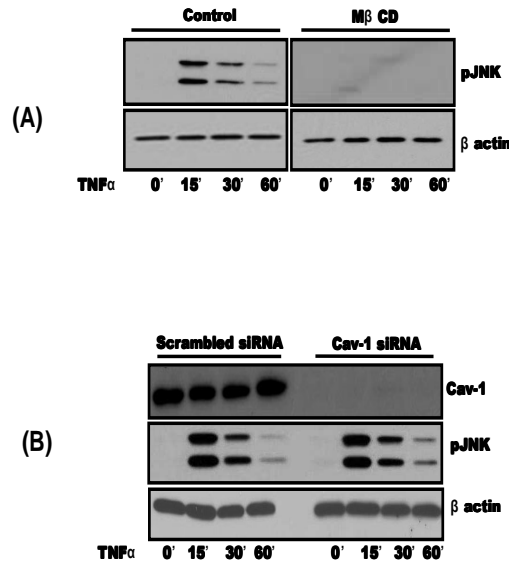
**Figure 39. Depletion of Caveolin-1/Caveolae attenuated TNF $\alpha$  induced proinflammatory nitroxidative signaling.** (A) BAECs were pretreated with Cav1 siRNA or scrambled siRNA prior to challenge with TNF $\alpha$ . Cell lysates were collected, processed for Western blot and probed for phosphorylation of the p65 subunit of NF $\kappa$ B, total p65, pSFK, total SFK and  $\beta$ -actin. TNF $\alpha$  induced phosphorylation of p65 as well as SFK at all-time points in scrambled siRNA pretreated cells, whereas pretreatments with Cav1 siRNA, attenuated these phosphorylation events. The blots here are representative of 3 independent experiments. (B), (D) Blots from 3 different experiments were quantified by densitometry and expressed as fold change compared to total p65 and total SFK \* $P$ <0.05. (E) BAECs were cultured on glass coverslips, pre-treated with Cav1siRNA and stimulated with TNF $\alpha$  for 2 hours. Cells were then fixed with 4% paraformaldehyde, permeabilized with TritonX100, blocked with normal goat serum and incubated with anti-p65 antibody followed by goat-anti-rabbit-Alexafluor568 secondary antibody and DAPI. Fluorescence signal was measured using identical laser wavelength (578/603nm) and microscope capture settings. BAECs treated with TNF $\alpha$  showed nuclear localization of p65 (white arrows), whereas p65 remained in the cytoplasm in cell depleted of Cav1.

*Depletion of Caveolin-1/Caveolae disrupts TNF $\alpha$  induced translocation of p65 subunit of NF $\kappa$ B into nucleus:*

In addition, p65 translocation from the endothelial cell cytoplasm to the nucleus was observed following TNF $\alpha$  exposure for 60 minutes. To evaluate Cav1/caveolae in the activation of p65 (NF $\kappa$ B) and its translocation into nucleus, cells were pretreated with Cav1 siRNA. Consistent with reductions in TNF $\alpha$ -induced phosphorylation of the p65 nuclear accumulation of p65 was not observed in BAECs depleted of Cav1 after TNF $\alpha$  challenge (Figure 39E).

*TNF $\alpha$  receptor associated stress activated protein kinase signaling remains intact upon Caveolin-1 depletion:*

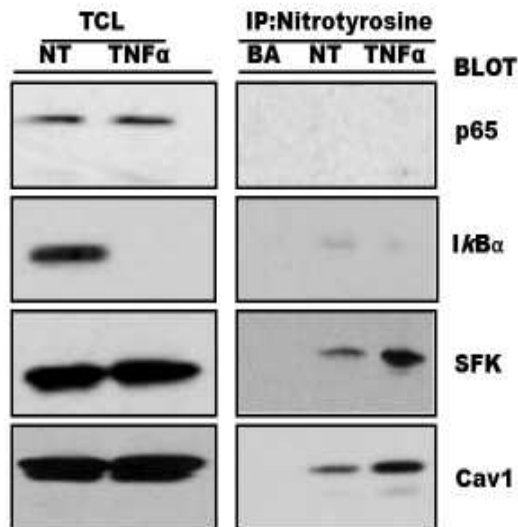
TNF $\alpha$ Receptor activation along with NF $\kappa$ B and SFK activation involves stress activated protein kinases (SAPKs), JNK phosphorylation. We tested alterations in JNK signaling upon disruption of both rafts and caveole (M $\beta$ CD) or only cav1 depletion (Cav1siRNA). Pretreatments with M $\beta$ CD blunted all TNF receptor mediated downstream signaling events (NF $\kappa$ B, SFK, and JNK), whereas depletion of Caveolin-1 only blocked NF $\kappa$ B and SFK and JNK signaling remained unaltered (Figure 40A,B).



**Figure 40. TNF $\alpha$  receptor activity remains unaltered upon depletion of Caveolin-1**  
 BAECs were pretreated with M $\beta$ CD, Cav1 siRNA or scrambled siRNA prior to TNF $\alpha$  stimulation. Cell lysates were processed for Western blot and probed for phosphorylation of JNK, Cav1 and  $\beta$ -actin. TNF $\alpha$ -receptor activation induces phosphorylation of JNK at indicated time points in control or scrambled siRNA pretreated cells. However, disruption of both lipid-raft and caveolae compartments (M $\beta$ CD) abolishes signaling events emanating from TNF $\alpha$  receptor activation. Similar to control or scrambled siRNA, Cav1 siRNA pretreatments prior to TNF $\alpha$  stimulation does not alter receptor-induced JNK pathway. Cav1 blots in B show protein levels in Cav1siRNA pretreated cells compared to scrambled siRNA. The blots here are representative of 3 independent experiments.

*RNS induces protein-tyrosine nitration of Src-family kinases:*

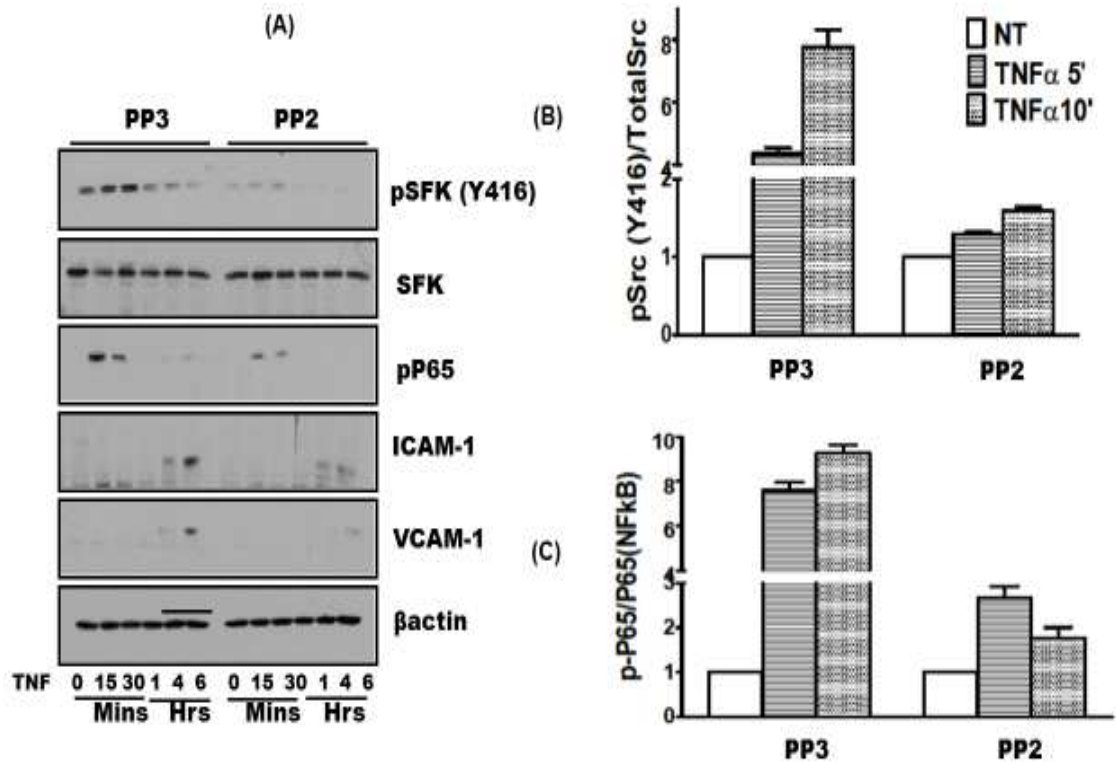
While SFK tyrosine phosphorylation can be enhanced by oxidative inactivation of protein phosphatases; SFK nitration has been reported to increase kinase activity (Mallozzi, Di Stasi, & Minetti, 1999). Based on our observation of the molecular weight pattern of proteins that are nitrated on tyrosine (chapter 1), we speculated that the proteins in the molecular weight range of 50-60kDa were members of the SFKs. To test this concept, proteins from TNF $\alpha$  treated and non-treated BAECs were immunoprecipitated using an anti-nitrotyrosine antibody. We found that SFKs and caveolin-1 but not p65 subunit of NF $\kappa$ B or its regulatory subunit I $\kappa$ B $\alpha$  were nitrated on a tyrosine residue in response to TNF $\alpha$  (Figure 41). Additionally there is recent evidence showing nitration of caveolin-1 protein in vessels derived from diabetic patients (Cassuto et al., 2013). Based on this, we looked at Caveolin-1 nitration under inflammatory conditions in endothelial cells. We found Cav1 nitration patterns similar to that of SFKs in response to TNF $\alpha$ .



**Figure 41. Src-family kinases are nitrated in response to TNF $\alpha$ .** (A) Proteins containing nitrotyrosine residues were immunoprecipitated with an anti-nitrotyrosine antibody from BAEC total cell lysates (TCL) collected after stimulation with TNF $\alpha$  (T) for 2 hours. Precipitates were probed with anti-I $\kappa$ B, anti-p65, anti-SFK, and anti-cav-1 antibodies by Western blot. TNF $\alpha$  induced nitration of SFKs and Caveolin-1 but not I $\kappa$ B or p65. The blots shown are representatives of 3 different independent experiments.

