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Redox-dependent calpain signaling in airway and pulmonary vascular and remodeling in COPD

Laszlo Kovacs¹, Yunchao Su^{1,2,3,4}

¹Department of Pharmacology & Toxicology, Medical College of Georgia, Augusta University, Augusta, GA 30912;

²Department of Medicine, Medical College of Georgia, Augusta University, Augusta, GA 30912;

³Vascular Biology Center, Medical College of Georgia, Augusta University, Augusta, GA 30912;

⁴Research Service, Charlie Norwood Veterans Affairs Medical Center, Augusta, Georgia 30912

Abstract

The calcium-dependent cytosolic, neutral, thiol endopeptidases, calpains, perform limited cleavage of their substrates thereby irreversibly changing their functions. Calpain have been shown to be involved in several physiological processes such as cell motility, proliferation, cell cycle, signal transduction and apoptosis. Overactivation of calpain or mutations in the calpain genes contribute to a number of pathological conditions including neurodegenerative disorders, rheumatoid arthritis, cancer and lung diseases. High concentrations of reactive oxygen and nitrogen species (RONS) originated from cigarette smoke or released by numerous cell types such as activated inflammatory cells and other respiratory cells cause oxidative and nitrosative stress contributing to the pathogenesis of COPD. RONS and calpain play important roles in the development of airway and pulmonary vascular remodeling in COPD. Published data show that increased RONS production is associated with increased calpain activation and/or elevated calpain protein level leading to epithelial or endothelial barrier dysfunction, neovascularization, lung inflammation, increased smooth muscle cell proliferation and deposition of extracellular matrix protein. Further investigation of the redox-dependent calpain signaling may provide future targets for the prevention and treatment of COPD.

Keywords

reactive oxygen and nitrogen species; calpain; remodeling; cigarette smoke; COPD

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive and life-threatening lung disease affecting more than 20 million individuals in the United States and predicted to become the third leading cause of death worldwide by 2030. COPD is characterized by irreversible airway obstruction mainly attributable to the pulmonary vascular and airway

remodeling which is the most critical process in the pathogenesis of COPD. The primary cause of COPD is inhaling air pollutants including cigarette smoke (CS) and secondhand smoke. Large amount of reactive oxygen and nitrogen species (RONS) from CS or released from lung cells can contribute to the development of airway and pulmonary vascular remodeling. Calpain is a conserved family of calcium-regulated, non-lysosomal, neutral cysteine endopeptidases which is involved in several physiological and pathological processes. Growing body of evidence indicates that CS and its constituent are able to regulate directly or indirectly the protein levels and activities of calpain indicating its implication in the pathological processes in COPD. This chapter focuses on the RONS production systems in the airway and pulmonary vasculature and the potential effect of RONS on calpain signaling in the pathogenesis of COPD.

2. Calpain signaling

Calpain is a family of neutral, calcium-dependent, non-lysosomal cysteine proteases which exist in a wide range of organisms such as mammals, invertebrates, plants, fungi, yeasts and bacteria [1]. They act as regulatory enzymes and perform precise and limited cleavage of substrate proteins irreversibly changing their functions [2]. The human genome contains 15 genes that encode the catalytic subunits of calpain, and two genes encoding the regulatory subunits [3]. Some of the calpains show ubiquitous expression (calpain-1, 2, 5, 7, 10, 13, 14, 15 and 16), while others are restricted to specific tissues (calpain 3, 6, 8, 9, 11 and 12) [4]. Classical or typical calpains contain penta-EF-hand motif in the calcium-binding domain (IV) in addition to the N-terminal domain (I), protease domain (II) and C2-like domain (III) in the catalytic subunit. Non-classical or atypical calpains include the domain I and II, but not all comprise the penta-EF-hand domain or C2-like domain and some of them have other types of domains [5]. Calpain-1 and calpain-2 are the two major typical and well-characterized members of the calpain family which differ in their sensitivity to calcium *in vitro*. Calpain-1 (μ -calpain) requires micromolar level of Ca^{2+} , while calpain-2 (m-calpain) requires millimolar Ca^{2+} concentration for half-maximal activation *in vitro* [6]. These ubiquitously expressed isoforms are heterodimers composed of a different larger catalytic subunit (80 kDa) including the N-terminal anchoring domain (DI), the catalytic domain (DII), the Ca^{2+} - and phospholipid binding domain (DIII), and the penta-EF-hand domain (DIV) as well as the smaller regulatory subunit (30 kDa) comprising of the N-terminal hydrophobic domain (DV) and the penta-EF-hand domain (DVI) [7]. Calpain is abundant in the cytoplasm therefore tight regulation of the protease is essential for normal cell function. The activity of calpain is modified by several mechanisms: (a) limited autolysis of the N-terminus of both subunits [8]; (b) binding to specific membrane phospholipids [9]; (c) posttranslational modifications such as phosphorylation of the calpain-2 at Ser50 by extracellular signal-regulated kinases 1/2 (ERK 1/2) which increases calcium sensitivity and the catalytic activity [10] or phosphorylation of the Ser369/Thr370 residues of calpain-2 by protein kinase A (PKA) inhibiting the calpain activity [11]; (d) interaction with the endogenous inhibitor calpastatin [12]; and (e) binding to activator proteins [13]. Calpain activity has been shown to be critical for wide range of basic cellular processes including cell motility [14], cell proliferation [15], cell cycle [16], signal transduction [17] and apoptosis [18]. Several pathological conditions are attributed to the over-activation of

calpain including Alzheimer's disease, Parkinson's disease, multiple sclerosis, stroke, traumatic brain injury, cataract formation, muscular dystrophies, myocardial infarcts, ischemia, rheumatoid arthritis, cancer and pulmonary hypertension. Genetic alterations in the calpain genes have been also shown to be associated with human diseases. For example, defects in calpain-3 inactivation cause limb girdle muscular dystrophy type 2A; downregulation of calpain-9 is linked to human gastric cancer; and mutations of the calpain-10 gene contribute to the development of type II or non-insulin-dependent diabetes mellitus [19–21].

