Doctor of Philosophy

The study for role and mechanisms of mitoNEET in mitochondrial dysfunction.

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Department of Biological science

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Contents

OVERVIEW

The function of mitoNEET in mitochondria.

Abbreviations

ROS	Reactive oxygen species
OXPHOS	Oxidative phosphorylation
ATP	Adenosine triphosphate
MMP	Mitochondria membrane potential
PTP	Permeability transition pore
MitoNEET	Mitochondrial protein containing NEET sequence
CISD1	CDGSH iron sulfur domain 1 protein
TZD	Thiazolidinediones
PPARγ	Peroxisome proliferator- activated receptor gamma
NADH	Nicotinamide adenine dinucleotide

Introduction

1. Mitochondria

Mitochondria, dynamic organelles, as the powerhouse of the cells have the role in the energy metabolism and the production of reactive oxygen species (ROS) through the oxidative phosphorylation (OXPHOS) and coordinate several metabolic pathways producing metabolites containing the macromolecules such as lipids, proteins and nucleotides. [1]. Therefore, mitochondria are a critical source for biosynthetic pathways including nucleotide synthesis, fatty acid and cholesterol synthesis, amino acid synthesis, and glucose and heme synthesis [2]. In addition, the maintenance of Ca2+ and ROS homeostasis in mitochondria is important for the regulation of cell survival and cell death [3]. That a complex interdependence exists between adenosine triphosphate (ATP) production, Ca2+ uptake, mitochondrial ROS generation, ROS detoxification, and redox signaling. During OXPHOS, electrons from reduced substrates are transferred to O_2- through a chain of respiratory electron transporters. And then, the electrochemical energy of a proton gradient across the mitochondrial inner membrane is used to generate ATP. However, electrons may react with oxygen to form reactive oxygen species (ROS) and induce oxidative stress. Overload of Ca2+ and ROS in mitochondria lower mitochondrial membrane potential ($\Delta\Psi m$, MMP) and trigger the opening of the permeability transition pore (PTP). That lead to mitochondrial cell death (apoptosis via mitochondrial pathway) by mitochondrial damage (Figure. 1) [4].

1.1. Mitochondrial dysfunction.

The integrity of mitochondrial function is fundamental to cell life and Mitochondria as a common energy source for organs affect many different organs. The cell demands for mitochondria and their

complex integration into cell biology, extends far beyond the provision of ATP. Therefore, Mitochondrial dysfunction is associated with human pathologies such as cancer, metabolic, and cardiovascular diseases. Disturbances of mitochondrial function leads to disruption of cell function, expressed as disease or even death. Mitochondrial diseases can affect almost any part of the body, including the cells of the brain, nerves, muscles, kidneys, heart, liver, eyes, ears or pancreas (Figure. 2) [5, 21]. Moreover, mitochondrial derived oxidative stress cause sepsis-induced organ failure and neurodegenerative diseases such as Huntington's disease, Parkinson's disease, Alzheimer's disease, as well as playing a role in the aging process and diabetes. Therefore, a number of studies have focused on therapeutic approaches targeting damaged mitochondria [6].

1.2. Mitochondrial oxidative stress.

Mitochondria are major producers of free radical species and also possibly of nitric oxide, and are, at the same time, major targets for ROS-induced oxidative damage [5, 21]. One of the possible ways of mitochondrial involvement in the cellular damage is excessive production of reactive oxygen and nitrogen species (ROS and RNS) that cannot be effectively neutralized by existing antioxidant systems [21]. In mitochondria, ROS and RNS are able to cause lipid peroxidation and damage to cell membranes, proteins and mitochondrial DNA (mtDNA) causing failure of enzymatic chains and mutations that can impair mitochondrial function. Defective proteins will in turn exacerbate mitochondrial dysfunction, increase production of ROS, and reduce mitochondrial energy production. These processes further lead to abnormal cell signaling, premature cell senescence, initiation of inflammation, and apoptosis [5].

1.3. Mitochondrial oxidative stress production system.

The oxidative stress protection system must be tightly controlled [24]. Antioxidant defenses are induced by different stimuli in response to stress condition. Several transcription factors have been proposed to modulate the expression of one or more protection genes and to be activated in response to oxidative

stress, but until recently a coordinated regulation had not been described. That the activation of oxidative metabolism and the mitochondrial function goes hand in hand with the induction of the ROS protection system [21, 22]. Peroxisome proliferator activated receptor gamma coactivator 1 alpha (PGC-1α), a transcriptional coactivator well known as a key regulator of mitochondrial biogenesis and liver gluconeogenesis and lipid catabolism, has been considered to be a master regulator of the mitochondrial oxidative metabolism and ROS protection system [25]. Signaling pathways such as the p38 mitogen-activated protein kinase (MAPK), AMP-activated protein kinase (AMPK), β-adrenergic receptor (β-AR)/cAMP, Ca2+-calmodulin kinase activate the gene expression of PGC-1α and then, induce the transcription of downstream target gene of PGC-1α. PGC-1 α coordinately regulates several antioxidant genes, including manganese-dependent superoxide dismutase 2 (Mn-SOD (SOD2)), catalase (CAT), Uncoupling protein 2 (UCP-2), Peroxiredoxin 3 (Prx3), thioredoxin 2 (Trx2), thioredoxin reductase 2 (TrxR2) [21]. Mn-SOD is the SOD isoform found in the mitochondrial matrix. SOD catalyze the dismutation of superoxide radicals (O2−) to molecular oxygen (O2) and hydrogen peroxide (H2O2). Therefore, that can cause a different type of oxidative stress in the absence of the corresponding H2O2 scavengers. Nevertheless, Mn-SOD appears to behave mainly as an antioxidant in most biological settings [22, 23].

2. mitoNEET

3.1 Discover of mitoNEET

mitoNEET has been identified as a mitochondrial target of thiazolidinediones (TZD), such as pioglitazone and rosiglitazone and referred to as CDGSH iron sulfur domain 1 protein (CISD1). MitoNEET contains two domains: a beta-cap region and a cluster-binding domain and it found on the outer mitochondrial membrane. A cluster-binding domain of mitoNEET is composed to CDGSH sequence and binds a redox-active [2Fe-2S] cluster (Figure. 3). mitoNEET protein was present in many tissue types, including insulin-responsive tissues such as the liver, adipose, skeletal muscle, and heart [7, 8]. Thiazolidinediones (TZD) as a class of medicines used in the treatment of diabetes mellitus type

2 through activating peroxisome proliferator- activated receptor gamma (PPARγ), affects in different tissues. [7]. Currently, many studies have been discovering the function of mitoNEET using modified TZD, a weaker affinity for PPARγ [9, 10, 11].

3.2 Function of mitoNEET

mitoNEET participates in a redox-sensitive signaling and in Fe-S (iron–sulfur) cluster transfer (Figure. 3). Therefore, mitoNEET suggests as a key regulator for mitochondrial metabolism, the mitochondrial oxidative capacity and iron homeostasis [8, 13]. Accordingly, mitoNEET is suggested as a potential drug target in several mitochondrial dysfunctional diseases [12, 14]. mitoNEET could act differently according to cell's redox condition. In reducing environment, mitoNEET is incapable of [2Fe-2S] cluster transfer by nicotinamide adenine dinucleotide (NADH) binding and the accumulation of iron in the mitochondria is abrogated. However, when cells are under oxidative stress, mitoNEET could induce transfer of mitoNEET [2Fe-2S] cluster and if not carefully regulated, this could lead to Fe overload stress in mitochondria (Figure. 4) [15, 16]. In addition, in oxidizing environment, the electron transfer function of mitoNEET contributes to oxidative stress and the production of superoxide radicals ($O_2^{\bullet -}$) via transferring electrons into oxygen by oxidizing NADH as the electron donor and alleviating the inhibitory effect on cluster transfer. (Figure 4) [15, 17]. Recent publications demonstrated that pioglitazone stabilizes the 2Fe–2S Cluster inhibits iron transfer from mitoNEET to mitochondria. Pioglitazone, which shows a strong preferential binding to mitoNEET in the oxidized state, may act to alleviate Fe overload stress (Figure. 5) [16]. Consequentially, mitoNEET is involved in a variety of human pathologies by mitochondrial dysfunction and is suggested as a therapeutic target for the regulation of mitochondrial dysfunction (Figure. 6) [18, 19, 20].