*TNF $\alpha$ -induced nitration of SFK is suggestive of gain-of-function effects regulating endothelial functioning in vitro:*

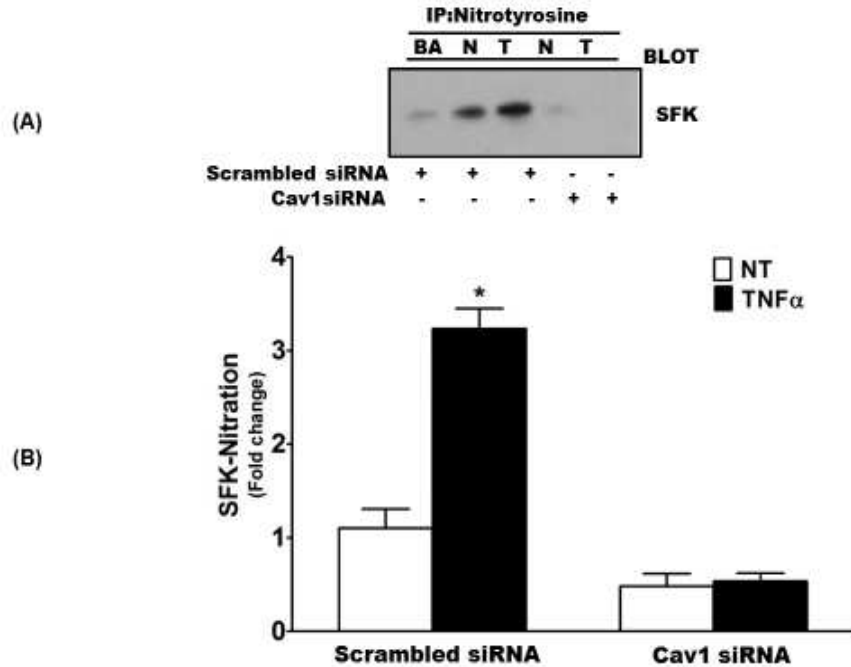
Based on previous studies and our data showing upstream localization of SFKs to NF $\kappa$ B pathway in mediating endothelial adhesion molecule expression, we determined whether SFK nitration renders it amenable to phosphorylation and is therefore linked to NF $\kappa$ B pathway activation and adhesion molecule expression. BAECs were pretreated with a pharmacological blocker of SFKs, PP2 and activation of both downstream NF $\kappa$ B pathway as well as adhesion molecules (ICAM-1/VCAM-1) was measured. We found that TNF $\alpha$ -induced SFK phosphorylation, NF $\kappa$ B activation and upregulation of ICAM-1 and VCAM-1 in cells pretreated with PP3, whereas PP2 pre-treatments attenuated each of these events at all-time points of observation (Figure 42).



**Figure 42. Inhibition of Src-family kinases blocks NF $\kappa$ B activation and adhesion molecule expression.** (A) BAECs were pre-treated with SFK inhibitor, PP2 (10 $\mu$ M for 60 mins) or its analog control, PP3 (10 $\mu$ M for 60 mins) and stimulated with TNF $\alpha$  for indicated time points. The cell lysates were collected and prepared for Western-blot analysis to detect tyrosine phosphorylated SFK (SFK-Y<sup>416</sup>), and expression of SFKs, phosphorylated p65 subunit of NF $\kappa$ B, ICAM-1, VCAM-1 and  $\beta$ -actin proteins. Representative blots from 3 different independent experiments are shown.

*Src-family kinase mediated nitrooxidative signaling is compartmentalized in caveolae microdomains:*

As discussed earlier, SFKs are proximally localized to caveolae compartments and their activation is dependent on the generation of ROS/RNS as well as presence of raft/caveolae microdomains. Additionally our present data also showed increased nitration of SFKs in response to TNF stimulations. Based on these, we tested if nitration of SFKs is compartmentalized in caveole domains. BAECs pretreated with Cav1siRNA prior to TNF $\alpha$  stimulation, showed abolishment of SFK nitration events compared to scrambled siRNA sequence (Figure 43A,B).



**Figure 43. Caveolin-1/caveolae depletion attenuates localized SFK nitration.** (A) BAECs pretreated with Cav1siRNA or Scrambled siRNA were stimulated with TNF $\alpha$  (T) for 2 hours and total cell lysates (TCL) immunoprecipitated with anti-nitrotyrosine antibody. Precipitates were probed with anti-Src family kinase (SFK), antibodies by Western blot (B) Blots from 3 independent experiments were quantified and plotted as fold change ( $*P < 0.05$ ) with respect to controls (N). BA: Beads Alone.

## Discussion

Tyrosine nitration of proteins is either of physiological or pathological significance and based on types of proteins involved, it can either change protein function or have no effect. Other possible effects include gain-of-function or loss-of-function of proteins (Balafanova et al., 2002; Gole et al., 2000) with evidence for possible steric hindrance and a distortion of the local protein structure (Ischiropoulos, 1998; Souza et al., 1999). Less well-characterized are PTN induced modulation of signal transduction through alteration of signaling molecule activity. The modulatory effects include direct or indirect interference with tyrosine phosphorylation/dephosphorylation of signaling pathways. The proposed mechanism by which PTN induces activation/inactivation of signaling molecules is through competition with phosphorylation/dephosphorylation events on the same or a neighboring tyrosine residue in a given protein (A. J. Gow, Duran, Malcolm, & Ischiropoulos, 1996).

NFκB is a transcription factor that regulates the expression of many proinflammatory genes including cellular adhesion molecules. Past reports demonstrating that depletion of membrane cholesterol or caveolin-1, both of which are necessary for maintaining caveolae structure, attenuated inflammatory mediator induced NFκB activation through a mechanism that involves ROS (Majkova, Toborek, & Hennig, 2010; Oakley, Smith, et al., 2009). Several studies also show that RNS can activate NFκB. In response to induction and/or exogenous application of peroxynitrite, NFκB is activated in both polymorphonuclear lymphocytes (Jozsef, Khreiss, El Kebir, & Filep, 2006) and skeletal myocytes (Bar-Shai & Reznick, 2006a, 2006b). In addition, IR radiation induced peroxynitrite-dependent activation of NFκB via IκBα-nitration (Y<sup>181</sup>) in CHO-K1 and MCF-7 cells (Matata & Galinanes, 2002; Yakovlev et al., 2007). However, data also exists

demonstrating that peroxynitrite can inhibit NF $\kappa$ B signaling. COS-1 and HEK293 cells exposed to peroxynitrite suppressed NF $\kappa$ B activation through nitration of p65 leading to faulty transport of the subunit into the nucleus (Levrant et al., 2005; Park et al., 2005). The concentrations of ONOO<sup>-</sup> given to the cells will determine the occurrence of positive or negative modulation. But the approach of infusing ONOO<sup>-</sup> externally, has pitfalls due to, a) arbitrary or non-physiological concentrations of ONOO<sup>-</sup>, and b) cell types involved, where different cells respond differently to same or different ONOO concentrations. Therefore, the physiologic occurrence and significance of signaling by tyrosine nitration and its relation with tyrosine phosphorylation could not be fully understood (Monteiro, Gruia-Gray, Peranovich, de Oliveira, & Stern, 2000). Our study in the past (Yang & Rizzo, 2007), and others (Neumann et al., 2006) has shown tyrosine nitration effects under physiological ONOO<sup>-</sup> conditions using TNF $\alpha$  as a stimulant. Similarly, studies here also looked at ONOO<sup>-</sup> effects under patho-physiological concentrations in response to TNF $\alpha$ . The study here found that TNF $\alpha$ -induced activation of NF $\kappa$ B, as measured by p65 subunit phosphorylation and nuclear translocation, was significantly attenuated in cells either treated with peroxynitrite scavengers or depleted of caveolin1/caveolae (Figure 39). We also found that neither I $\kappa$ B nor p65 were nitrated in response to TNF $\alpha$ , indicating a mechanism other than direct tyrosine nitration in the regulation of NF $\kappa$ B activity. In general, our data is in agreement with those suggesting that peroxynitrite functions as a proinflammatory oxidant and that caveolae serve as an important cellular component for regulation of NF $\kappa$ B in endothelial cells challenged with inflammatory cytokines.

To determine factors that relay signals from caveolae-compartmentalized RNS to NF $\kappa$ B and adhesion molecule expression, we focused on proteins which were nitrated in

response to  $\text{TNF}\alpha$  and localized to caveolae membranes (Yang & Rizzo, 2007). While the signaling pathway upstream of NF $\kappa$ B is complex, Src family kinases have a demonstrated role in the activation process through direct phosphorylation of the I $\kappa$ B $\alpha$  subunit of I $\kappa$ B/NF $\kappa$ B heterodimer (Huang, Chen, & Chen, 2003). Given that SFKs are enriched in caveolae (Liu et al., 1997) and our observation of nitrated proteins in the molecular weight range of that of SFKs (50-60kDa), we reasoned that members of SFKs are targets of PTN. Indeed, immunoprecipitation of cell lysates with an anti-nitrotyrosine antibody revealed SFKs in the precipitated fraction (Figure 41A). As mentioned, tyrosine nitration can have varied effects on protein function. In the case of SFKs, exogenous application of peroxynitrite induces SFK Tyr<sup>416</sup> phosphorylation resulting in enzyme activation (Mallozzi et al., 1999; Mallozzi et al., 2001c). Here, we show that Tyr<sup>416</sup> phosphorylation events are dependent on RNS production as well as caveolae domains (Figure 43). In addition, blockade of SFK activity (PP2 pretreatment) attenuates  $\text{TNF}\alpha$  induced NF $\kappa$ B activation and adhesion molecule expression (Figure 42). These findings indicate that caveolae-based signaling molecules such as SFK are available targets for the locally produced RNS and once nitrated participate in nitroxidative signaling events. Although we did not determine the mechanism by which SFK nitration enhance enzyme activity, it has been postulated that the nitration of Tyr<sup>527</sup> would disable Tyr<sup>527</sup> phosphorylation. In effect, Try<sup>527</sup> nitration would mimic Tyr<sup>527</sup> dephosphorylation and induce a conformational change facilitating Try<sup>416</sup> phosphorylation and enzyme activation (Mallozzi et al., 2001c; Minetti et al., 2002). Whether Tyr<sup>527</sup> is amenable to nitration and alters enzyme function in our experimental system is the subject of future evaluation.