3. RONS in the airway and pulmonary vasculature

RONS can be produced by many types of cells including inflammatory, endothelial, adventitial and smooth muscle cells in the airway and lung vasculature. Under normal conditions, generation of these species are well controlled by enzymatic and non-enzymatic reactions. RONS at physiological level contribute to a number of physiological processes such as cell proliferation, differentiation, migration, apoptosis, cytoskeletal movement and vascular contraction and relaxation [22, 23]. However, overproduction of RONS due to disturbed balance between oxidants and antioxidants leads to oxidative and nitrosative stress causing pathological responses [24–28].

3.1 Production of reactive oxygen species (ROS)

ROS are relatively unstable small molecules formed as by-products in reactions involving oxygen. They consist of free oxygen radicals and non-radicals. Free radicals contain one or more unpaired electrons in atomic or molecular orbitals such as superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), peroxy ($RO_2^{\cdot}$), lipid peroxy ($LOO^{\cdot}$) and alkoxy ($RO^{\cdot}$). Non-radicals are created from free radicals by sharing their unpaired electrons such as hydrogen peroxide (H_2O_2), hypochlorous acid ($HOCl$), ozone (O_3), singlet oxygen (1O_2) and lipid peroxide ($LOOH$) [29]. At low to moderate concentrations, ROS have beneficial effects and play important role in normal airway and vascular physiology as well as maintain vascular integrity. However, at high concentrations they can damage lipids, proteins, carbohydrates and nucleic acids contributing to the pathogenesis of lung diseases [30–32]. ROS can also act as a second messenger in the signal transduction pathways [33, 34]. There are three major ROS including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) in the airway and pulmonary vasculature. The initial reaction in the ROS production is usually one electron transfer to molecular oxygen to create superoxide anion ($O_2^{\cdot-}$). Superoxide is water soluble, highly unstable and has a short half-life. Superoxide can be dismutated into more stable and lipid soluble hydrogen peroxide (H_2O_2) via a spontaneous reaction or catalyzed by superoxide dismutase (SOD). Superoxide can react with nitric oxide to form extremely reactive peroxynitrite ($ONOO^-$). Superoxide can also react with hydrogen peroxide in iron ion catalyzed process via Haber-Weiss reaction to generate hydroxyl radicals ($OH^{\cdot}$) [35]. Biological sources of ROS include the mitochondrial electron transport chain and several enzymatic system such as NADPH oxidases, xanthine oxidoreductase (XOR), and nitric oxide synthase (NOS).

3.2 ROS production from mitochondria

Mitochondria represent a major source for ROS production in the cardiovascular system. During oxidative phosphorylation, the mitochondrial respiratory chain governs the electron transfer to the molecular oxygen which is reduced to water at the terminal cytochrome oxidase (Complex IV). Superoxide can also be generated as a by-product at the proximal sites. Under physiological conditions, approximately 1–3% of the electrons can leak from the electron transport chain (ETC) yielding ROS. The production of superoxide occurs at complex I, II, and III. The complexes I and III (NADH:ubiquinone oxidoreductase and coenzyme Q: cytochrome c oxidoreductase, respectively) are considered as the major sites of ROS production in the mitochondria [36–38]. Superoxide is converted into more stable hydrogen peroxide by manganese superoxide dismutase (Mn-SOD) and then further converted to water by catalase or thiol peroxidases (e.g. glutathione and thioredoxin peroxidases) in the mitochondrial matrix. Superoxide can also be dismutated by copper/zinc superoxide dismutase (Cu,Zn-SOD) or scavenged by cytochrome c in the mitochondrial intermembrane space, or released from the mitochondria into cytosol through voltage-dependent anion channels (VDAC) [39, 40]. Mitochondria is also a source of reactive nitrogen species such as nitric oxide (NO $\cdot$) which is produced by nitric oxide synthases (NOS) during the conversion of L-arginine to citrulline. Nitric oxide can block the respiration by binding to the heme groups of cytochromes contributing to elevated superoxide formation. Superoxide and nitric oxide can form peroxynitrite. These RONS cause oxidative and nitrosative damage of numerous mitochondrial structures such as proteins, DNA and lipids leading to the development of pathological processes [41–43]. In patients with COPD excessive mitochondrial RONS levels are also associated to mitochondrial dysfunction contributing to the pathophysiology of COPD [44]. Mitochondria also produce hydroxyl radicals (OH $\cdot$) which are one of the strongest oxidants and can easily interact with unsaturated lipids causing lipid peroxidation. Lipid peroxidation generates hydroperoxides (LOOH) as primary products and reactive carbonyl species, e.g., malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE) as end-products of the reaction. They induce irreversible modification of cellular components causing lipoxidative damage to mitochondrial protein, phospholipids and DNA which is associated with a variety of pathophysiological states [45–47].

3.3 ROS from NADPH oxidases (NOX)

The NADPH oxidases are membrane-associated proteins that catalyze the superoxide production via electron transfer from NADPH to molecular oxygen [48]. The NADPH oxidase family has seven members: five mammalian NOX isoforms such as Nox1, Nox2 (gp91phox), Nox3, Nox4 and Nox5 and two related enzymes dual oxidase 1 and 2 (DUOX1 and 2). The family members have a common core structure consisting of six transmembrane domains at the N-terminal part including two hemes, and a relatively long C-terminal cytosolic part containing FAD and NADPH-binding domains [49, 50]. Nox5 and Duox 1/2 have additional domains at their N-terminal region. For example, Nox5 contains four Ca²⁺-binding EF-hand domains; and DUOX enzymes have two Ca²⁺-binding EF-hand domains, an additional membrane-spanning domain, and a peroxidase-like domain. Due to the EF-hand domains these oxidases are regulated by Ca²⁺ signaling [51]. The major NADPH oxidase isoforms are DUOX1 and 2 in the lower and upper airways and Nox1, Nox2, Nox4,