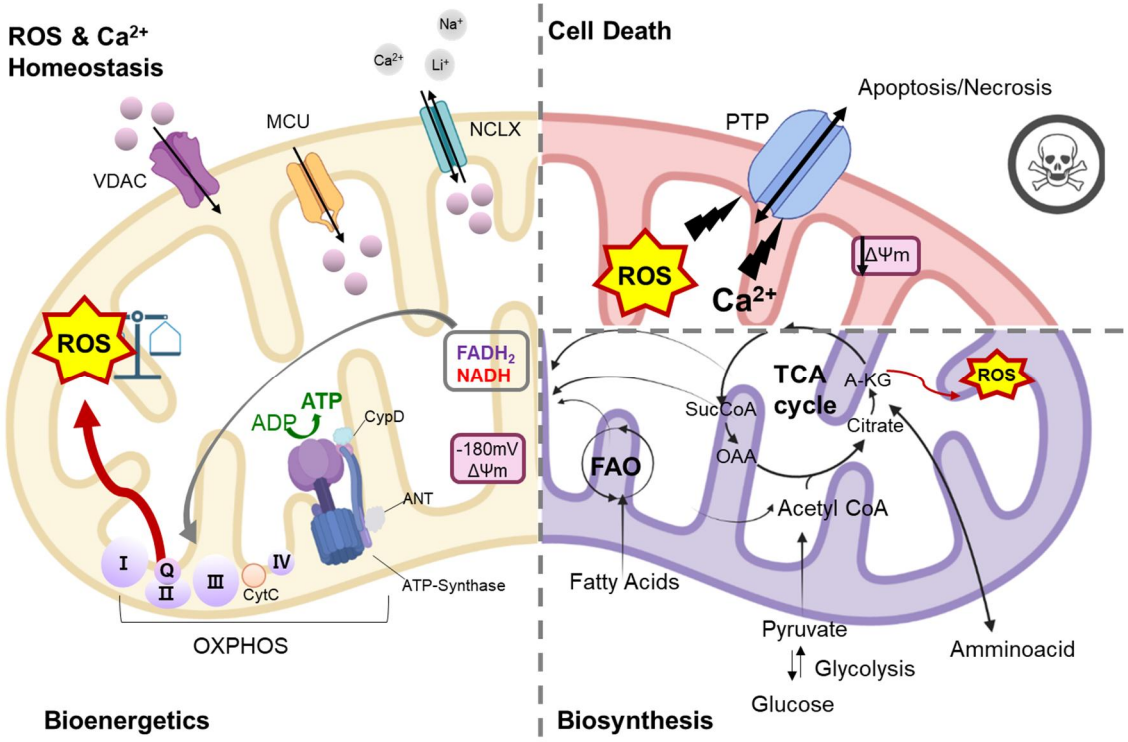

Figure 1. Schematic representation for mitochondrial function [1].

Left panel: under physiological conditions. Mitochondria play an important role for bioenergetics activities. Mitochondria are Ca2+ -buffering organelles. *Right top panel*: pathological conditions. Mitochondrial ROS (mtROS) burst and mitochondrial Ca2+ overload activate cell death through the opening of the mitochondrial permeability transition pore (mPTP). *Right-bottom panel*: Ca2+ uptake by mitochondria activates mitochondrial metabolism. (Created with BioRender.com)

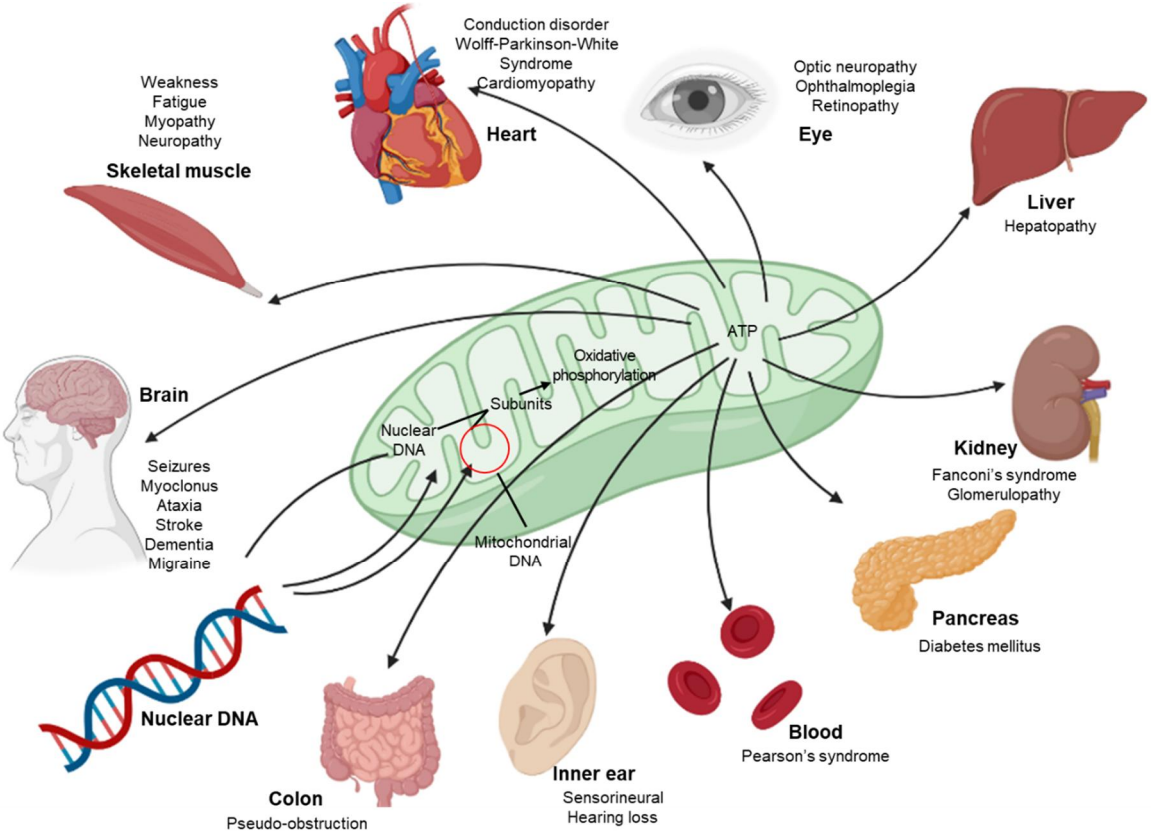

Figure 2. Mitochondria-related diseases and affected body parts [26].

Mitochondrial dysfunction is involved in the pathophysiology of a variety of metabolic and neurodegenerative disorders affecting important organs of body including brain, muscles, eyes, heart, liver, and pancreas. (Created with BioRender.com)

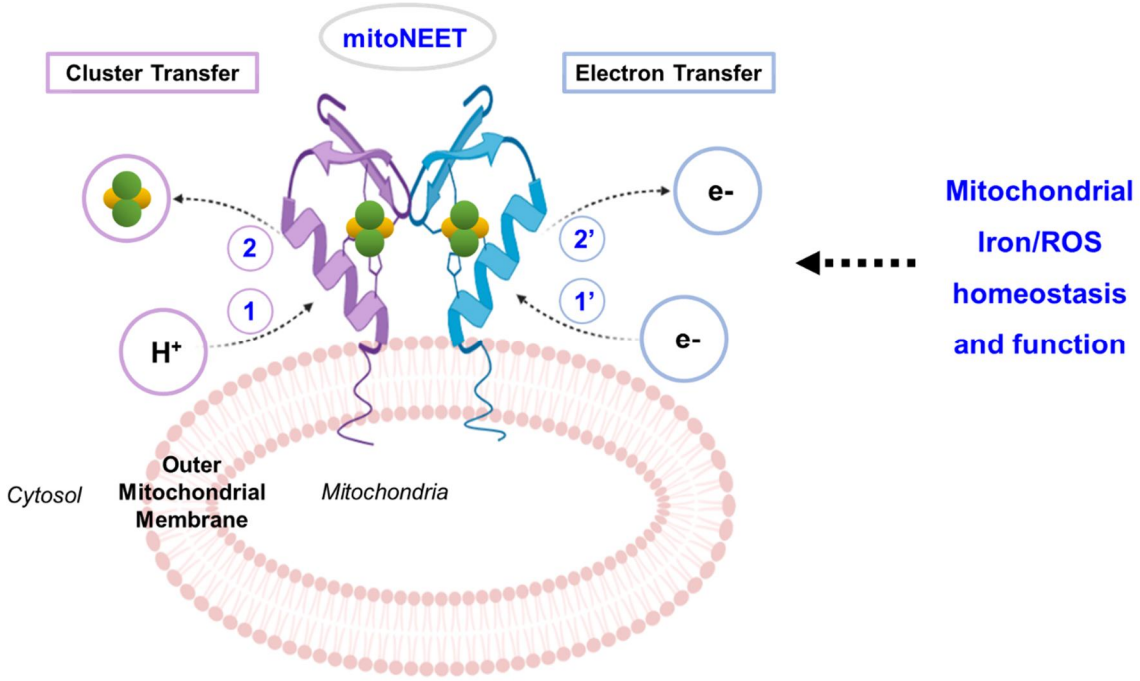

Figure 3. Structure and biophysical properties of mitoNEET [27, 28, 32].