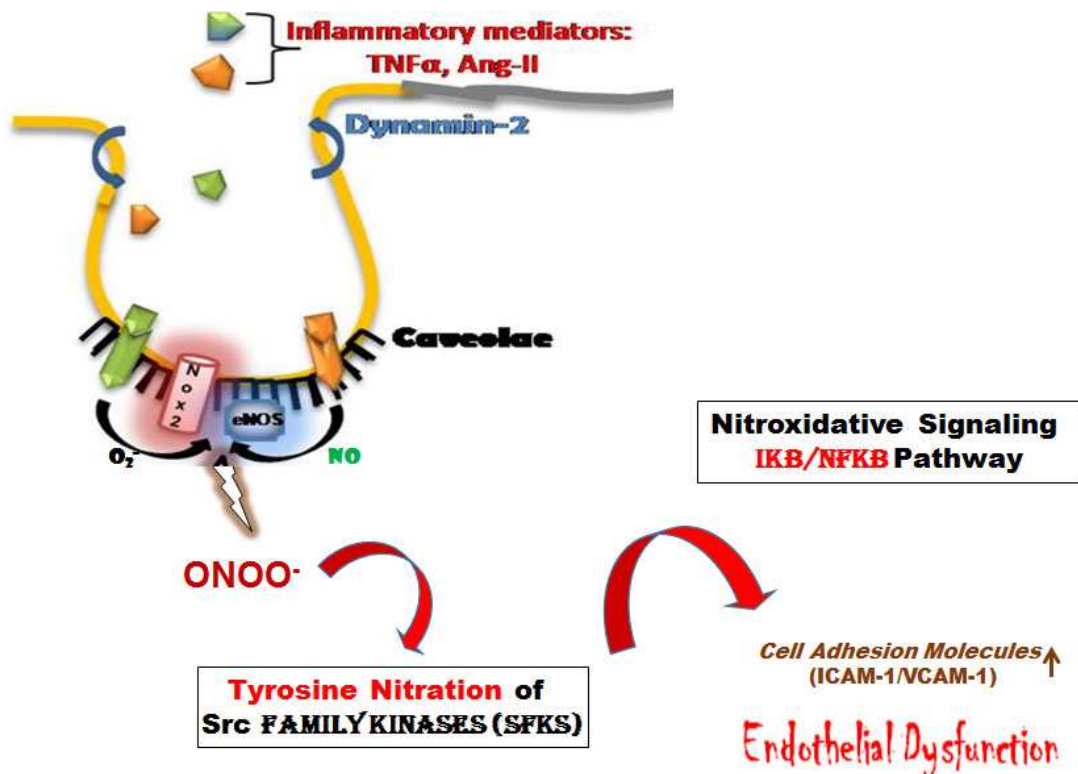
In support of our findings of localized PTN in caveolae, a very recent study (Cassuto et al., 2013) showed co-localization of 3-nitrotyrosine labeling with caveolin-1 in the endothelium of coronary arterioles derived from diabetic patients. The nitration events were associated with reduced flow mediated dilation and loss of caveole structures from plasma membrane. We discovered similar nitration of caveolin-1 in response to TNF $\alpha$  stimulation (Figure 41). While we did not pursue the functional significance of caveolin-1 nitration in this study, the findings suggest that increased caveolin-1 nitration is a feature of endothelial dysfunction. Mechanistically, internalization of caveole is implicated in the formation of a proinflammatory “redoxosome” (Singleton et al., 2009) and phosphorylation of the Tyr<sup>14</sup> residue of caveolin-1 is a determinant of caveolae endocytosis. Considering these known functions of caveolin-1/caveolae, we envision a mechanism where RNS-induced caveolin-1 nitration influences Cav1 tyrosine phosphorylation state and subsequent caveolae trafficking which impacts redoxosome formation and redox signaling in the endothelium. This concept needs further exploration.

TNF $\alpha$  induces inflammatory signaling via activation of raft/caveolae localized TNFReceptor (TNFR1) which along with NF $\kappa$ B and SFK involve, activation of stress activated protein kinases (SAPKs) such as JNK. Our observations here showed that disruption of raft and caveolae compartments resulted in loss of both NF $\kappa$ B as well as SFK signaling events; therefore it is highly possible that attenuation in these signaling events is result of TNFR1 activity disruption. Disruption of both lipid-raft and caveole domains resulted in attenuation of all TNFR associated downstream signaling events, whereas specific depletion of caveolae compartments (Cav1siRNA) only inhibited Src and NF $\kappa$ B signaling, leaving JNK pathway, important for cell growth and survival, intact

(Figure 40). The unaltered JNK signaling is suggestive of intact TNF $\alpha$  receptor activity upon membrane domain disruption.

Our findings here show that loss of caveolin1/caveolae attenuates cytokine-induced production of RNS and subsequent redox signaling pathways that activate the endothelium. These findings are consistent with the concept that depletion of caveolae, especially in the endothelium, guards against development of vascular disease (Fernandez-Hernando et al., 2010; Pavlides, Gutierrez-Pajares, Iturrieta, Lisanti, & Frank, 2014; Takayanagi et al., 2013). However, this notion does not appear to extend to the lung vasculature where marked pulmonary hypertension observed in Cav1<sup>-/-</sup> mice is associated with impaired PKG function rendered by tyrosine nitration of the enzyme (Zhao et al., 2009). In addition, lungs of Cav1<sup>-/-</sup> mice display enhanced endothelial permeability due to p190RhoGAP-A nitration resulting in impaired GAP activity, RhoA activation and disruption of adherens junctions (Siddiqui et al., 2011). It was suggested that increased nitration events in Cav1<sup>-/-</sup> lungs are due to enhanced eNOS activity and NO generation, which then cross-reacted with available superoxide. While the source of ROS was not identified in those studies, our group (Yang & Rizzo, 2007) and others (Milovanova et al., 2008; Oakley, Smith, et al., 2009; A. Y. Zhang et al., 2006) show a lack in the assembly of NADPH oxidase upon caveolae disruption and a decrease in superoxide production in response to inflammatory stimuli in aortic endothelial cells. Given the observed hypercellularity in lung tissue of Cav1 null mice (Drab et al., 2001; Jasmin et al., 2004), immune cell infiltrate may be contributing ROS necessary for peroxynitrite formation and account for protein tyrosine nitration events observed in this organ.

In conclusion, the present study revealed the importance of caveolae as essential spatial compartments in simultaneous generation of superoxide and peroxynitrite, resulting in PTN of proximally located Src family kinases in TNF $\alpha$  induced endothelial cell dysfunction. It also identifies the components of nitroxidative signaling (Src family kinases, NF $\kappa$ B) in response to localized activation of NADPH oxidase and eNOS (Figure 44). Our study demonstrates the possibility of inhibiting ROS/RNS induced pathological signaling, preventing nitration of SFKs via down-regulating of caveolae proteins, and opening the possibility to therapeutically modulate the adverse effects of inflammation in vascular diseases.



**Figure 44. Hypothesized model of caveolin-1/caveolae regulation in TNF $\alpha$ -induced proinflammatory nitrooxidative signaling.** Caveolae localized Nox2 and eNOS undergo simultaneous activation resulting in generation of peroxynitrite and localized protein tyrosine nitration. The structure of the caveolae facilitates organization of signaling by regulation SFKs. These kinases are targets of protein-tyrosine nitration, which attains gain-of-function in activation NF $\kappa$ B pathway induced nitrooxidative signaling. The activation of signaling mediators lead to overexpression of adhesion molecules, ICAM-1/VCAM-1 that induces endothelial dysfunction.