and Nox5 in human vascular cells. The tissue expression, subcellular localization, regulatory mechanisms, activation and physiological function of these enzymes are fairly different [52–55]. Nox2, also known as the phagocyte NADPH oxidase, is the prototype Nox. The enzyme complex consists of the plasma membrane spanning flavocytochrome b558 heterodimer that is composed of the gp91phox and p22phox proteins, and cytosolic protein subunits such as p67phox, p47phox, p40phox, and the small GTP-binding protein Rac. During stimulation, p47phox becomes phosphorylated and translocates to the membrane-bound cytochrome b558 with the activator subunit p67phox and p40phox. Rac also translocates to the membrane independently of p47phox and p67phox. Association of the cytosolic subunits with the transmembrane heterodimer leads to the activation of the enzyme. The assembled and activated NADPH oxidase catalyzes the electron transfer from NADPH via FAD and two hemes to molecular oxygen resulting in the formation of $O_2^{\cdot-}$ [56–58]. Nox1 requires two cytosolic subunits such as the p47phox homolog, NADPH oxidase organizer subunit 1 (NOXO1) and the p67phox homolog, NADPH oxidase activator subunit 1 (NOXA1) for its enzyme activity [59, 60]. In contrast, Nox4 is different from Nox1 and Nox2, and seems to be constitutively active enzyme, and its activation does not depend on the cytosolic subunits p47phox and p67phox or their homologues. ROS production of Nox4 requires association with membrane subunit p22phox and primarily generate H_2O_2 rather than superoxide [61–63]. Nox5 is directly activated by calcium binding to the EF-hand motifs at the N-terminus and by subsequent conformational changes. The enzymatic activity of Nox5 does not require p22phox or the cytosolic subunits. Binding of calcium-bound calmodulin or phosphorylation increase sensitivity to calcium [64–66]. DOUX enzymes contain two EF-hand motifs between the NADPH oxidase and peroxidase homology domains indicating the role of calcium in the regulation of the enzyme activities. Interestingly, the NADPH oxidase domain generated superoxide is quickly converted to H_2O_2 by the peroxidase-like domain, therefore the H_2O_2 production theoretically occurs without formation of detectable amounts of superoxide [67–69]. The NADPH oxidases play a critical role in the physiological responses of the airway and vascular cells such as host defense, mucin expression, maintenance of barrier function as well as cell growth, proliferation, migration, differentiation and apoptosis. They are also implicated in several airway and vascular pathologies including pulmonary diseases such as COPD, pulmonary hypertension, asthma as well as atherosclerosis, inflammation, hypertension, restenosis, diabetes and metabolic syndrome [48, 54, 55, 70].

3.4 ROS from xanthine oxidoreductase (XOR)

Xanthine oxidoreductase is a molybdenum-containing flavoprotein and a potential source of ROS in the airway and pulmonary vasculature. XOR is a homodimer with a molecular mass of approximately 300 kDa, and each subunit of the enzyme comprises of four redox centers: two non-identical [2Fe–2S] centers at the N-terminus, a central flavin adenine dinucleotide (FAD) cofactor and one molybdopterin cofactor (Mo-pt) at the C-terminus [71]. XOR has two isoforms: xanthine oxidase (XO) and xanthine dehydrogenase (XDH) which are encoded by the same gene. The enzyme primarily exists as a dehydrogenase which can be converted into an oxidase by reversible oxidation of cysteine residues or by irreversible proteolysis. XOR governs the final two steps in purine degradation. It also catalyzes the oxidation of hypoxanthine and xanthine to uric acid and transfers electrons from xanthine to NAD^+ and molecular oxygen to produce NADH, hydrogen peroxide and superoxide. XDH

mostly contributes to the generation of NADH, whereas XO is more likely responsible for the production of ROS. For this reason, the absolute amount of XO and the ratio of XO to XDH is very important to determine the ROS generation in the vasculature [71–76]. XOR can also act as a nitrate/nitrite reductase by catalyzing the reduction of nitrate to nitrite and nitrite to nitric oxide ($\text{NO}\cdot$) via electron transfer supplied by either xanthine or NADH under hypoxic and inflammatory conditions [77–81]. Superoxide can rapidly interact with nitric oxide to form the powerful and cytotoxic non-radical oxidant species peroxynitrite (ONOO^-) [82, 83]. XOR is mainly expressed in the endothelium, and its expression and activity are significantly increased in various pathological conditions such as ischemia/reperfusion injury, endothelial dysfunction, metabolic syndrome, airway inflammation and vascular remodeling in PAH and COPD [84–91].

3.5 Production of reactive nitrogen species (RNS)

RNS are nitrogen-centered molecules including free radical nitric oxide ($\text{NO}\cdot$) and nitric dioxide ($\text{NO}_2\cdot$), as well as several non-radicals, such as peroxynitrite (ONOO^-), its protonated form peroxynitrous acid (ONOOH) and alkylperoxynitrite (ROONO). Nitric oxide is a small, uncharged molecule which has a short half-life and contains one unpaired electron. Production of nitric oxide is catalyzed by the nitric oxide synthases (NOSs) which convert the L-arginine to L-citrulline and nitric oxide [92, 93]. It serves as a second messenger in various physiological processes including vasodilation, neurotransmission, apoptosis and immune response [94]. Nitric oxide is relatively unreactive, but it has the ability to react with other radical species to generate more reactive derivatives. For example, diffusion-limited reaction between nitric oxide and superoxide radical produces the toxic peroxynitrite [95]. Peroxynitrite is a strong oxidant which can react with a wide range of biological substrates causing oxidation of DNA, lipids, and proteins and nitration of tyrosine residues of the proteins [96]. It can also react with CO_2 leading to the formation of a reactive intermediate nitrosoperoxocarbonate (ONOOCO_2^-), the decomposition of which provides additional two radicals, nitrogen dioxide and carbonate radical anion ($\text{CO}_3\cdot^-$). The protonation of peroxynitrite generates peroxynitrous acid (ONOOH) that homolyzes dioxide and hydroxyl radical to yield nitrogen. It can also undergo nitration on free or protein bound tyrosine to form 3-nitrotyrosine which is a detectable *in vivo* marker for the RNS formation. Nitric oxide can rapidly react with O_2 to form the very reactive oxidant nitric dioxide ($\text{NO}_2\cdot$). Peroxidases can also produce nitric dioxide as an intermediate product by catalyzing the nitrite oxidation which is also able to induce nitration on protein tyrosine residues [96–99]. Overproduction of RNS, especially high levels of peroxynitrite and its derivatives contribute to nitrosative stress which is associated with several pathological conditions including cancer, neurodegenerative disorders, chronic inflammatory diseases, circulatory shock, diabetes, vascular diseases and chronic diseases of the airways such as asthma and COPD [100–103].