MitoNEET is a novel iron–sulfur (Fe–S) protein in mitochondrial outer membrane (MOM), with the cluster binding domain facing the cytosolic side. This protein was initially discovered as mitochondrial target for thiazolidinedione (TZD) including rosiglitazone as a peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist as a drug for type 2 diabetes. Many of the beneficial effects of thiazolidinedione (TZD), could not be explained by PPAR-γ activity alone. That two possible functions of mitoNEET are suggested by its biophysical properties involves cluster transfer and electron transfer. (Created with BioRender.com)

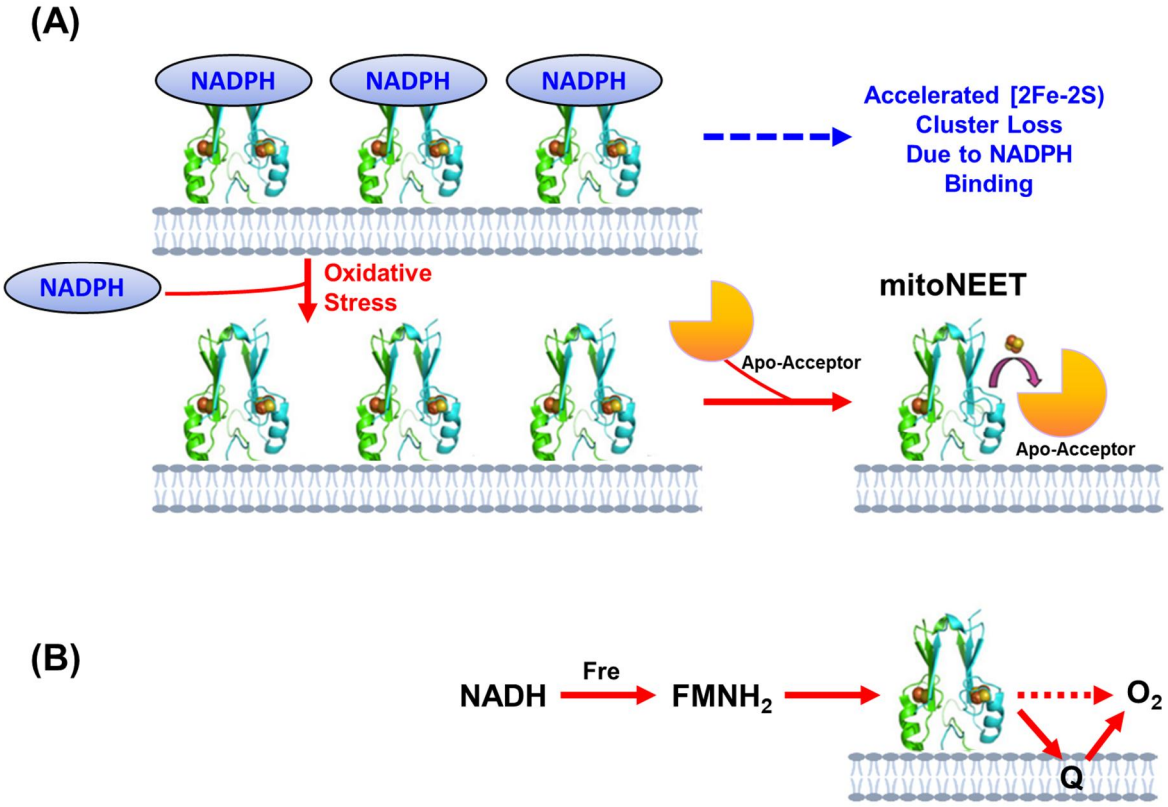

Figure 4. Model for the regulation of mitoNEET stability and cluster transfer or electron transfer properties in mitochondrial outer membrane [15, 17].

(**A**) Under normal conditions, NADPH inhibits [2Fe-2S] cluster transfer from mitoNEET to an apo-acceptor protein and sustains mitochondrial iron homeostasis by causing accelerated loss of the [2Fe-2S] cluster in mitoNEET. However, when oxidative stress, [2Fe-2S] clusters of mitoNEET become oxidized and thus capable of [2Fe-2S] cluster transfer. (Created with BioRender.com)

(**B**) In cytosol, FMNH2 is reduced by flavin reductase using NADH as the electron donor. FMNH2 mediates the reduction of mitoNEET [2Fe-2S] clusters which in turn transfer electrons to oxygen or ubiquinone in the mitochondrial outer membrane. Together with flavin reductase and FMN, mitoNEET as a redox-sensitive protein promotes NADH oxidation with a concomitant reduction of oxygen or ubiquinone in mitochondria. (Created with BioRender.com)

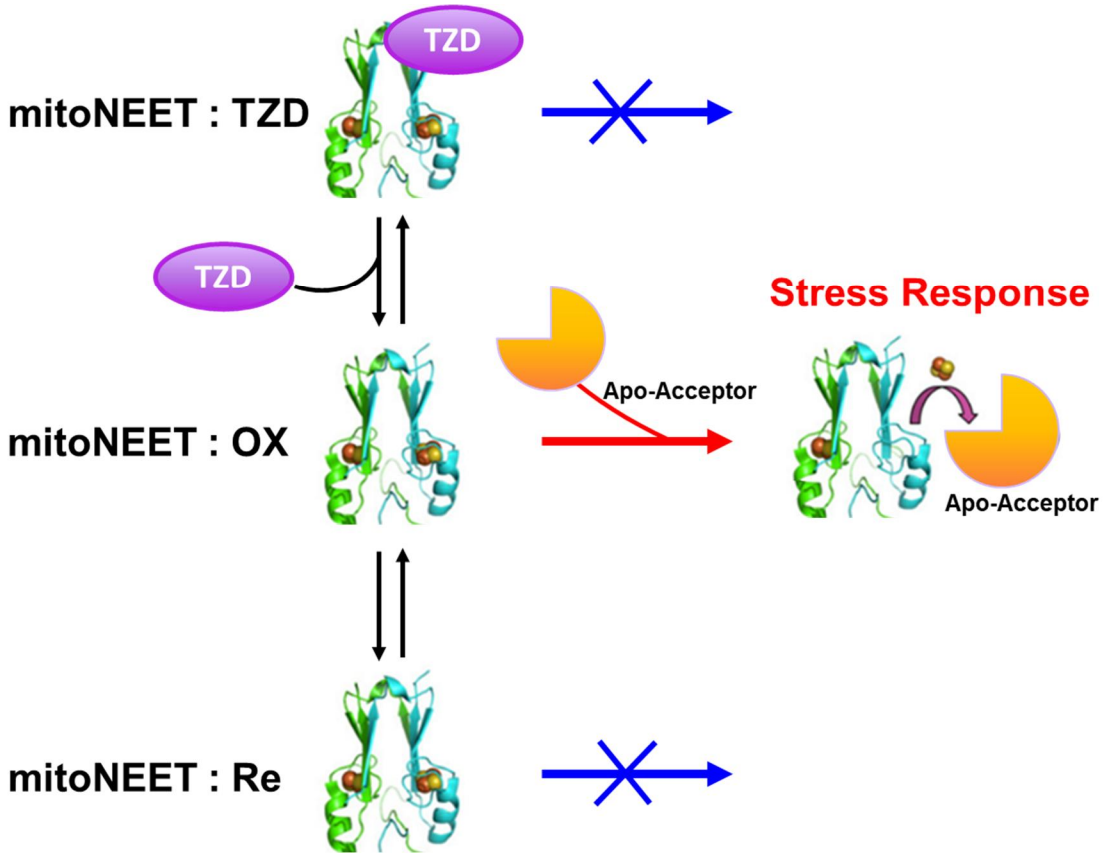

Figure 5. Model describing a possible therapeutic mode of action for pioglitazone [16].

When cells are under oxidative stress, mitoNEET could transfer [2Fe-2S] cluster and if not carefully regulated, this could lead to Fe overload stress in mitochondria. MitoNEET is a novel target of pioglitazone as a type 2 diabetes drug. Pioglitazone shows a strong preferential binding to mitoNEET in the oxidized state and may interfere the FMNH2 binding in mitoNEET and inhibit the transfer activity of mitoNEET in mitochondria. Therefore, pioglitazone is capable of regulating Fe accumulation in mitochondria may act to alleviate iron overload stress.