CHAPTER 4  
CAVEOLAE INTERNALIZATION IS AN ESSENTIAL MECHANISM FOR NADPH  
OXIDASE ENZYME ACTIVATION AND REDOX SIGNALING

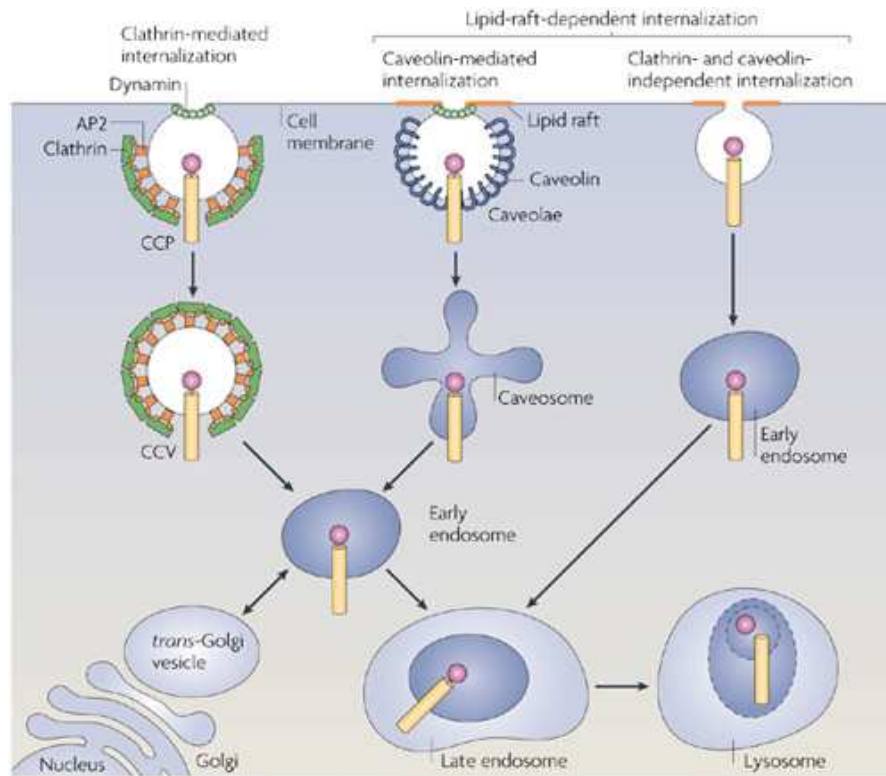
Introduction

Other than the ligand induced activation, variety of receptors and enzymes can also undergo internalization/endocytosis mediated activation. Although traditionally considered a deactivation mechanism for receptor tyrosine kinases, increasing evidence has identified an active signaling role of endosomes. Endosome-based signaling can more intricately modify the duration and magnitude of the response initiated by ligand binding at the cell surface. In addition, endosomal membranes are biochemically suited to act as specialized signaling platforms promoting selective recruitment of assorted scaffold and signaling proteins (Trejo, 2003; von Zastrow & Sorkin, 2007). It is also believed that compartmentalization of signaling molecules is required to provide the appropriate molecular proximity necessary for rapid, efficient, and specific activation of downstream signaling events (Okamoto, Schlegel, Scherer, & Lisanti, 1998; Quest, Leyton, & Parraga, 2004)

Caveolae, like clathrin coated pits (CCPs), are dynamic structures and are known to undergo internalization/endocytosis, however their internalization is independent of clathrin coat proteins (Figure 45). In endothelial cells, caveolae represent the major route of transcytosis-transporting albumin. The albumin docking protein gp60 localizes to caveolae in endothelial cells and thereby activates plasmalemmal vesicle formation and the directed migration of vesicles. Caveolae endocytosis is a cause as well as effect of specific signaling cascade in endothelial cells. For example, several endocytotic stimuli are known to activate signaling cascade of G-protein-coupled Src family kinases

(Minshall & Malik, 2006). The substrate for one of the Src family kinases, c-Src, is Cav1 at Y-14 and it can bind to the scaffolding domain of Cav1 resulting in further endocytosis.

The internalization of raft/caveolae domains and their role in endothelial functioning is of great pathophysiological significance. However, it is not known if activation of NADPH oxidase enzyme and compartmentalized ROS generation also involve caveolae internalization via Dynamin-2 mechanism. If true, these caveolae endosomes can act as spatial-temporal compartments for ROS generation in endothelial cells. Although discussed in previous chapter (chapter II) showing caveolae roles as signaling platforms for activating proximal signaling cascades, the mechanism of serially activating downstream inflammatory signaling mediators were not tested. Therefore, elucidating functional significance of caveole endocytosis will help greatly in understanding steps involved in the activation of endothelium and probable interventional therapies to correct it.



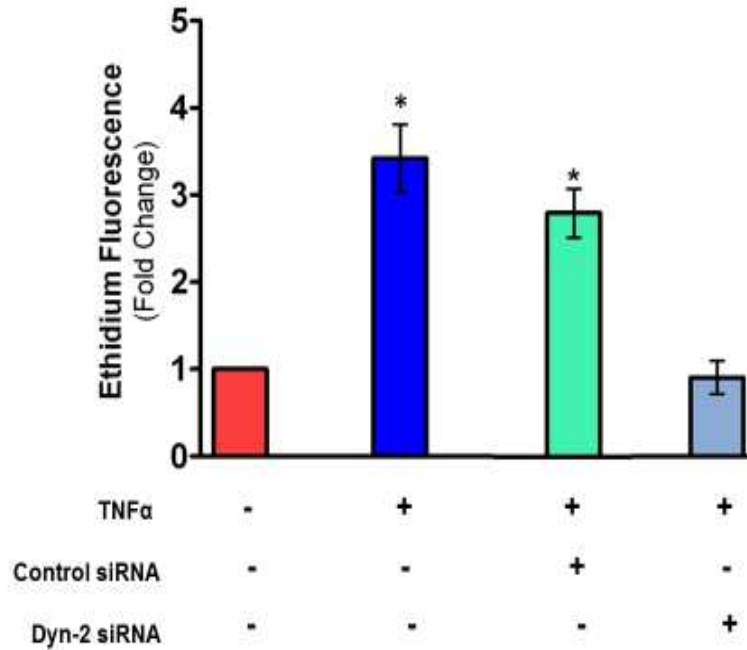
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**Figure 45. Caveolae undergo clathrin-independent internalization.** Different mechanisms of membrane internalization are known. These include, Clathrin-mediated, Clathrin and caveolae-independent, and caveolae and Dynamin-dependent (Clathrin-independent). Caveolae internalization or caveolae endocytosis involves fusion with other internalized caveolae vesicles to form, caveosomes. Internalization of enzyme eNOS via caveolae is well established and is an important event in activation of enzyme and NO production, whereas role of these internalization events in NADPH oxidase enzyme functioning are not clear (Schutze S et al., 2008).

## Results

### *TNF $\alpha$ -induced compartmentalized ROS generation involves endocytosis of Caveolae microdomains:*

Previously it has been shown that caveolae compartments undergo dynamin-2 dependent or clathrin-independent endocytosis. Given our previous evidence that Nox2 enzyme is enriched in caveolae compartments (Yang & Rizzo, 2007) and work from other groups showing internalization of Nox2 as an important event in enzyme activation, we tested if caveole compartmentalized ROS generation is dependent on dynamin-2-dependent endocytosis of caveolae domains. BAECs were pre-treated with either Dynamin-2 siRNA or Dynasore, prior to treatment with TNF $\alpha$  for 2 hours. Inhibition of internalization events showed significant attenuations in ROS generations compared to scrambled siRNA pretreated controls (Figure 46).



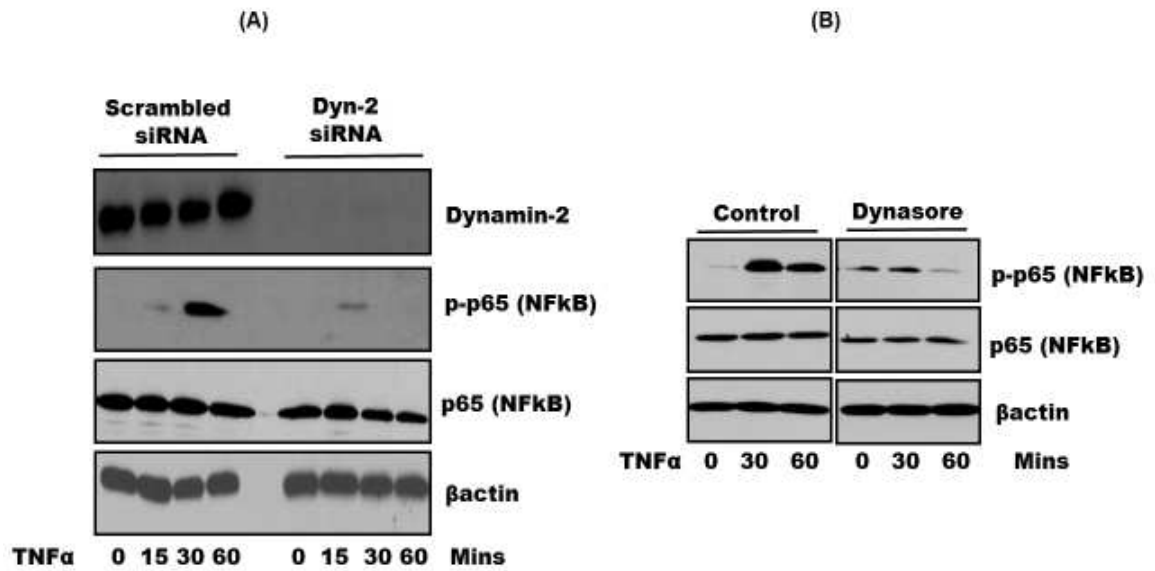
**Figure 46. Compartmentalized ROS generation is dependent on caveolae endocytosis.** BAECs were pretreated with Dynamin-2 siRNA, scrambled siRNA or (B) Dynasore and labeled with dihydroethidium. The cells were then stimulated with TNF $\alpha$  for additional 2 hours. The superoxide production was detected by ethidium fluorescence. TNF $\alpha$  treatments induced significant increases in ROS generation in 2-hour time points, whereas pretreatments with either Dynamin-2 siRNA or Dynasore, attenuated ROS generation. Fluorescence values were by equalized by sample protein concentration and presented as fold difference in Experimental/control \* $P < 0.05$

*Caveolae internalization via Dyamin-2 is essential for activation of ROS mediated NFkB signaling serving as redox generating endosomes (Redoxosomes):*