3.6 Nitric oxide synthase (NOS)

Nitric oxide synthases are a family of enzymes which catalyze the formation of nitric oxide ($\text{NO}\cdot$) and L-citrulline from L-arginine via five electron oxidative reactions. Cofactors such as flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), iron protoporphyrin IX (heme) and (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) are essential for the proper enzyme

function. There are three different isoforms of the family: neuronal NO synthase (nNOS or NOS1), inducible NO synthase (iNOS or NOS2) and endothelial NO synthase (eNOS or NOS3). All NOS isoforms are expressed in the airways and vasculature. nNOS and eNOS are calcium-dependent and are constitutively expressed in neurons and endothelial cells, respectively. iNOS is independent of calcium concentration and is inducible by endotoxin or inflammatory cytokines in various cell types [104–106]. Active NOS is a homodimeric enzyme complex in which each monomer consists of an oxygenase domain in the N-terminus and a reductase domain in the C-terminus that are connected via a regulatory calmodulin-binding domain. The homodimer is structurally stabilized by the zinc tetrathiolate (ZnS_4) cluster which is formed by a zinc ion and two cysteines from each monomer. The oxygenase domain contains binding sites for heme, cofactor BH_4 and substrate L-arginine as well as forms the active site of the enzyme. The reductase domain binds the two flavinic cofactors FAD, FMN and cosubstrate NADPH [92, 107, 108]. During NO synthesis, electron transfer occurs from NADPH via FAD and FMN to the heme which catalyses the substrate reaction. This electron flow is triggered by calcium-induced calmodulin binding in case of nNOS and eNOS. In contrast, iNOS binds the calmodulin at low calcium level, therefore it does not depend on calcium [109]. At low concentrations, NO diffuses into adjacent cells and stimulates the soluble guanylate cyclase (sGC) which converts guanosine triphosphate (GTP) to second messenger cyclic guanosine monophosphate (cGMP). The increased level of cGMP can modify the activity of numerous target proteins such as protein kinases and ion channels. NO produced by eNOS plays a role in the regulation of vascular tone and platelet aggregation. NO generated by nNOS is a very important neurotransmitter in the central nervous system and contributes to the regulation of synaptic transmission. Higher concentrations of NO produced by iNOS in response to endotoxin or inflammatory stimuli has cytotoxic effect and it is involved in the host defense [110–112]. Under some circumstances, the absence or limited availability of the substrate L-arginine or the critical cofactor BH_4 , the enzymes become in a dysfunctional state termed NOS uncoupling. In these conditions, electrons transferring from the NADPH to the molecular oxygen to produce superoxide rather than to L-arginine to generate nitric oxide leading to pathogenic processes [95, 113–115].

3.7 Antioxidant Defenses

Antioxidant can be defined as “any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate” [116]. Under physiological conditions, RONS are generated as a normal product of aerobic metabolism and play an important role in a wide range of cellular processes. The levels of RONS are tightly controlled by the regulation of the RONS-generating enzymes and by antioxidant defenses. In pathophysiological circumstances, excessive production and accumulation of RONS and/or decreased ability of antioxidant defenses create an imbalance in the RONS homeostasis. Elevated RONS bioavailability causes irreversible changes of biological molecules including proteins, lipids and DNA, inhibiting their normal function as well as leading to oxidative and nitrosative stress [117–119]. These stress conditions contribute to the development of a number of diseases, including cancer, ischemia/perfusion, diabetes, neurological disorders, atherosclerosis, hypertension, asthma, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, COPD, as well as ageing

[31]. Therefore, the main purpose of the antioxidants is to counterbalance the production of RONS. They can be classified into two groups: enzymatic and non-enzymatic. The major enzymatic antioxidants in pulmonary vasculature include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). There are three forms of SOD: Cu/ZnSOD (SOD1) in the cytoplasm, MnSOD (SOD2) in the mitochondria, and the extracellular SOD in the extracellular space (EC-SOD or SOD3). SODs catalyze the conversion of superoxide into hydrogen peroxide and oxygen. Catalase decomposes hydrogen peroxide to water and oxygen. Glutathione peroxidases catalyze the reduction of lipid hydroperoxides to alcohols and the decomposition of hydrogen peroxide to water and oxygen. In addition, heme oxygenases and reductases including thioredoxins (TRXs), peroxiredoxins (PRXs), and glutaredoxins (GRXs) are also involved in the enzymatic antioxidant defense. Non-enzymatic antioxidants are small molecular-weight substances including ascorbic acid (vitamin C), alpha-tocopherol (vitamin E), glutathione (GSH), uric acid, carotenoids (beta-carotene), flavonoids and trace metals such as selenium which serve as direct scavengers of ROS [120–122].

4. Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory disease of the respiratory system which is a major cause of morbidity and mortality throughout the world and represents the third leading cause of death in the United States [123]. It is characterized by airflow limitation which is associated with an abnormal inflammatory response of the lungs to noxious particles or gases [124]. Spirometry is used for diagnosis and evaluation of COPD. Post-bronchodilator forced expiratory volume in one second (FEV_1) is less than 80 % of predicted as well as the ratio of FEV_1 to forced vital capacity (FVC) (FEV_1/FVC ratio) less than 0.7 confirm the presence of airflow obstruction [125]. In contrast to asthma, the airflow limitation in COPD is largely irreversible [126]. Pathological changes of COPD include chronic bronchitis, emphysema, as well as inflammation and remodeling of small/large airways and vasculature. Several processes are implicated in the development of COPD including oxidative stress (disturbed balance between oxidants and antioxidants), inflammation, degradation of extracellular matrix (imbalance between proteases and antiproteases), defects in tissue repair mechanisms and apoptosis of lung cells [127, 128]. The major risk factor for the development of COPD is the exposure to cigarette smoke (CS) which contains more than 4700 chemical components including about 10^{15} reactive species per puff in the gas phase. These are short-lived oxidants such as superoxide, nitric oxide and peroxynitrite. The tar phase of the CS contains several relatively stable free radicals such as quinone/hydroquinone (Q/QH₂) complex, hydrogen peroxide and hydroxyl ions. The tar has more than 10^{18} free radicals per gram [129, 130]. In addition to CS, increased burden of oxidants originated from exogenous sources such as environmental air pollutants and from endogenous sources such as RONS generated by inflammatory cells also contribute to the pathogenesis of COPD. Numerous inflammatory cells including neutrophils, macrophages, T-lymphocytes and eosinophils are involved in the chronic inflammatory processes of airways which are important features of COPD. Increased number of inflammatory cells in COPD produce and release several inflammatory mediators and tissue-degrading enzymes which play critical role in the lung tissue destruction, bronchoconstriction, airway