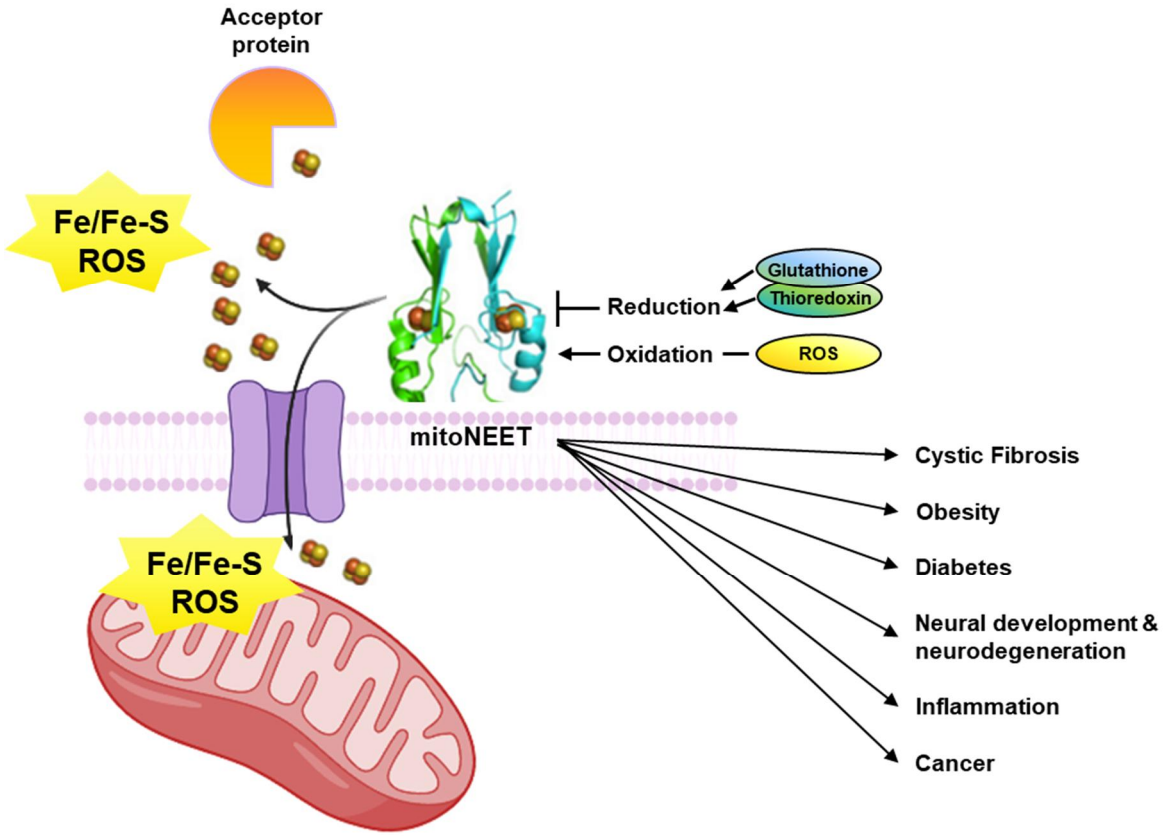

Figure 6. The role of mitoNEET involved in human disease [31].

Function of mitoNEET for cluster transfer leads in some cases to the enhanced generation of reactive oxygen species (ROS) in mitochondrial dysfunction and that lead to different diseases. (Created with BioRender.com)

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PART 1.

The role of mitoNEET under LPS-induced mitochondrial dysfunction in inflammatory disease.

Abbreviations

ROS	Reactive oxygen species
LPS	Lipopolysaccharide
MOM	Mitochondrial outer membrane
MMP	Mitochondria membrane potential
MitoNEET	Mitochondrial protein containing NEET sequence
NL-1	NEET Ligand-1
TZD	Thiazolidinediones
PPARγ	Peroxisome proliferator- activated receptor gamma
NADH	Nicotinamide adenine dinucleotide
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
TNF-α	Tumor necrosis factor-alpha
iNOS	inducible nitric oxide synthase
COX2	Cyclooxygenase-2
TLRs	Toll-like receptors
SIRS	systemic inflammatory response syndrome
RNS	reactive nitrogen species
HO-1	Heme oxygenase-1
SOD	superoxide dismutase

Abstract

MitoNEET (mitochondrial protein containing Asn-Glu-Glu-Thr (NEET) sequence) is a 2Fe-2S cluster-containing integral membrane protein that resides in the mitochondrial outer membrane (MOM) and participates in a redox-sensitive signaling and Fe-S (iron-sulfur) cluster transfer. Thus, mitoNEET is a key regulator of mitochondrial oxidative capacity and iron homeostasis. Moreover, mitochondrial dysfunction and oxidative stress play critical roles in inflammatory diseases such as sepsis. Increased iron levels mediated by mitochondrial dysfunction lead to oxidative damage and generation of reactive oxygen species (ROS). Increasing evidence suggests that targeting mitoNEET to reverse mitochondrial dysfunction deserves further investigation. However, the role of mitoNEET in inflammatory diseases is unknown. Here, we investigated the mechanism of action and function of mitoNEET during lipopolysaccharide (LPS)-induced inflammatory responses in vitro and in vivo. Levels of mitoNEET protein increased during microbial or LPS-induced sepsis. Pharmacological inhibition of mitoNEET using mitoNEET Ligand-1 (NL-1) decreased the levels of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in animal models of sepsis, as well as LPS-induced inflammatory responses by macrophages in vitro. Inhibition of mitoNEET using NL-1 or mitoNEET shRNA abrogated LPS-induced ROS formation and mitochondrial dysfunction. Furthermore, mitochondrial iron accumulation led to generation of LPS-induced ROS, a process blocked by NL-1 or shRNA. Taken together, these data suggest that mitoNEET could be a key therapeutic molecule that targets mitochondrial dysfunction during inflammatory diseases and sepsis.

Introduction

1. Inflammation

Inflammation is critical for healing and immune response enabling survival during infection or injury and underlies a wide variety of physiological and pathological processes [1]. The inflammatory response is orchestrated by various modulators and pathways. A typical inflammatory response consists of inducers, sensors, mediators, and target tissues. inflammatory exogenous or endogenous inducers initiate the inflammatory response and active the sensors. Sensors, such as Toll-like receptors (TLRs), are expressed on specialized sentinel cells, such as tissue-resident macrophages, dendritic cells, and mast cells. They induce the production and secretion of the inflammatory mediators, including cytokines, chemokines, bioactive amines, eicosanoids, and products of proteolytic cascades, such as bradykinin, thereby affecting the functions of target tissues. These inflammatory mediators play the role optimizing adaptation to the noxious condition (e.g., infection or tissue injury) associated with the particular inducers that elicited the inflammatory response (Figure. 1) [2, 3]. Inflammation is an adaptive response to noxious conditions. Under normal conditions, tissue-resident macrophages largely exhibit an M2 phenotype and maintain tissue homeostasis and repair by removing dead cells and other debris and by producing growth factors. Under noxious conditions, the cellular stress response is activated and results in a cell-autonomous adaptation controlling intracellular adaptations. A tissue-level stress response, or parainflammation as a tissue adaptive response, is elicited by the resident macrophages. if the condition is severe enough (e.g., infection or injury), an acute inflammation as the immediate response ensues. Acute inflammatory response recruit neutrophils and specialized subsets of monocytes protecting the host from infection and promote tissue repair and restoration of homeostasis (Figure. 2) [3, 4].

1.1. Inflammatory disease, sepsis

Uncontrolled and dysregulated inflammation can increase the risk of developing various diseases (e.g. obesity, cardiovascular disease, type 2 diabetes mellitus, cancer, sepsis) [5, 6]. Especially, sepsis, caused mainly by bacterial infection, is a highly inflammatory disorder that, in severe cases, can cause organ dysfunction and death as the one of the most serious causes of mortality worldwide (Figure. 3) [7]. Sepsis threatens life and causes injury to its own tissues and organs through an inflammatory immune response triggered by an infection. Most commonly, the infection is bacterial, but it may also be fungal, viral, or protozoan. Sepsis is defined as a systemic inflammatory response syndrome (SIRS) to infection, that is associated with an increased production of both pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-lβ, and IL-6 promote inflammation and cause fever, tissue destruction, and, in some cases, shock and death. Anti-inflammatory cytokines, IL-10 suppress the activity of proinflammatory cytokines and is essential for homeostasis of the immune system [8]. Cytokine storm is considered to be one of the major causes of multiple-organ failure and increase mortality [9].

1.2. Sepsis and mitochondrial dysfunction

Sepsis is characterized by systemic and organ-specific changes in metabolism. Alterations of oxygen consumption, increased levels of circulating substrates, impaired glucose and lipid oxidation, and mitochondrial dysfunction are all associated with organ dysfunction and poor outcomes in both animal models and patients. The link between sepsis-associated organ failure and mitochondrial dysfunction is increasing interest to researchers [10, 14]. Sepsis-induced mitochondrial dysfunction mediates hyper-inflammation through cellular metabolic disorders, insufficient energy production, and oxidative stress; as such, it plays a key role in the development of sepsis-related multi-organ failure (Figure. 4) [11, 12, 13]. Inflammatory processes trigger a chain of events including increased production of ROS and reactive nitrogen species (RNS), mitochondrial iron accumulation by disruption of iron metabolism and mitochondrial dysfunction [15] Mitochondria, dynamic organelles that serve as the

powerhouse of the cell, are a major source of reactive oxygen species (ROS); they are also the site where iron is transformed into its bioactive form [16, 17, 18, 19]. Increased mitochondrial iron accumulation due to pro-inflammatory signaling promotes oxidative damage by catalyzing generation of ROS and causing mitochondrial dysfunction [15, 18]. These processes develop into a vicious inflammatory cycle [20]. Therefore, the mitochondrial iron level must be strictly regulated to avoid iron-mediated damage and maintain mitochondrial function (Figure. 5). Several studies demonstrate that targeting mitochondrial iron accumulation using iron chelators has the potential to improve the prognosis of sepsis [17, 21, 22].