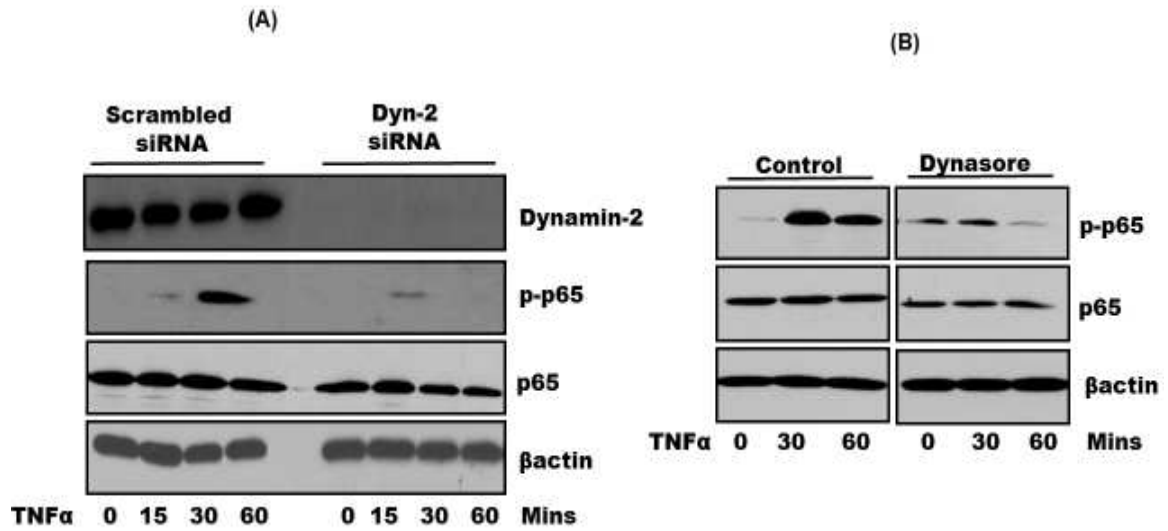
Previously we showed that inflammatory cytokine induced ROS generation is compartmentalized in caveolae and inhibition of either ROS generation (gp91ds-tat peptide) or disruption of raft/caveolae compartments (M $\beta$ CD or Cav1siRNA), resulted in attenuations in NFkB redox signaling. Based on these and our present observations showing caveolae internalization roles in ROS generation (Figure 46), Dynamin2 siRNA or pharmacological agent, Dynasore, blocking Dynamin-2 internalization, pretreatments prior to TNF $\alpha$  treatments were probed phosphorylation of p65. Cell lysates collected from either Dynamin-2 or Dynasore pretreatment showed abolishment of p65 (NFkB) phosphorylation at indicated TNF time points (Figure 47). These ROS mediated redox signaling endosomes are often times referred to as “redoxosomes” based on “redox signaling endosomes”.

*Caveolae redoxosome formation is important for endothelial adhesion molecule expression:*

Based on our previous observations showing caveolae internalization as a mechanism for compartmentalized ROS generation and associated redox signaling, we tested the functional consequences of ROS generating caveolae organelles in inducing adhesion molecule expression. Similar to previous findings, pretreatment of BAECs with either Dynamin-2 siRNA or Dynasore followed by TNF $\alpha$  showed significant decreases in expression of adhesion molecule, ICAM-1/VCAM-1 compared to either scrambled siRNA or non-Dynasore treated cells, respectively (Figure 48).



**Figure 47. Proinflammatory redox signaling (ROS) requires internalization of caveolae organelles.** (A) BAECs were pre-treated with Dynamin-2 siRNA, scrambled siRNA or (B) Dynasore and stimulated with TNF $\alpha$  for indicated time points. The cell lysates were collected and prepared for Western-blot analysis to detect phosphorylation of p65 subunit of NF $\kappa$ B, JNK and  $\beta$ -actin proteins. The blots were also probed for Dynamin-2 to measure expression levels upon siRNA knockdown compared to scrambled siRNA pretreatments. Representative blots from 3 different independent experiments are shown.



**Figure 48. Caveolae endocytosis as an essential mechanism for endothelial inflammatory phenotype.** (A) BAECs were pre-treated with Dynamin-2 siRNA, scrambled siRNA or (B) Dynasore and stimulated with TNF $\alpha$  for indicated time points. The cell lysates were collected and prepared for Western-blot analysis to detect expression of ICAM-1 and  $\beta$ -actin proteins. Representative blots from 3 different independent experiments are shown.

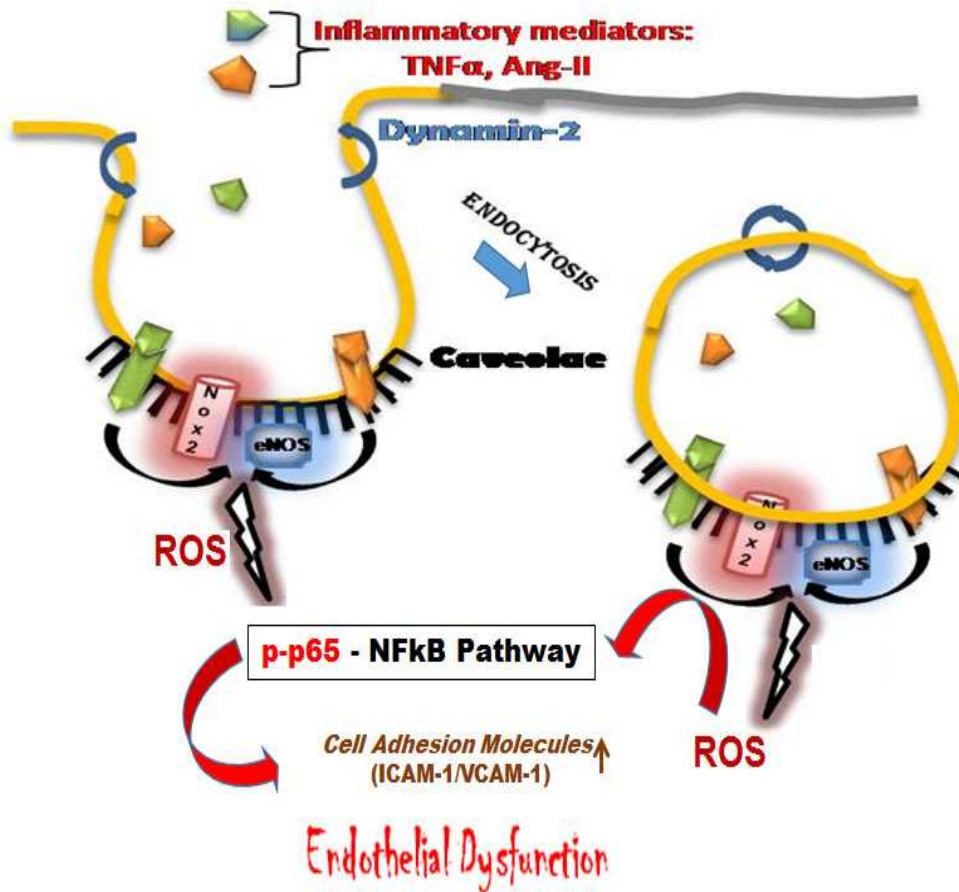
## Discussion

The ligand-induced internalization of enzyme eNOS has been demonstrated as an important event in its activation (Maniatis et al., 2006; Sanchez et al., 2008). Since the enzyme is localized in caveolae, blockade of endocytosis was used to study enzyme activation via phosphorylation (Ser1177, Thr495), NO production and PI3K/Akt nitrate signaling. Dominant negative Cav1 (CavY14F) and dynamin-2 (DynK44A) prevented enzyme NO production and eNOS phosphorylation via inhibition of PI3K/Akt activation, whereas prevention of superoxide generation and pathological consequences of these events in endothelial dysfunction (ICAM-1/VCAM-1) are not known. The only available evidence for dynamin-2 dependent internalization of lipid-raft/caveolae localized NADPH oxidase comes from studies in human pulmonary artery endothelial cells in response to hypoxia (Singleton et al., 2009). The study showed important roles of both dynamin-2 and Caveolin-1 in the Nox2-lipid-raft/caveolae in ROS production. The phosphorylation of p47 subunit of NADPH oxidase and dynamin-2 (tyrosine phosphorylation) via c-Abl, a tyrosine kinase, was found to be important event in hypoxia induced enzyme activation. These results are particularly important in pathophysiology associated with lung inflammation, ischemia/reperfusion, sepsis, hyperoxia, and ventilator associated lung injury. The exact ligand-receptor mediated mechanisms of Nox-Caveolae activation during inflammation (TNF $\alpha$ ) are still not clear. Consistent with eNOS and NADPH oxidase activation via Dynamin-2-dependent caveolae internalization, our data here in endothelial cells depleted of either Dynamin-2 (Dyn2 siRNA) or pretreatments with pharmacological blocker of Dynamin-2 GTPase activity, Dynasore, blocked ROS generation in response to inflammatory cytokine, TNF $\alpha$  (Figure 46). The functional consequences of Dynamin-2 mediated caveolae internalization was tested using

expression of adhesion molecules. Inhibition of endocytosis events using both methods blunted expression of ICAM-1 and VCAM-1 molecules, suggesting importance of these events in endothelial cell functioning (Figure 48). Similar to observations in endothelial cells, studies in vascular smooth muscle cells (VSMCs) under inflammatory conditions show dynamin-2-dependent activation of Nox1 and generation of ROS in early endosomes (Miller et al., 2010). The role of endosomal compartments in ROS generation was taken further by testing endocytosis roles in activating redox-signaling mediators. In MCF-7 cells, it was shown that endocytosis events activate TNF $\alpha$ /TNFR1 dependent TRAF2/TRADD and NADPH oxidase subunit recruitment in a dynamin-2 dependent manner to induce I $\kappa$ B/NF $\kappa$ B signaling (Q. Li, Spencer, Oakley, Buettner, & Engelhardt, 2009). The endosome structures producing ROS and regulating redox signaling are thus termed as “**redoxosomes**” from **redox** signaling end**osomes**. However, caveolae roles as redoxosomes in endothelial cells were not known. Based on the observations in endothelial cells showing caveolae endocytosis mechanism for ROS generation, and data from MCF-7 cell redoxosomes, we believed that ROS producing caveolae vesicles may also serve as redox signaling endosomes in endothelial cells. Indeed, blockage of dynamin-2 dependent endocytosis of caveolae (Dyn2 siRNA or Dynasore) attenuated phosphorylation of p65 subunit of NF $\kappa$ B redox signaling pathway (Figure 47). These studies here provide an additional mechanism of compartmentalized ROS generation in caveole vesicles to activate localized signaling cascades under inflammatory conditions in endothelial cells (Figure 49).