remodeling and excess mucus production [126, 131]. Other risk factors related to COPD are environmental tobacco exposure, age, body mass index, education level, history of tuberculosis, hospitalization for respiratory problems before the age of 10 years, family history of COPD and years worked in dusty jobs [132]. Treatments for COPD such as short-acting/long-acting bronchodilators, long-acting anticholinergic agents and corticosteroids are slightly able to improve symptoms, quality of life and exacerbation rates, but not the mortality rate [133]. However, long term oxygen therapy and pulmonary rehabilitation may improve mortality and morbidity [131].

4.1 Remodeling in COPD

One of the main pathological feature of COPD is the remodeling and thickening of airway walls which can contribute to the airway lumen narrowing and airflow obstruction. Structural changes of the airways comprise increased airway smooth muscle (ASM) mass, disproportionate deposition of extracellular matrix (ECM) proteins, neovascularization, mucous gland and goblet cell hyperplasia and epithelial disruption [134, 135]. Significant increase in the thickness of the ASM layer is a key event in the airway remodeling which is more dominant in the small airways compared to the large airways. Variety of stimuli including inflammatory mediators and growth factors induced signaling cause hyperplasia and hypertrophy of ASM cells as well as evoke the accumulation and deposition of ECM components such as collagens, elastin, fibronectin, laminin, and proteoglycans around the smooth muscle cells leading to the thickening of small airway walls and reduction of lumen calibre. Moreover, ECM can influence the phenotypic switch of ASM cells from contractile to proliferative/synthetic phenotype contributing to the proliferation and migration of the ASM cells and the secretion of chemokines, cytokines and ECM proteins [136, 137]. Inflammation is also involved in the airflow limitation in the small airways. Infiltration of the airway wall by inflammatory cells such as neutrophils, macrophages, CD8 cells and B cells and accumulation of inflammatory mucous exudates in the lumen are associated with luminal narrowing and progression of COPD [138]. In addition, the number of the goblet cells is elevated in the epithelium of peripheral airways which are mostly represented in the central airways [139]. Structural changes of the lung parenchyma is also presented in COPD. Destruction of alveolar walls and parenchymal tissue due to the excessive proteolysis and/or inappropriate repair mechanisms are associated with chronic infiltration of inflammatory cells and inflammation which are responsible for the airflow obstruction [140]. Remodeling of the large airways also occurs in patients with COPD including thickening of epithelial, subepithelial and ASM layers as well as reticular basement membrane (RBM). Hyperplasia of goblet cells and mucus-secreting submucosal glands, metaplasia of squamous cell and loss of ciliated epithelial cells are important pathological features of the large airways in COPD [141]. COPD is also associated with abnormal deposition of ECM components such as collagen I and versican in the subepithelial layer as well as increased depositions of collagens (e.g. collagen I, III and IV), fibronectin and laminin can be detected in the surface epithelial basement membrane (SEBM) of the bronchial walls and vasculature [142, 143]. In addition, RBM thickness is increased due to the ECM deposition apposing the lamina reticularis as well as inflammation by eosinophils [144, 145]. Moreover, the ASM mass is significantly greater in the central airways attributable to the growth and proliferation of AMC cells [142].

Pulmonary vascular remodeling is another critical feature of COPD which is characterized by thickening of the vessel wall leading to narrowing of the vascular lumen, elevation of vascular resistance and development of pulmonary hypertension [146]. All vessel wall layers are affected in COPD, though the enlargements of the intima and media are the major contributors to the remodeling. Degree of intimal and medial thickening is correlated with the deterioration in lung function and the severity of the disease [147]. Augmentation of the intima thickness is associated with intense deposition of collagen and intimal hyperplasia including increased proliferation of a subpopulation of less differentiated vascular smooth muscle cells that express smooth muscle alpha-actin and vimentin, but not desmin (VSMC) [148] [149]. Endothelial dysfunction also occurs in pulmonary arteries of COPD patients [150]. Hypertrophy and hyperplasia of VSMC as well as medial accumulation of fibroblasts and inflammatory cells contribute to the muscularization and thickening of the media layer. Furthermore, over-deposition of ECM proteins such as collagen III and laminin in vascular media and adventitia occurs [147]. Infiltration of increased number of inflammatory cells mostly activated T lymphocytes can be observed in the adventitia of pulmonary arteries in COPD patients which play an important role in the chronic inflammatory response and pathogenesis of the disease [151].

Angiogenesis is an important event in the development of COPD which governs the formation of new blood vessels from a preexisting vasculature. Wide range of stimuli including inflammation, shear stress, numerous angiogenic factors such as growth factors (e.g. vascular endothelial growth factor [VEGF], fibroblast growth factors [FGFs], epidermal growth factor [EGF], platelet-derived growth factor [PDGF], angiopoietin-1 [Ang-1] as well as chemokines and cytokines promote the angiogenesis. Variety of cell types such as macrophages, lymphocytes, epithelial cells, fibroblasts, and SMC can produce and release angiogenic factors regulating the critical steps in angiogenesis including survival, proliferation, differentiation, and migration of the endothelial cells and tubes formation [152–154]. Nevertheless, the regulation of angiogenesis in COPD is not fully understood yet. VEGF is one of the crucial player in the angiogenesis. It was reported that expression of VEGF and its receptors are significantly increased in smokers and patients with moderate COPD compared to the nonsmokers [155, 156]. In contrast, patients with severe emphysema showed reduced VEGF and VEGFR-2 receptor expression which was associated with increased endothelial apoptosis [157, 158]. These observations suggest that expression levels of VEGF highly depend on the severity of COPD and may be implicated in the pathogenesis of the disease [159, 160].