2. The role of mitoNEET in mitochondrial dysfunction

The mitochondrial protein containing Asn-Glu-Glu-Thr (NEET) sequence, mitoNEET [also referred to as CDGSH (C-X-C-X2-(S/T)-X3-P-X-C-D-G-(S/A/T)-H) iron sulfur domain 1 (CISD1)] is a 2Fe-2S cluster-containing, redox-sensitive protein that resides on the outer mitochondrial membrane; as such, it is a powerful regulator of mitochondrial iron content [23, 24, 25]. Only when the mitoNEET [2Fe-2S] clusters are oxidized do they transfer apo-proteins and electrons from FMNH2 (reduced 1,5-dihydro form of flavin mononucleotide) to oxygen or ubiquinone in mitochondria [26, 27, 28]. Therefore, mitoNEET exerts marked effects on cellular and systemic metabolic homeostasis by acting as a powerful regulator of mitochondrial iron content. Early studies showed that mitoNEET plays a key role in regulating cellular energy use, lipid metabolism, and cancer cell proliferation and tumor formation [29, 30, 31]. Recent studies on the effects of redox regulation by mitoNEET demonstrate that mice overexpressing mitoNEET exhibit reduced ROS generation by mitochondria; however, oxidative phosphorylation and electron transport are significantly upregulated in the absence of mitoNEET (Figure. 6) [13, 32]. This is associated with generation of ROS by mitochondria, along with mitochondrial dysfunction [33]. Thus, mitoNEET is involved in a variety of human pathologies, including cystic fibrosis, diabetes, muscle atrophy, and neurodegeneration [33, 34, 35]. Initially, mitoNEET was identified as a mitochondrial target of thiazolidinediones such as pioglitazone and

rosiglitazone, a class of medicines used to treat type 2 diabetes [36, 37]. Thiazolidinediones show anti-oxidative and anti-inflammatory activity in different disease models, including sepsis [38, 39, 40]. Overproduction of ROS during sepsis is thought to be a central part of the disease process [13, 41]. However, the role of mitoNEET in sepsis is unknown. Here, we have used a mitoNEET Ligand (NL-1), modified TZD as a weaker affinity for PPARγ [36] and show that inhibiting expression or activity of mitoNEET reduces inflammation and oxidative stress during inflammatory responses and sepsis.

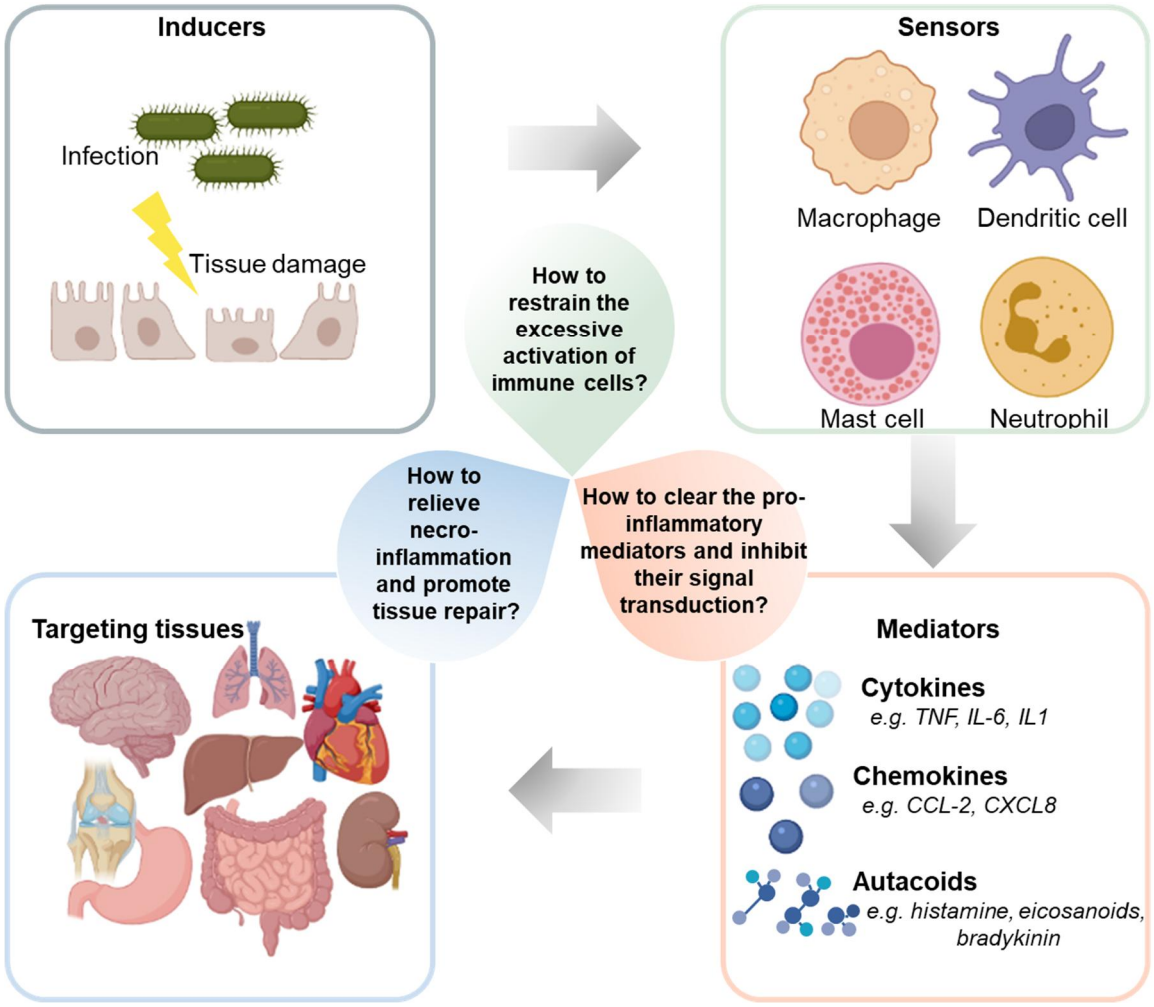

Figure 1. Inflammatory Pathway Components [50, 51].

Inflammatory pathway consists of inducers, sensors, mediators and effectors. Inducers initiate the inflammatory response and are detected by sensors. Sensors, such as Toll-like receptors (TLRs), are expressed on the surface of sentinel cells, such as tissue-resident macrophages and dendritic cells, mast cells. They induce the production of a variety of inflammatory mediators, including cytokines, chemokines, bioactive amines, eicosanoids and products of proteolytic cascades, such as bradykinin. In response to infection of tissue injury, these inflammatory mediators act on various target tissues to elicit changes in their functional states. (Created with BioRender.com)