Similar to our results, other groups have also demonstrated the importance of Dyn2 in generation of ROS (Singleton et al., 2009) or role of caveolae endocytosis in IL-1 $\beta$  dependent redox signaling (Oakley, Abbott, et al., 2009), whereas the exact molecular

mechanism of Dyn2-dependent caveolae internalization in activating NADPH oxidase enzyme, are not clearly understood. Various explanations have been proposed to understand this mechanism; one of them involves Src-dependent tyrosine phosphorylation of p47<sup>phox</sup> (Chowdhury et al., 2005) and its recruitment to membrane Dyn2 as an important event in inducing enzyme activation and initiation of caveolae internalization. Since, c-Src also phosphorylates Dyn2 (DynY231/DynY597) (Shahjahan et al., 2004), we believed that p-Dyn2 and p-p47 interaction may be is a requirement for NADPH oxidase enzyme activation. Although we did not test this concept in our studies, the future directions for this aim will involve testing Dyn-2-phosphorylation roles in serving as an adaptor molecule in bringing p47<sup>phox</sup> subunit with membrane gp91<sup>phox</sup>/p22<sup>phox</sup> to facilitate enzyme activation upon caveolae internalization.



**Figure 49. Hypothesized model of caveolae endocytosis as an essential mechanism for activation of inflammatory redox signaling and endothelial functioning.** Inflammatory cytokine, TNF $\alpha$ -induced activation of Nox2 (ROS) requires Dyanmin-2-dependent internalization of caveolae organelles. The caveoale vesicles compartmentalize ROS generation, thus activating proximally localized NF $\kappa$ B signaling mediators (redox signaling), resulting in endothelial dysfunction upon overexpression of adhesion molecule, ICAM-1/VCAM-1.

CHAPTER 5  
INHIBITION OF CAVEOLAE COMPARTMENTALIZED ROS AS A THERAPEUTIC  
STRATEGY FOR LIMITING VASCULAR OXIDATIVE STRESS

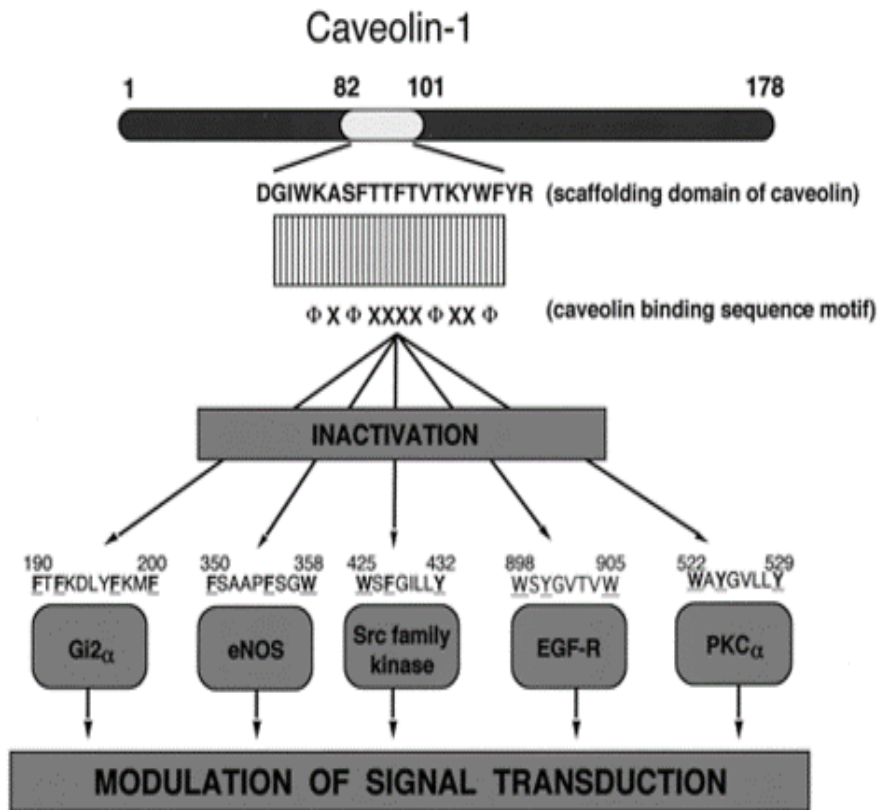
Introduction

Our observations described in chapter I and II show that caveolae compartmentalize ROS production, peroxynitrite (RNS) generation and localized protein-tyrosine nitration, first step in development of vascular disease, atherosclerosis. The importance of localized ROS and RNS generation includes activation of specialized nitroxidative signaling pathways central to endothelial activation and dysfunction. As discussed previously, various generalized antioxidant therapies and strategies have been employed in the past to block total ROS generation, but most seem to have either limited or no effect at all in reducing oxidative and nitrate stress induced progression of diseases. Based on our observations here, we developed a caveolin-1 targeting compound to specifically block Nox2 produced ROS localized in caveolae domains. In the past, few caveolin-1 targeting therapeutic peptides have been generated but mechanism of action involved blockage of caveolin-1 binding to enzyme eNOS, inducing sustained release of beneficial NO in endothelial cells. Although, these targeting peptides have mostly been beneficial, but their negative effects under high NO and cross-reaction with superoxide during inflammation to generate ONOO, cannot be ruled out. Herein we developed a caveole targeting peptide with NADPH oxidase enzyme inhibiting properties. The peptide can be of dual beneficial effects, firstly by blocking ROS production and secondly, it can also increase bioavailability of beneficial NO by preventing eNOS uncoupling, a pathological modification under inflammatory oxidative stress.

## Results

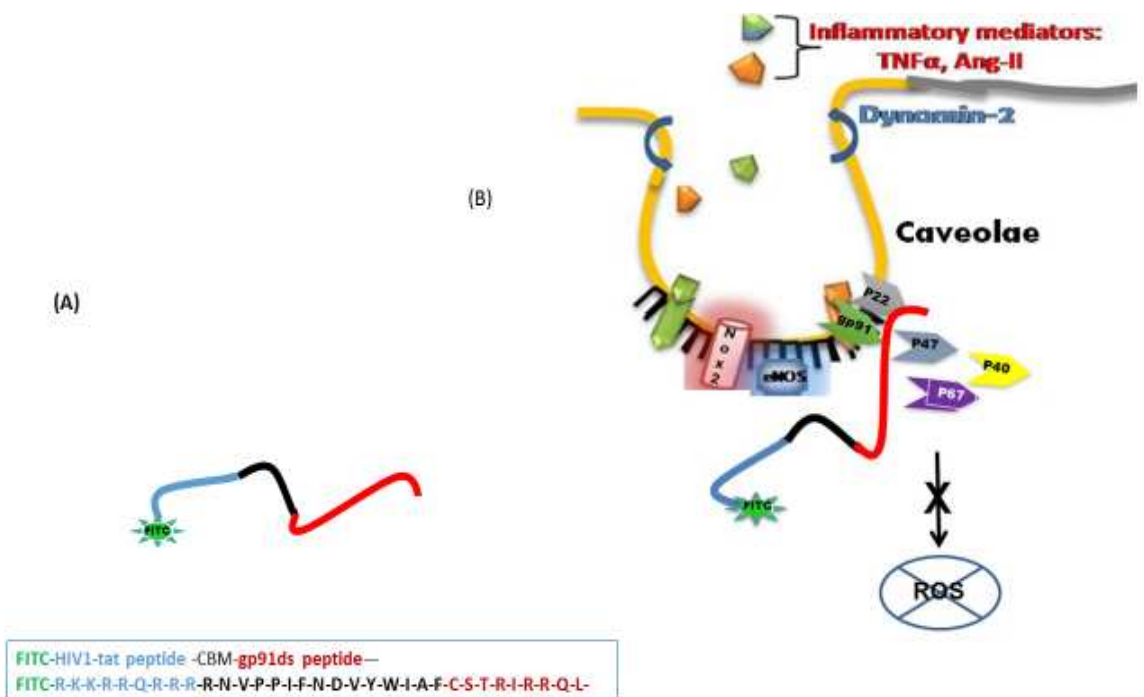
### *Designing Caveolin-1 Scaffolding Domain binding (CBD) peptide with NADPH oxidase inhibiting-properties: FITC-Tat-CBM-gp91ds*

Caveolae compartmentalized ROS generation inhibiting peptide was designed by exploiting receptor and enzyme binding properties of Caveolin-1 scaffolding domain (CSD). Aside from roles in caveolae formation, caveolins recruit, retain and regulate many caveolae-associated signaling molecules such as enzyme eNOS,  $G_{i2\alpha}$ , SFKs, Ha-Ras (S. Li et al., 1995; Lisanti et al., 1994; Lisanti et al., 1995; Lisanti, Tang, & Sargiacomo, 1993). Caveolin-protein interactions are commonly considered to occur between a ~20 amino acid regions within caveolin-1, the caveolin scaffolding domains (CSD), and an aromatic-rich caveolin binding motif (CBM) on the binding partner ( $\phi X\phi XXXX\phi$ ,  $\phi XXXX\phi XX\phi$  or  $\phi X\phi XXXX\phi XX\phi$ , where  $\phi$  is an aromatic and X an unspecified amino acid) (Figure 50).



**Figure 50. Caveolin-1 scaffolding domain and caveolin binding motif reciprocal interactions.** Sequence of the caveolin-1 scaffolding domain and caveolin binding sequence motifs ( $\Phi X \Phi XXXX \Phi XX \Phi$ ) with several caveolae-localized signaling molecules are shown. These include G-proteins subunits (Gi2 $\alpha$ ), eNOS, Src-family tyrosine kinases, receptor tyrosine kinases (EGF-R), and PKC isoforms. In most cases, the caveolin-1 interaction is inhibitory, leading to inactivation of the signaling molecules and modulation of downstream signal transduction (Adapted from Razani et al., 2002).