4.2 RONS on calpain in COPD

Oxidative/nitrosative stress, due to the oxidant/antioxidant imbalance, have a critical role in the pathogenesis of COPD. RONS are exogenously derived from cigarette smoke (CS) or air pollutants, or endogenously released from activated inflammatory cells such as neutrophils, alveolar macrophages and eosinophils or other respiratory cells such as epithelial, endothelial, and smooth muscle cells. They are implicated in various pathological processes including chronic inflammation; oxidation and nitration of cellular substances such as lipids, proteins, carbohydrates and nucleic acids causing damage to the lung cells and apoptosis; activation of redox-sensitive transcriptions factors and chromatin regulation contributing to

increased gene expression of inflammatory mediators and cytokines; inactivation of antiproteases and activation of metalloproteinases leading to imbalance of the protease/antiprotease system and ECM degradation; defects in tissue repair mechanisms, as well as abnormal proliferation of ASM cells. [161, 162]. The main enzyme systems which are responsible for the production of RONS such as mitochondria, NADPH oxidase, xanthan-oxidase and iNOS are upregulated in COPD patients [88, 163, 164]. Several intracellular signaling pathways are implicated in the RONS-mediated processes involving extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 kinase, and PI3K/Akt, in addition, activation of these pathways can contribute to the pathological responses in COPD [165].

CS and its components are major risk factors in COPD and are implicated in the development of airway and pulmonary vascular remodeling. One of the main effector protein which mediates these processes is the calcium-activated enzyme namely calpain. Calpain is a family of calcium-dependent, cytoplasmic neutral cysteine endopeptidases that act through limited proteolysis of substrate proteins in mammalian cells. Heijink and colleagues revealed that cigarette smoke extract (CSE) causes disruption of cell-cell contacts leading to epithelial barrier dysfunction in bronchial epithelial cells via degradation of tight junction (TJ) proteins such as zonula occludens-1 (ZO-1) and occludin by EGFR-dependent calpain activation. Inhibition of EGFR tyrosine kinase and/or inhibition of calpain prevent the CSE-induced permeability increase as well as the cleavage of TJ components, respectively, emphasizing their participation in the reduced epithelial integrity evoked by CSE [166]. One report demonstrated that CSE induces calpain-1 expression via activation of ERK signaling pathway in bronchial epithelial cells contributing to distributional abnormalities and increased cleavage of adherens junction proteins such as E-cadherin and β -catenin consequently leading to transepithelial electrical resistance reduction and barrier disruption [167]. Calpain-1 specific inhibitor ALLM and ERK inhibitor U0126 diminish the CSE-induced epithelial barrier disruption. Moreover, active form of vitamin D, 1 α ,25-Dihydroxyvitamin D₃, reduces these pathological changes via inhibiting ERK phosphorylation and activation and calpain-1 expression suggesting its protective role against CSE damage in the airway epithelium by down-regulating the ERK signaling pathway [167]. It was recently published by Wang and colleagues that exposure to particulate matter (PM) air pollution causes tight junction protein ZO-1 degradation and increases in vascular permeability via calpain activation in human lung microvascular endothelial cells (HLMVECs). They found that PM induces ROS production, ROS-dependent endothelial permeability increase and degradation of ZO-1 as well as provokes calcium leakage via ROS-activated transient receptor potential cation channel member M2 (TRPM2) leading to elevated calpain activation. Using either the ROS scavenger, N-acetylcysteine (NAC) or calpain inhibitors such as calpeptin and ALLN or calcium chelator BAPTA-AM, the PM-induced decrease in transendothelial electrical resistance and ZO-1 degradation are attenuated. These data suggest that ROS-mediated calpain activation has a crucial role in PM-induced endothelial barrier disruption [168]. Chun and Prince reported that calpain can also alter the epithelial junctions to promote the polymorphonuclear leukocytes (PMNs) transmigration via activation of toll-like receptors (TLR) and interleukin-8 (IL-8) production in response to inhaled pathogens. Patients with COPD are

highly susceptible for infections which may have significant role in acute exacerbations. Inflammation and infiltration of inflammatory cells to the airways may contribute to the airflow obstruction in COPD. They found that activation of TLR2 signaling due to the inhaled pathogens causes calpain activation, which in turn, cleaves the transmembrane components such as occludin and E-cadherin leading to elevated PMN transmigration. The selective calpain inhibitor calpeptin almost completely blocks the PMN mobilization into the airway lumen indicating the relevance of calpain in the PMN transmigration and pulmonary inflammation [169]. The role of calpain in lung inflammation was further strengthened by Liu and colleagues. They demonstrated that mechanical ventilation causes rapid elevation in calpain activation as well as enhances the neutrophil infiltration into the lung causing pulmonary vascular barrier disruption and edema formation. Calpain inhibition by either calpain inhibitor I or specific siRNAs against calpain-1 and 2 prevented the inflammatory responses evoked by hyperinflation of the lung. In addition to this, inhibition of calpain also abolishes the eNOS-mediated NO production and subsequent phosphorylation of the transmembrane protein ICAM-1 after mechanical ventilation. These findings indicate that activation of calpain regulates early lung inflammation through eNOS/NO-dependent ICAM-1 phosphorylation and recruitment of neutrophils in response to lung hyperinflation [170]. Activated calpain can also cleave other critical cytoskeletal proteins such as myristoylated alanine rich C-kinase substrate (MARCKS) which plays a role in regulation of airway mucin secretion. Hypersecretion of mucin has a significant role in the pathophysiology of COPD. It has been shown that phorbol-12-myristate-13-acetate (PMA) induces the activation of protein kinase C (PKC) pathway, contributing to elevated calpain activity and mucin secretion in human bronchial epithelial cells. Calpain inhibitors ZLLY-FMK (FMK) and Z-LLY-CHO (CHO) significantly diminish the calpain activity and mucin secretion induced by PMA. PMA also increased the cleavage of MARCKS. They proposed that calpain-evoked MARCKS cleavage may have an important role in regulation of airway mucin secretion [171].