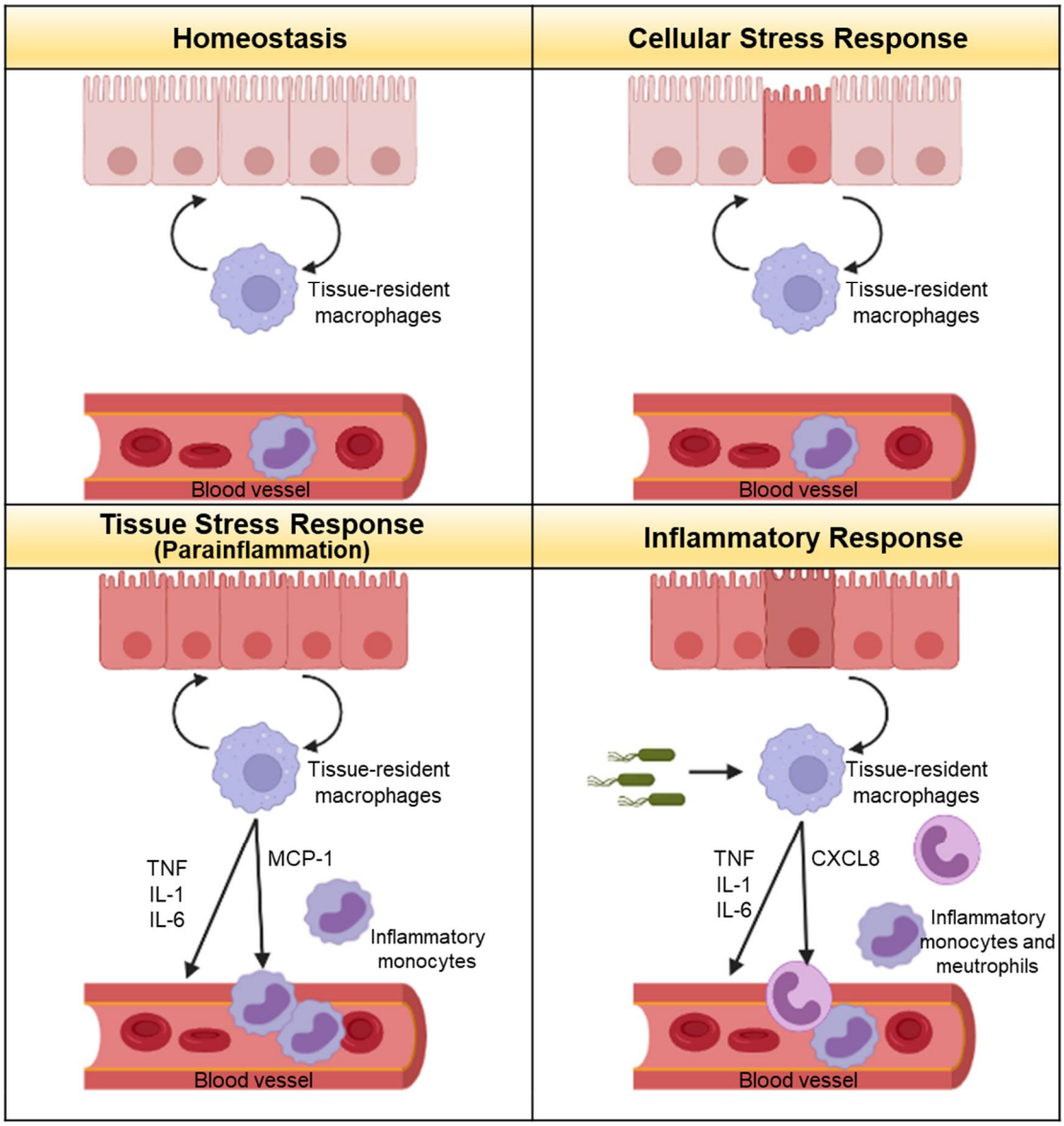

Figure 2. Inflammation and the stress response [51].

(A) Under normal conditions, tissue-resident macrophages maintain tissue homeostasis by removing dead cells and by producing growth factors. (B) Under noxious conditions, the cellular stress response is activated and mediates a cell-autonomous immune response. (C) Stress affects not only individual cells but the entire tissue, then, a tissue-level stress response, or parainflammation, is elicited. (D) Finally, an acute inflammatory response is induced against sever injury and infection. (Created with BioRender.com)

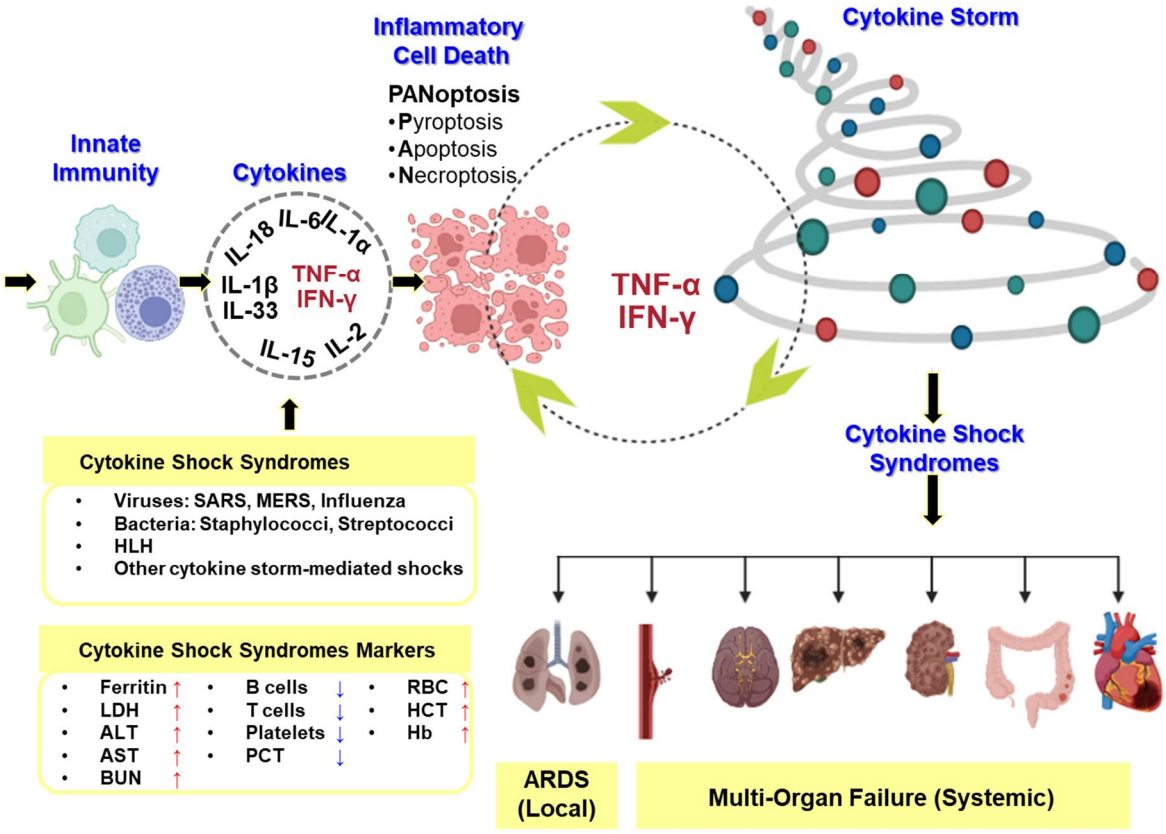

Figure 3. Schematic diagram of sepsis [52].

The activation of the immune system and production of inflammatory cytokines are essential for the natural anti-viral immune responses. However, hyperactivation of the immune system results in an acute increase in circulating levels of pro-inflammatory cytokines, leading to a "cytokine storm". While a mechanistic definition of cytokine storm is largely lacking, this storm is clinically characterized by systemic inflammation and hyper-ferritinemia, hemodynamic instability, multiple organ failure. The process of cell death is a possible cause mechanism of organ damages by cytokine storm. Among the programmed cell death pathways including apoptosis, pyroptosis and necroptosis have been proposed. Pyroptosis and necroptosis have been defined as inflammatory cell death processes, which are characterized as lytic forms of death that release cytokines and other cellular factors to drive inflammation and alert immune cells to a pathogenic or sterile insult. (Created with BioRender.com)

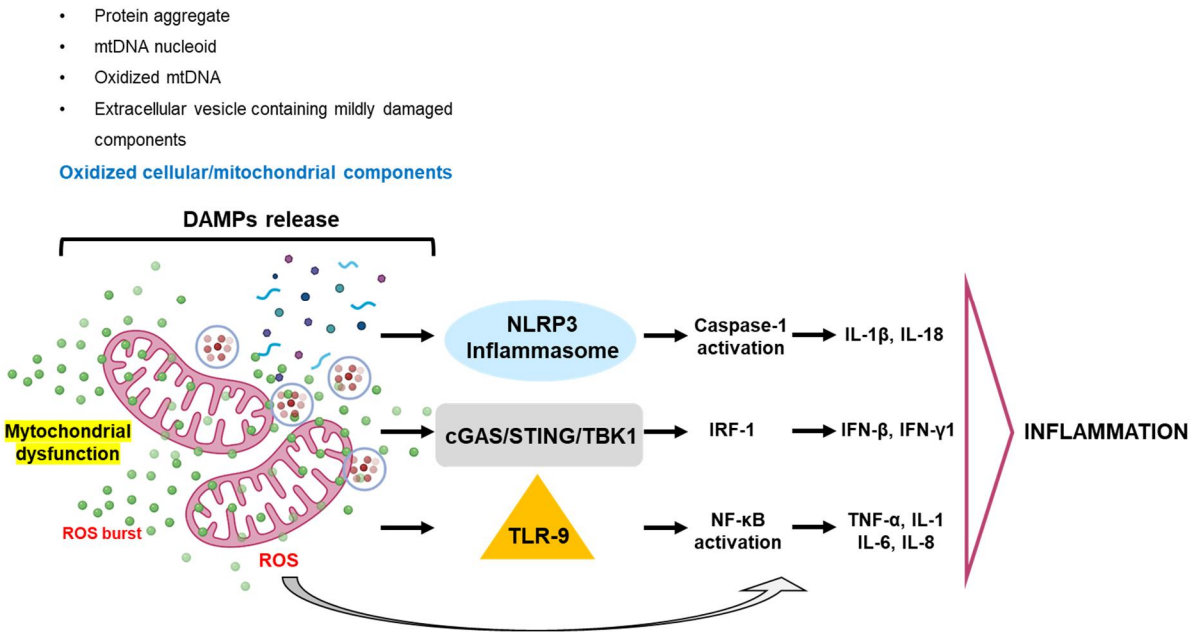

Figure 4. Mitochondrial oxidative stress can promote inflammation [53].

Failing mitochondrial quality control processes may lead to release damaged-associated molecular patterns (DAMPs). Acting as a DAMP, oxidized mitochondrial DNA (mtDNA) either in the form of mitochondrial transcription factor A (TFAM)-bound nucleoids or free mtDNA can trigger inflammatory response via interacting with (1) toll-like receptors (TLRs), (2) nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, and (3) cytosolic cyclic-GMP-AMP synthase (cGAS)–stimulator of interferon genes (STING) DNA-sensing system. (Created with BioRender.com)

Abbreviations: IFN, interferon; IL, interleukin; IRF-1, interferon regulatory factor 1; mtDNA, mitochondrial DNA; NF-κB, nuclear factor κB; ROS, reactive oxygen species; TBK1, TRAF family member-associated NF-κB activator (TANK)-binding kinase 1; TNF-α, tumor necrosis factor alpha.