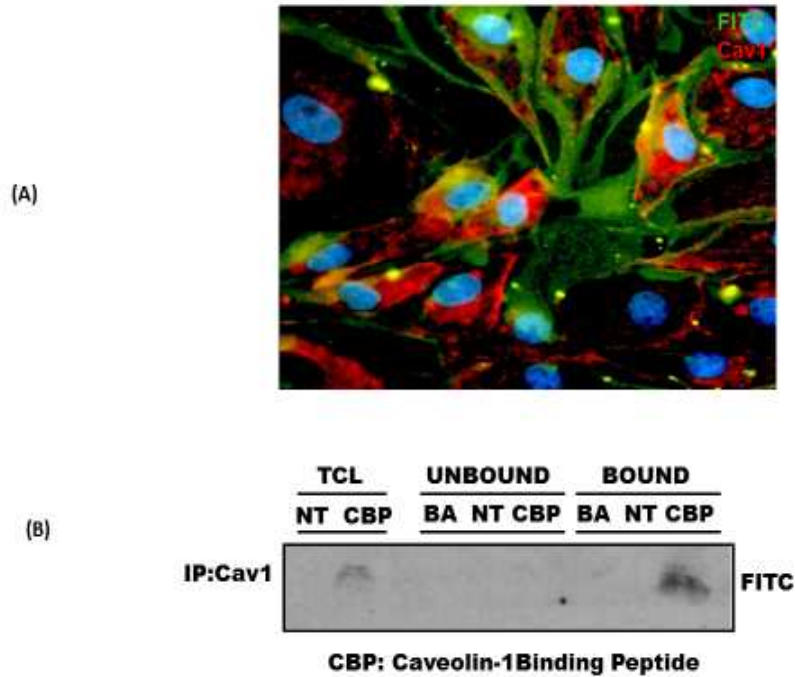
Based on these we generated a peptide containing CSD binding motif (CBM) with sequence, -R-N-V-P-P-I-F-N-D-V-Y-W-I-A-F-. The next step was to attach NADPH oxidase enzyme inhibiting peptide, gp91ds, used previously to block ROS generation in endothelial cells (gp91ds-tat peptide), to the C-terminal of CBM peptide. The peptide contains a sequence, -C-S-T-R-I-R-R-Q-L-, identical to that of p47 subunit of NADPH oxidase, preventing docking of p47 to the gp91 cytoplasmic face, therefore called as gp91ds (docking site) peptide. The next step was to attach a cell-permeable peptide sequence for intracellular delivery of the CBM-gp91ds peptide. This was accomplished by attaching RGD domain sequence, -R-K-K-R-R-Q-R-R-R-, of HIV coat protein, Tat, to the N-terminal of CBM-gp91ds peptide. The peptide was further tagged with a fluorescent probe, FITC for tracking intracellular delivery as well as targeting to the caveolae domain (CSD binding). The final peptide, **FITC-Tat - Caveolin binding motif (CBM) - gp91ds**, sequence was, **FITC-R-K-K-R-R-Q-R-R-R-N-V-P-P-I-F-N-D-V-Y-W-I-A-F-C-S-T-R-I-R-R-Q-L-** (Figure 51A). Similarly, a control peptide containing Tat-CBM-Scrambled-gp91ds sequence (**Scrambled CBM peptide**) was designed to account for cell penetration (Tat sequence) and caveolae targeting (CBM) peptide-induced changes in ROS generation. The peptide design was sent to Bio-synthesis, Inc, (Lewisville, Tx), for synthesis and 1M stock solutions were prepared in DDH<sub>2</sub>O based on nature of amino acids in the peptide. The expected mechanism of action of peptide at caveolae domains of endothelial cells is shown in illustration (Figure 51B).



**Figure 51. Designing NADPH oxidase inhibiting Caveolae binding domain motif (CBM) peptide**(A) Designing peptide involved attaching FITC fluorescent tag followed by HIV-Tat peptide (R-K-K-R-R-Q-R-R-R) and downstream to it we added, caveolae scaffolding domain binding sequence motif (R-N-V-P-P-I-F-N-D-V-W-I-A-F), gp91ds-peptide (-C-S-T-R-I-R-R-Q-L-). (B) Schematic shows possible binding of the peptide to CSD of caveolin-1 (Caveolae) and mechanism of action of NADPH oxidase inhibiting, gp91ds-peptide in inhibiting ROS generation.

*CBD peptide targets to caveolae compartments and was found associated with caveolin-1 protein:*

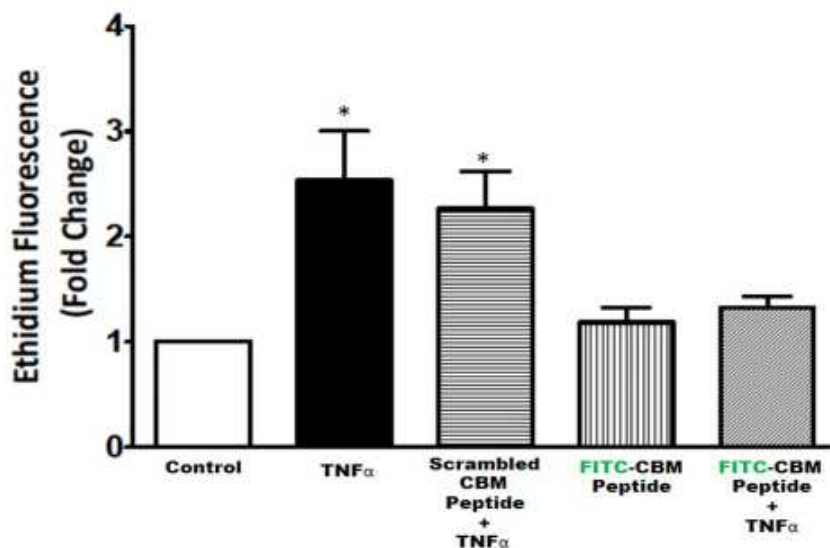
Localization of FITC-Tat-CBM-gp91ds peptide to caveole domains in endothelial cells was measured using 2 different methods. BAECs were pre-treated with 10uM of peptide and cells were processed for immunocytochemistry by observing co-localization of caveolin-1 signal (Alexa596-Red) with green, FITC. Peptide (1-FITC-Tat-CBM-gp91ds). The peptide treated cells showed membrane localization of both caveolin-1 and FITC signal (Figure 52A). Similarly, when cell lysates collected from BAECs pretreated with both peptide were immunoprecipitated using caveolin-1 antibody, bound fractions showed FITC in precipitates (Figure 52B).



**Figure 52. CBM peptide targets to the caveolae domain** (A) BAECs were cultured on glass coverslips, treated with CBM. Cells were then fixed with 4% paraformaldehyde, permeabilized with TritonX100, blocked with normal goat serum and incubated with anti-Cav1 antibody followed by goat-anti-rabbit-Alexafluor568 secondary antibody and DAPI. Fluorescence signal was measured using identical laser wavelength (578/603nm) and microscope capture settings. CBM peptide with tagged fluorophore, FITC co-localizes with red signal from membrane caveolin-1. (B) Total cell lysates collected from BAECs treated with CBM peptide were immunoprecipitated with anti-caveolin-1 antibody. Precipitates were probed with anti-FITC antibody by western blotting. Bound fraction showed CBM peptide targeting to caveolin-1 protein. The blots shown here are representative of three different independent experiments.

*CBD peptide pre-treatment of endothelial cells attenuates TNF $\alpha$  induced ROS generation:*

In order test inhibitory effects of the CBM peptide for preventing compartmentalized ROS production, we treated BAECs with peptides prior to TNF stimulations and ROS was measured using DHE assay. CBM peptide treatment (10uM for 45 mins each) followed by TNF $\alpha$  stimulations, showed marked decreases in ROS generation compared to Scrambled peptide (Figure 53).



**Figure 53. CSD binding peptide with gp91-ds inhibits TNF $\alpha$  stimulated superoxide generation in endothelial cells.** BAECs were pretreated with FITC-Tat-CBM-gp91ds peptide or scrambled peptide for 45 mins and then labeled with dihydroethidium. The cells were then stimulated with TNF $\alpha$  at 100U/ml for 2 hours, and superoxide production was detected by ethidium fluorescence. TNF $\alpha$  stimulation showed increases in superoxide generation at 2 hours, whereas cells pretreated with the FITC-Tat-CBM-gp91ds peptide abolished ROS generation compared to scrambled peptide. Fluorescence values were by sample protein concentration and presented as fold difference in Experimental/control \* $P < 0.05$ .

## Discussion

Endothelial NADPH oxidase, enriched in caveolae domains appears to be primary source of pathological ROS during disease conditions. Therefore, it was thought that inhibition of caveolae localized NADPH oxidase enzyme can prove to be important therapeutic strategy in preventing endothelial oxidative stress. Disruption of lipid-rafts/caveole or depletion of caveolin-1 does not appear to be a feasible method of blocking Nox2 enzyme activation due to the variety of beneficial roles of caveolae/caveolin-1 in endothelial as well as other cell-organ system normal functioning. Thus, development of specialized therapeutic strategy to block caveolae compartmentalized ROS generation, is of great importance.