CSE can inhibit endothelial monolayer wound repair and angiogenesis. We previously reported that CSE results in dose-dependent inhibition of tube formation, proliferation and migration of pulmonary artery endothelial cells (PAEC) as well as decreases in calpain activity. Calpain specific inhibitor, calpain inhibitor-1 potentiates inhibitory effect of CSE. Downregulation of calpastatin which is an endogenous calpain inhibitor prevents CSE-induced reduction in calpain activity and angiogenesis. Taken together, these data suggest that CSE diminishes angiogenesis through calpain inhibition which may contribute to the remodeling in COPD [172]. Edirisinghe and colleagues revealed that inhibition of vascular endothelial growth factor receptor-2 (VEGFR-2) enhances cigarette smoke-induced oxidative stress and inflammatory responses causing endothelial dysfunction. They found that CS and VEGFR-2 inhibitor NVP-AAD777 increases oxidative stress, macrophage infiltration and the levels of proinflammatory mediators as well as decreases the levels of VEGF and VEGFR-2, phosphorylation of VEGFR-2, and levels of phosphorylated and total eNOS protein in mouse lung. In addition, they showed that CS exposure or VEGFR-2 inhibition results in increases in ROS production as well as reduction in VEGFR-2 expression, eNOS levels, and VEGF-induced VEGFR-2 phosphorylation in human lung microvascular endothelial cells (HMVEC-Ls) contributing to diminished endothelial cell

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migration and angiogenesis induced by VEGF [173]. More importantly, we recently reported that VEGF causes dose- and time-dependent increases in calpain activity and protein levels of calpain-2 in human pulmonary microvascular endothelial cells (PMEC). Downregulation of the calpain activity with either calpain-2 specific siRNA or by overexpressing the endogenous calpain inhibitor calpastatin prevented the increases in angiogenesis induced by VEGF, which further support the idea that calpain may have role in the CSE-induced inhibition of endothelial angiogenesis [174]. Calpain-calpastatin system also play a role in the cigarette smoke-induced inhibition of endothelial NOS in pulmonary artery endothelial cells (PAECs). Decreases in the NO production and impaired endothelium-dependent vasodilation evoked by cigarette smoke may lead to the progression of pulmonary vascular diseases in smokers. Our group previously published that CSE causes an irreversible, time- and dose-dependent inhibition of eNOS activity as well as causes time-dependent decreases in mRNA and protein levels of eNOS [175]. We also revealed the implication of calpain in the regulation of eNOS activity in hypoxic PAEC. Exposure to hypoxia reduces the eNOS activity, but not the protein levels, which are prevented by calpain specific inhibitors, calpain inhibitor I and calpeptin. Hypoxia also decreases the protein content of heat shock protein 90 (HSP90) which is attenuated by both calpain inhibitors. Moreover, HSP90 specific inhibitor geldanamycin mimicked the effect of hypoxia via diminishing the eNOS activity. These findings show that hypoxia-induced decrease in eNOS activity is attributable to the calpain-mediated reduction of HSP90 protein level [176]. Our additional study discovered the involvement of calpain-calpastatin system in the inhibition of eNOS induced by CSE in PAECs. We found that CSE exposure results in decreases in the transcription of eNOS gene and calpain activity as well as increases the protein levels of calpastatin. We also found that overexpression of calpastatin can mimic the inhibitory effects of CSE. Diminished activities of calpain and eNOS as well as lower mRNA and protein levels of eNOS were observed in calpastatin-overexpressing PAECs. In addition, downregulation of the calpastatin protein level by antisense oligodeoxyribonucleotides prevented the CSE-induced reduction in calpain activity as well as it prevented the decreases in eNOS gene transcription rate, eNOS protein levels, eNOS activity, and NO release induced by CSE. Together, these data indicate that CSE-induced inhibition of calpain activity and consequently reduced eNOS expression and activity are associated with an increase in the protein levels of calpastatin [177]. More recently, we reported that nitric oxide can affect the angiogenesis of lung microvascular endothelial cell in a dose-dependent manner via regulation of calpain activity. At lower concentrations, NO enhances angiogenesis, however, higher levels of NO inhibit angiogenesis and calpain activity as well as increase the calpain-1 nitrosylation. Decreased calpain activity is attributable to the nitrosylation of calpain which may have role in the anti-angiogenic effect of NO [178].

A recent report shows that acrolein which is a highly reactive, unsaturated aldehyde in cigarette smoke can induce the ROS production in different cell types and it is associated with COPD [179, 180]. Tanel and colleagues found that calpain and other proteases have role in the ER-mediated apoptosis of lung cells induced by acrolein. They showed that acrolein increases the activities of calpain and calpase-7 leading to the activation of ER initiator caspase-4 and apoptosis. The elevated calpain activity was prevented by calcium chelator BAPTA-AM or calpain inhibitor. In addition, acrolein reduced the protein levels of

calpain inhibitor calpastatin during calpain activation. Moreover, acrolein-induced apoptosis was partially inhibited by calpain inhibitors and completely blocked by BAPTA-AM. These findings revealed the participation of calpain as well as caspase-7 and -4 in acrolein-induced apoptosis in human lung cells [181]. Ryter and colleagues investigated the pathogenic implication of autophagy in cigarette smoke-induced COPD. They found that autophagy is increased in lung tissue derived from COPD patients and revealed that activation of autophagic proteins is associated with epithelial cell apoptosis in response to CS [182]. Other group reported the implication of calpain in the autophagy/apoptosis switches. Yousefi and colleagues demonstrated that apoptosis is related to calpain-mediated cleavage of autophagy protein 5 (Atg5) which is responsible for the autophagosomes formation and increased the susceptibility towards apoptosis. They found that calpain cleaved fragment of Atg5 facilitates apoptotic cell death by translocating from the cytosol to mitochondria and then binding to and consequently inhibiting the anti-apoptotic Bcl-xL molecule leading to cytochrome c release and caspase activation [183].