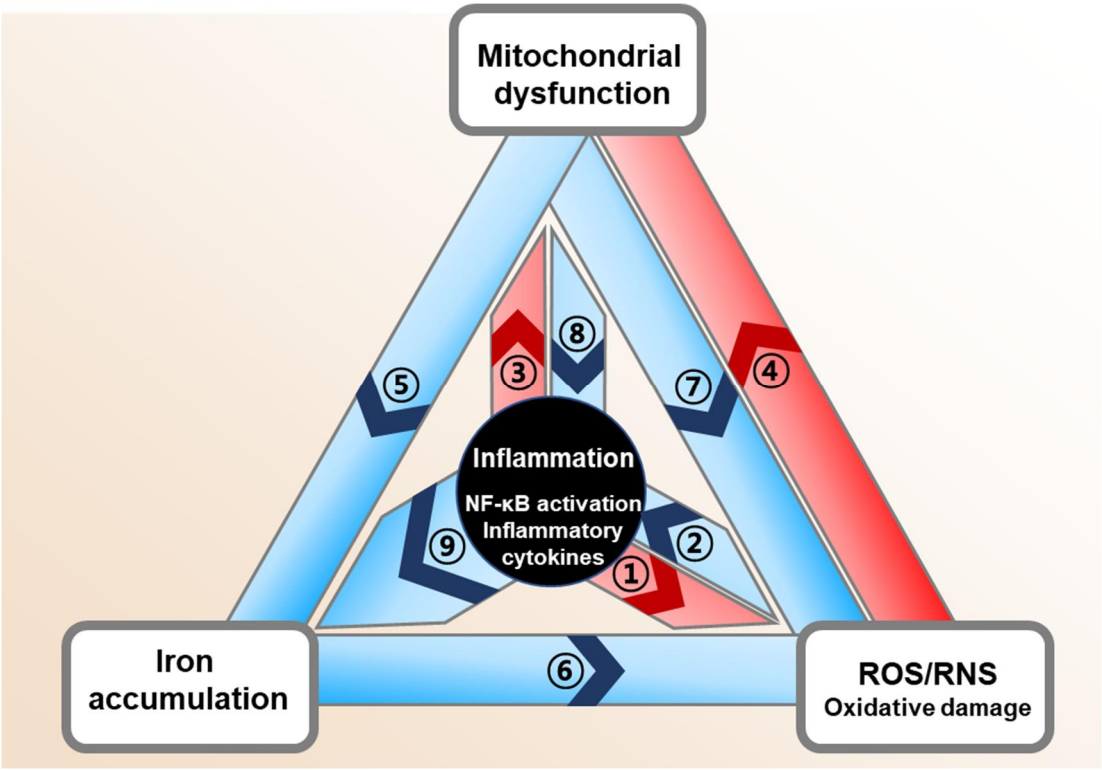

Figure 5. Inflammation causes ROS/RNS production, mitochondrial dysfunction, and iron accumulation [54].

Inflammation, oxidative damage, and mitochondrial dysfunction are common features of inflammatory diseases. These generate self-feeding cycle that could lead to organ failure in sepsis. In this cycle, (1) inflammation induces ROS/RNS generation by activation of the NOX and iNOS enzymes in turn, (2) ROS/RNS induce the expression of inflammatory cytokines. Additionally, (3) inflammation induces mitochondrial dysfunction through activation of TLR signaling. (4) ROS in turn induce mitochondrial dysfunction which results in the inactivation of several mitochondrial iron–sulfur (Fe-S) enzymes. (5) Mitochondrial dysfunction leads to IRP1 activation and increased iron uptake. (6) Iron increases oxidative damage by transforming superoxide and hydrogen peroxide into the hydroxyl radical. (7) Electron transport chain inhibition generates ROS production by electron leak, and (8) could activate the innate immune response by TLR signaling. Finally, (9) inflammation is likely to cause iron accumulation through induction of divalent metal transporter 1 (DMT1) expression and transient ferroportin (FPN) decrease.

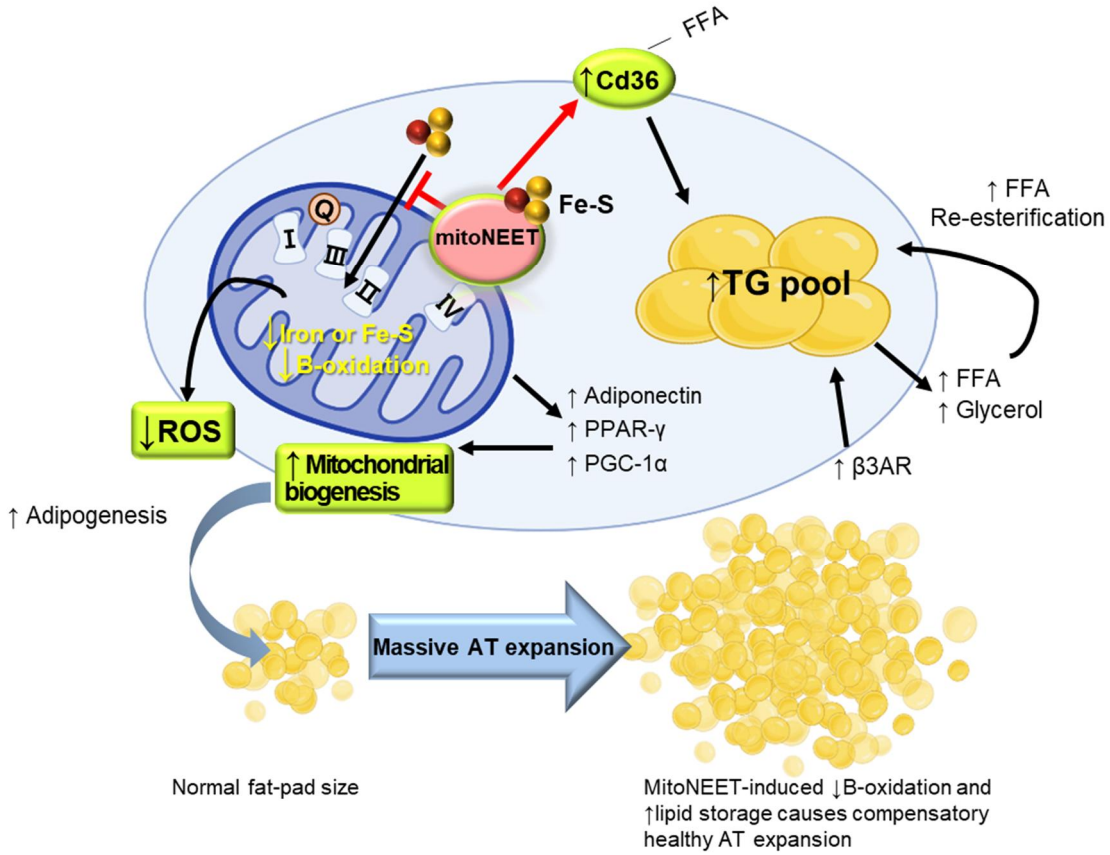

Figure 6. Proposed mechanism of mitoNEET action for the regulation of mitochondrial Iron and ROS [29].

MitoNEET inhibits mitochondrial iron transport into the matrix and, because iron is a rate-limiting component for electron transport, lowers the rate of b-oxidation. This effect is associated with lower levels of reactive oxygen species (ROS)–induced damage. Conversely, a reduction in mitoNEET expression enhances iron content in the matrix and causes heightened oxidative stress and glucose intolerance. (Created with BioRender.com)