Caveolae membrane domains are associated with hair-pin loop like caveolin-1 protein with a scaffolding domain (CSD), important for anchoring Cav1 protein to membrane as well as in acting as a site for interactions with caveolae regulated enzymes and proteins such as L-type calcium channels, Nox2, eNOS and tyrosine kinases. All proteins interacting with CSD were found to be composed of a specific sequence pattern of aromatic and non-aromatic residues in full length ( $\phi X\phi XXXX\phi$ ,  $\phi XXXX\phi XX\phi$  or  $\phi X\phi XXXX\phi XX\phi$ , where  $\phi$  is an aromatic and X an unspecified amino acid) (Couet et al., 1997). In the past various groups have synthesized caveolae-targeting peptides with ability to inhibit/activate localized enzymes and proteins. One of the first caveolae targeting peptide was developed by Bernatchez P et al., 2005, where caveolin-1 scaffolding domain interacting peptide was composed of sequence, **W-K-A-S-F-A-A-AT-V-TK-W-Y-FY-R** (residues in **red** are aromatic amino acids), with a cell permeable, Antennapedia sequence (P. N. Bernatchez et al., 2005). The full-length sequence of peptide is termed as **Cavtratin**. A slight modification of Cavtratin with amino acid substitutions is called **Cavnoxin**. The peptide functions by blocking CSD to prevent

binding of enzyme eNOS, rendering it constitutively active to produce beneficial NO. Cavnoxin when administered in Wt mice, showed increased eNOS-derived NO synthesis, vasodilation and reduced inflammation (P. Bernatchez, Sharma, Bauer, Marin, & Sessa, 2011; Bucci et al., 2000). Another study by Makarewich CW et al., 2012, tagged L-type calcium channel blocker, Rem peptide to CSD-binding peptide. The peptide works by targeting to caveole domains and blocks localized L-type Calcium ion channels in cardiomyocytes. The beneficial effects of peptide included inhibition of calcium ion induced hypertrophic signaling in cardiomyocytes without reducing cardiac motility. Based on these studies, we synthesized a caveolae targeting (CSD binding domain) peptide with a sequence identical to that of gp91ds, a blocker of NADPH oxidase enzyme. The cell membrane permeable HIV1-Tat sequence was attached to the C-terminal with a fluorophore, FITC, helpful in visualizing the peptide targeting to caveole domains (Figure 51). Similar to Rem-CSD binding peptide, the FITC-Tat-CBM-gp91ds peptide localized to caveole domains and showed protein-protein interaction with caveolin-1 (Figure 52). Interestingly, endothelial cells treated with peptide showed significant decreases in ROS generation when stimulated with TNF $\alpha$ . The results here strongly suggest the importance of caveolae domains in localized ROS generation (Figure 53).

In future we would like to test roles of this peptide in inhibiting localized nitration events, nitroxidative signaling pathways important for endothelial functioning. Similarly, clinical significance of peptide can be tested in animal models showing increased vascular oxidative/nitrative stress induced development of atherosclerosis.

## CHAPTER 6 SUMMARY AND CONCLUSIONS

Endothelial responsiveness to physiological ROS levels plays a critical role in vascular homeostasis. However, under inflammatory conditions, excess ROS and RNS, peroxynitrite, generation, results in initiation of vascular disease such as atherosclerosis. Describing the mechanism of ROS/RNS generation and its effects on signaling proteins and associated pathways is, therefore, of significant importance in understanding the etiology of vascular disease and ultimately developing effective treatment regimes in the future.

Previously, the role of endothelial NADPH oxidase enzyme isoforms (Nox1, Nox2, Nox4) has been shown in vascular oxidative stress, whereas contribution of individual enzyme isoform in disease progression was lacking. Herein, based on our data from caveolae residing enzyme isoforms, Nox2, negligible expression levels of Nox1 and lack of cytoplasmic subunits for activation of Nox4, we show that gp91ds-tat peptide functions specifically to block Nox2 activation by blocking the docking site of p47 subunit on gp91 membrane subunit. Although, role of other sources such as mitochondrial respiration, Xanthine oxidase and uncoupled eNOS in endothelial ROS cannot be ruled out, our data here discusses caveolae localized Nox2 as one of the major source involved in endothelial oxidative stress.

Mechanistic studies from our group and others have shown localization of Nox2 and eNOS in lipid-raft domains and functional significance of these spatial compartments in producing ROS and peroxynitrite induced tyrosine nitration of proteins. The data from the present study shows specific roles of caveole domain in ROS production but also their importance in serving as spatio-temporal compartments for dual activation of Nox2,

eNOS to induce RNS generation and localized protein-tyrosine nitration. ROS and RNS-induced intracellular effects involve activation of variety of signaling cascades, whereas directionality component to reactive radical induced nitroxidative signaling pathways was least understood. Therefore, elucidating ROS/RNS induced downstream signaling cascade will be of great importance in not only understanding mechanism of activation of enzymes, signaling pathways but also to develop specific interventional strategies to prevent oxidative and nitrative stress. The studies here showed activation of inflammatory NF $\kappa$ B as well as Src-family kinases in response to increased ROS and RNS levels. Based on the short half-life of both ROS/RNS, mediators of both the signaling cascades were believed to be proximally located to caveolae domain and findings that RNS generation involves total protein-tyrosine nitration, signaling protein targets of nitration reaction were probed. Our data here showed nitration of SFKs but not NF $\kappa$ B signaling components. Furthermore, spatio-temporal regulation of caveolae compartments was tested upon depletion of caveolin-1/caveolae, which showed attenuations in SFK tyrosine nitration events. Similar to SFKs, we observed increases in caveolin-1 nitration in response to inflammatory cytokine, but were not pursued further. SFKs perform diversity of functions inside cells in phosphorylating variety of signaling proteins and enzymes. Previously, upstream roles of SFKs in phosphorylating IKK $\beta$ , responsible for phosphorylating I $\kappa$ B $\alpha$  and NF $\kappa$ B, were shown, whereas positive or negative effects of SFK nitration in regulating downstream NF $\kappa$ B pathways was unknown. Our studies here by blocking SFK activation and lack of p65 subunit (NF $\kappa$ B) phosphorylation, strongly suggest gain-of-function roles of SFK tyrosine nitration. Although not tested here, the future studies will include elucidation of exact tyrosine residues in SFK undergoing nitration. Site directed mutagenesis by substituting tyrosine residues undergoing phosphorylation with phenylalanine amino acid will reveal exact

sites and their roles in phosphorylation/dephosphorylation events. Furthermore, functional studies need to be conducted to bolster SFK pharmacological inhibition results by specific kinase assays using both native as well as mutated (Y→F) SFKs.

The consequences of increased oxidative stress involve endothelial activation and dysfunction by overexpression of adhesion molecules, ICAM-1/VCAM-1, whereas roles of RNS induced protein tyrosine nitration and caveolae compartments in driving expression of adhesion molecules, is not known. The *in vitro* as well as *in vivo* studies here provide evidence for this. Treatments with TNF $\alpha$  showed increased expression of both ICAM-1 and VCAM-1 in both primary bovine endothelial cells as well as endothelium of aortas derived from Wt mice. These increases correspond to higher levels of ROS as well as PTN in both cells and aortic tissue, suggesting that endothelial adhesion molecule expression is dependent on both ROS and RNS. Similar to these findings, lipid-raft and caveolae compartments also showed regulation of adhesion molecule expression. Caveole compartmentalize ROS/RNS generation, specific nitroxidative signaling events, which translated into ICAM-1/VCAM-1 expression in both *in vitro* studies in primary endothelial cells as well as in Cav1KO mice under inflammatory conditions.

We have additionally provided mechanistic insights in to role of dynamin-2 dependent caveolae internalization in regulating ROS generation. Based on previous evidence showing lipid-raft endocytosis as an important mechanism for NADPH oxidase activation, we pursued this concept further by testing caveolae roles in Nox2 mediated ROS generation and downstream redox signaling events. The findings indicated caveolae endocytosis as an essential step in ROS generation and activation of downstream redox signaling events. Correspondingly, blockage of caveolae endocytosis also showed lack of ICAM-1/VCAM-1. These observations show additional mechanism

involved in caveolae mediated ROS formation, which can be probed further for RNS generation and localized protein-tyrosine nitration events. Based on these findings, it is possible that these caveole vesicles serve as intracellular compartments for localized nitration and activation of other signaling molecules, proteins, and enzymes involved in other cardiovascular diseases.

In the final set of studies presented, we tested applications of our findings so far to see if intervention at any of the mechanistic steps can be developed as a therapeutic strategy in limiting vascular oxidative and nitrate stress. Knowing practical limitations of depleting membrane cholesterol or altering expression levels of caveolin-1 protein as a drug therapy in patients, we focused on abolishing caveolae localized ROS production. Unlike generalized anti-oxidant therapies or enzyme inhibitors, development of synthetic cell permeable peptide containing CSD binding motif with Nox2 inhibitor, gp91ds, proved very successful in blunting endothelial ROS. This kind of specific inhibition of caveole Nox2 has been shown for the first time here. Our findings therefore are novel as well as more holistic by abolishing harmful oxygen radicals instead of increasing bioavailability of beneficial NO, as shown previously.

Future efforts will serve to fill in many of the questions that arise from presented work. These include, an in-depth analysis of SFK protein structure to determine nitrated tyrosine residues and conducting substitution mutations to assess importance of identified tyrosine residue in ROS/RNS induced endothelial dysfunction. Furthermore, studies are needed to further probe roles of caveolin-1 nitration in regulating caveolin-1 phosphorylation, caveolae endocytosis and its importance in signaling mechanisms. Presently, this concept is under investigation in our research group. Additionally, we would like to explore further the mechanism of dynamin-2 and caveolae endocytosis in activation of Nox2 subunits. Questions to address include determining phosphorylated

dynamin-2 as an adaptor molecule for caveolin-1 and p47 subunit of NADPH oxidase regulating compartmentalized endosome ROS/RNS generation and activation of associated signaling events. Lastly, we would like to explore *in vivo* beneficial roles of CBM peptide in mouse models of inflammation, atherosclerosis and abdominal aortic aneurysms; all involving increased oxidative and nitrative stress as common intermediate event. This can be addressed by measuring ROS, PTN, ICAM-1/VCAM-1 levels in Wt animals exposed to stimuli such as TNF $\alpha$  or Ang-II by comparing these events to animals injected with CBM peptide. All these future studies discussed here will greatly increase our understanding of process of vascular diseases such as atherosclerosis and coming up with other strategies to prevent it.

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