Increased ROS production and activation of proteolytic system such as calpain pathway, ubiquitin-proteasome (UP), the caspase and autophagy-lysosomal system (AL) are associated with skeletal muscle atrophy in COPD [184]. Chronic hypoxemia is one of the contributors to skeletal muscle dysfunction [185]. Chaudhary and colleagues revealed the involvement of calpains and UP pathway in the chronic hypobaric hypoxia-induced skeletal muscle atrophy. They found that exposure to hypoxia simultaneously elevates the activities of chymotrypsin-like enzyme of the UP pathway and calpains as well as enhances the expressions of ubiquitinated proteins and calpain-1 leading to increased myofibrillar protein degradation. They proposed that chronic hypoxia-induced oxidative stress may play an important role in the skeletal muscle loss via ub-proteasome and calpains pathways [185]. It was also demonstrated that hypoxia increases both activity and mRNA levels of calpain in pulmonary artery endothelial cells (PAECs). Inhibition of the transcription by actinomycin D prevented the increases in calpain gene expression and activity suggesting that acute hypoxia enhances calpain activity via transcriptional process in PAECs [186]. The implication of calpain in the development of skeletal muscle atrophy in response to ROS was further evidenced by other groups. McClung and colleagues demonstrated that calpain-1 is essential for myotube atrophy induced by hydrogen peroxide. They showed that exposure to H₂O₂ induces C2C12 myotube oxidative damage and atrophy. Downregulation of calpain-1 by siRNA, but not calpain-2 or caspase 3 proteases, attenuated the H₂O₂-induced myotube atrophy suggesting the requirement of calpain-1 in this process [187]. In addition, it was reported by Dargelos and colleagues that H₂O₂ administration causes up-regulation of both calpain-1 and calpain-2 expression and increases their proteolytic activities in LHCN-M2 human myoblasts. Interestingly, they also found that natural antioxidant extracted from pine bark (Oligopin®) prevents the H₂O₂-induced effects and up-regulation of calpain. These findings indicate that ROS-induced oxidative stress is involved in the skeletal muscle dysfunction via calpain-dependent pathways [188]. Recent study also revealed that oxidative stress is required for the activation of calpain and caspase-3 in the diaphragm during prolonged mechanical ventilation (MV) resulting myofiber atrophy and contractile dysfunction. They also showed that the prevention of MV-induced oxidative stress via the antioxidant Trolox abolishes calpain and caspase-3 activation, as well as prevents

diaphragmatic atrophy and contractile dysfunction evoked by MV [189]. Smuder and colleagues also confirmed the role of calpain and/or caspase-3 in the oxidative stress-induced myofibrillar protein breakdown. They demonstrated that oxidative stress causes augmented oxidative modification of myofibrillar proteins leading to their accelerated proteolytic degradation by calpain-1, calpain-2 and caspase-3. They conclude that oxidation of myofibrillar proteins increases their susceptibility to degradation through calpain and caspase-3 in response to oxidative stress [190]. Taking together, these reports show that elevation in ROS production in the skeletal muscle is associated with the activation of calpain.

Increased ROS formation and calpain activation are also associated with pulmonary hypertension (PH), a common complication of COPD. The critical mechanisms leading to PH in COPD are hypoxic vasoconstriction and remodeling of pulmonary arteries which is characterized by increased smooth muscle cell proliferation [191]. Elevated expressions of genes encoding smooth muscle mitogens such as PDGF have been found in COPD. Xing and colleagues demonstrated that CS exposure significantly increased the protein and mRNA levels of PDGF isoform B (PDGF-B) and PDGF receptor beta (PDGFR β) in pulmonary artery of rats. In addition, they also showed that CSE induces the rat PASMC proliferation which was inhibited by PDGFR inhibitor imatinib. This finding indicates that PDGF signaling is one of the major contributor to the CSE-induced PAH [192]. More importantly, our publication provides evidence that calpain mediates the proliferation and collagen synthesis of PASMCs induced by EGF and PDGF in pulmonary vascular remodeling. We found that EGF and PDGF cause significantly elevation in the calpain activity, cell proliferation and collagen synthesis in PASMCs which were attenuated by specific calpain inhibitor MDL28170 or using siRNAs targeted against calpain-1 and calpain-2. Moreover, we revealed that inhibition of the calpain activity by conditional knockout of calpain 4 or by calpain inhibitor MDL28170 prevents the hypoxia and monocrotaline (MCT)-induced progression of pulmonary vascular remodeling demonstrating the critical role of calpain in this process [193]. More recently, we further confirmed that mediators of PAH including PDGF, serotonin, H₂O₂, endothelin-1 and interleukin-6 markedly enhance the calpain activity, cell proliferation and protein levels of collagen-I of PASMCs. These processes were abolished by downregulation of calpain-2 by specific siRNA or using extracellular signal-regulated kinases (ERK) inhibitor PD98059 indicating the involvement of ERK1/2 in the PAH mediators induced activation of calpain-2 in pulmonary vascular remodeling. Furthermore, we demonstrated that smooth muscle-specific knockout of calpain 4 diminishes and knockout of calpastatin potentiates the chronic hypoxia-induced pulmonary vascular remodeling suggesting that calpain activation is an important signaling pathway in the pathogenesis of PH [10]. The implication of the calpain/calpastatin system in the progression of PH was further confirmed by Wan and colleagues. They found elevated lung expression of calpastatin, calpain-1 and calpain-2 proteins and activities occurring both intracellularly and extracellularly in patients with iPAH and in mouse models of PH induced by either hypoxia or overexpression of the serotonin transporter (5HTT) in SMCs. Overexpression of calpastatin or calpain inhibition by calpain inhibitor PD150606 decreased the protein levels of calpain-1 and 2 as well as reduced the intracellular and extracellular calpain activities in mouse models which were associated with

attenuated PH severity. In addition, in PASMCs derived from WT and calpastatin overexpressing transgenic mice, exogenous calpastatin or calpain inhibitor PD150606 diminished the cell proliferation and migration suggesting the inhibitory role of extracellular calpastatin in the proliferation and migration of PASMC induced by various stimuli. Thus calpastatin acts as an endocrine factor which markedly decreases the severity of PH in mice mainly via the inhibition of extracellular calpain activity [194].

5. Summary

In conclusion, there are convincing evidence that RONS and the members of the calpain family, primarily the two major isoforms calpain-1 and calpain-2, have mediating role in pathogenesis of COPD. Targeting the redox-dependent calpain signaling pathways using multidisciplinary approaches could provide therapeutic intervention in the pathogenesis and the progression of airway and pulmonary vascular remodeling in COPD.

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