Results

Inflammatory stimuli induce expression of mitoNEET during sepsis

Mitochondrial damage or dysfunction is the major cause of the multiple organ failure during sepsis [29]. Prior studies show that mitoNEET, an outer mitochondrial membrane protein, plays an important role in regulating mitochondrial function, especially oxidative capacity [33, 39, 49]. In this study, we hypothesized that mitoNEET plays a role in inflammation and oxidative stress during sepsis. To identify the role of mitoNEET during sepsis, we assayed expression of mitoNEET after induction of sepsis. Wild-type mice on a pure C57BL/6 genetic background were subjected to cecal ligation and puncture (CLP) to induce poly microbial peritonitis, bacteremia, and sepsis. We then examined expression of mitoNEET mRNA and protein in the spleen 48 hours later (Fig. 1A and 1B). Expression of mitoNEET mRNA and protein increased significantly (by 4.3-fold (Fig. 1A) and 4.1-fold (Fig. 1B), respectively) in mice with CLP-induced sepsis compared with sham mice (n=4 per group). In addition, expression of mitoNEET mRNA and protein increased by 2.2-fold (Fig. 1C) and 3.4-fold (Fig. 1D), respectively, in mice with Escherichia coli (Gram-stain negative)-induced sepsis compared with sham mice (n=4 per group). These data suggest that mitoNEET plays a critical role in sepsis caused by Gram-negative bacteria. To investigate the significance of mitoNEET induction by Gram-negative bacteria, we assessed its mRNA and protein levels in the spleen after induction of lipopolysaccharide (LPS)-mediated sepsis. LPS is a common pathogenic component of the outer membrane of Gram-negative bacteria. We harvested total RNA and protein from the spleen at 6, 12, 24, 48, and 72 hours after LPS injection. We found that mitoNEET mRNA levels began to increase by 6 hours after LPS injection, and marked induction of mitoNEET was evident at 24 hours (compared with vehicle) (Fig. 1E); protein

levels increased at 48 hours (Fig. 1F). Furthermore, expression of mitoNEET protein in bone marrow-derived macrophages (BMDMs) increased in the presence of LPS compared with vehicle (Fig. 1G). To identify the signaling pathway involved in regulating mitoNEET expression in LPS-stimulated macrophages, we used specific inhibitors Bay 11-7085 (an NF-kB inhibitor), SP600125 (a JNK MAP kinase inhibitor), SB203580 (a p38 MAP kinase inhibitor), U0126 (a mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor), LY294002 (a PI3 kinase inhibitor), and NAC (N-acetyl-L-cysteine, cytosolic ROS scavenger, A7250). BMDMs were treated with these kinase inhibitors in the presence of LPS, and levels of mitoNEET protein were assessed 6 hours later. Bay 11-7085 blocked LPS-induced mitoNEET expression (Fig. 1H); however, the other inhibitors had no effect. These data suggest that mitoNEET may have a critical role in inflammation during sepsis, and that the LPS-induced NF-kB signaling pathway is involved in induction of mitoNEET expression under inflammatory conditions.

Inhibition of mitoNEET reduces inflammatory responses during LPS stimulation of macrophages

Macrophages play critical roles in various inflammatory diseases through release of inflammatory mediators and cytokines such as IL-1β (Interleukin-1β), IL-6 (Interleukin-6), and TNFα (Tumor necrosis factor-alpha). To investigate the role of mitoNEET during inflammatory responses, we analyzed LPS-stimulated expression of cytokines and mediators by RAW264.7 cells in the presence or absence of a mitoNEET inhibitor, mitoNEET Ligand-1 (NL-1) (Fig. 2). We found that NL-1 reduced expression of mRNA encoding pro-inflammatory cytokines IL-1β, IL-6, and TNFα, and of mRNA encoding inflammatory mediators iNOS (inducible nitric oxide synthase) and COX2 (Cyclooxygenase-2), in cells exposed to LPS for 12 hours (Fig. 2A–E). This decrease was not seen in control cells (treated with LPS alone). In addition, IL-1β, IL-6, TNFα, iNOS, and COX2 protein levels decreased in the presence of NL-1 (Fig. 2F–I).

To investigate whether LPS-induced expression of mitoNEET alters inflammatory responses, we generated mitoNEET shRNA or control shRNA-expressing cells. Real-time PCR and western blot analyses were performed to assess expression of mitoNEET mRNA and protein, respectively (Fig. 3A and B). Expression of mRNA encoding IL-1β, IL-6, TNFα, iNOS, and COX2 decreased in mitoNEET shRNA-expressing cells compared with control shRNA-expressing cells (Fig. 3C–G). This was also the case for protein expression (Fig. 3H–K). These data indicate that LPS-stimulated expression of mitoNEET is involved in inflammatory responses by macrophages via release of pro-inflammatory cytokines and mediators.

To assess the effect of mitoNEET inhibition during LPS-induced sepsis, we injected NL-1 intraperitoneally into wild-type C57BL/6 mice 12 hours prior to injection of LPS. Blood was collected from the right atrium 48 hours after LPS injection, and the concentration of IL-6 and TNFα in serum was measured. Control mice received vehicle alone. The levels of IL-6 and TNFα fell markedly in the presence of NL-1 (Fig. 4A and 4B). Liver- and spleen-mediated immune responses are responsible for clearing bacteria and toxins, but they can also cause inflammation and organ damage [31, 32]. We found

that NL-1 reduced expression of mRNA encoding iNOS (Fig 4C) and COX2 (Fig. 4D) in the spleen and liver of mice with LPS-induced sepsis. Taken together, these data demonstrate that mitoNEET is a key regulator of inflammatory responses during LPS-induced sepsis.

Inhibition of mitoNEET attenuates LPS-induced oxidative stress and mitochondrial dysfunction

Inflammatory processes induce oxidative stress and alter mitochondrial function [20]. When cells are under oxidative stress, mitoNEET acts as a redox-sensitive protein to induce transfer of the [2Fe-2S] cluster in mitochondria and plays a role in production of ROS [33]. Therefore, we analyzed whether inhibiting mitoNEET protects RAW264.7 cells from LPS-induced oxidative stress and mitochondrial dysfunction. Cells were treated with LPS in the presence or absence of NL-1 for 24 hours. Next, total ROS and superoxide were assayed by flow cytometry using a ROS/Superoxide Detection kit. LPS-induced total ROS and superoxide fell in the presence of NL-1 (Fig. 5A and B). Furthermore, to investigate whether downregulation of mitoNEET regulates oxidative stress, we stimulated control shRNA- or mitoNEET shRNA-expressing RAW264.7 cells for 12 hours with LPS (1 μg/mL), and measured total ROS and superoxide levels by flow cytometry. Total ROS and superoxide levels fell in LPS-treated cells expressing mitoNEET shRNA (Fig. 5C and D). In addition, confocal microscopy clearly demonstrated that NL-1 or mitoNEET shRNA suppressed LPS-induced cytosolic ROS when compared with LPS alone (Fig. 5E and F). Treatment of cells with the iron chelator DFO (deferoxamine) in the presence of LPS showed results comparable to those observed after mitoNEET inhibition by NL-1 or mitoNEET shRNA (Fig. 5E and F). To verify whether inhibition of mitoNEET regulates mitochondrial dysfunction, we examined the mitochondrial membrane potential (MMP), a hallmark of mitochondrial dysfunction. Cells were treated NL-1 in the presence or absence of LPS, and the loss of MMP was measured by flow cytometry using MitoProbe JC-1. Inhibition of mitoNEET rescued LPS-induced depolarization of the mitochondrial membrane (Fig. 6A). LPS-induced loss of MMP was also rescued by mitoNEET shRNA (Fig. 6B). To verify the effects of NL-1 on the MMP, we stained LPS-stimulated RAW264.7 cells with mitochondrial probes MitoTracker Red CMXRos, DiOC6(3), or TMRM (tetramethylrhodamine, methyl ester) in the presence or absence of NL-1. TMRM staining is used widely to monitor MMP. The MMP in LPS-treated cells fell but was rescued by NL-1 (Fig. 6C). In addition, confocal microscopy clearly demonstrated that mitoNEET shRNA rescued LPS-induced loss of MMP when compared with LPS alone (Fig. 6D). Treatment of cells with the iron chelator DFO

(deferoxamine) in the presence of LPS showed results comparable to those observed after mitoNEET inhibition by NL-1 or mitoNEET shRNA (Fig. 6C and D). To verify that inhibition of mitoNEET decreases the mitochondrial iron content, we stained mitochondrial iron using the mitochondrial probes Mitochondrial Marker Deep Red and Mito-ferroGreen by, which allow visualization of ferrous ion (Fe2+) by confocal microscopy. We found that NL-1 depleted mitochondrial Fe2+ in the presence of LPS. Similar results were obtained using DFO (Fig. 6E). Furthermore, mitochondrial Fe2+ was depleted in LPS-treated cells expressing mitoNEET shRNA (Fig. 6F). DFO showed similar effects. Taken together, these data suggest that inhibiting mitoNEET in RAW264.7 cells reduces mitochondrial iron content, thereby preventing oxidative stress and mitochondrial dysfunction during LPS-induced inflammation.

Oxidant-induced injury during inflammatory processes such as sepsis induce organ failure [9]. To confirm the anti-inflammatory effects of NL-1 during LPS-induced oxidative stress, we examined expression of HO-1 (heme oxygenase-1), SOD2 (superoxide dismutase 2), and SOD1 (superoxide dismutase 1) mRNA and protein in LPS-stimulated RAW264.7 cells in the presence or absence of NL-1 (Fig. 7A–D). NL-1 increased expression of HO-1 and SOD2 mRNA and protein, but not that of SOD1 mRNA and protein. Consistent with this, HO-1 and SOD2 mRNA and protein levels in cells expressing mitoNEET shRNA were higher than those in control shRNA-expressing cells (Fig. 7E–H). These results demonstrate that NL-1 or mitoNEET shRNA may attenuate oxidative-induced organ injury during LPS-induced inflammation by upregulating expression of antioxidant defense genes.

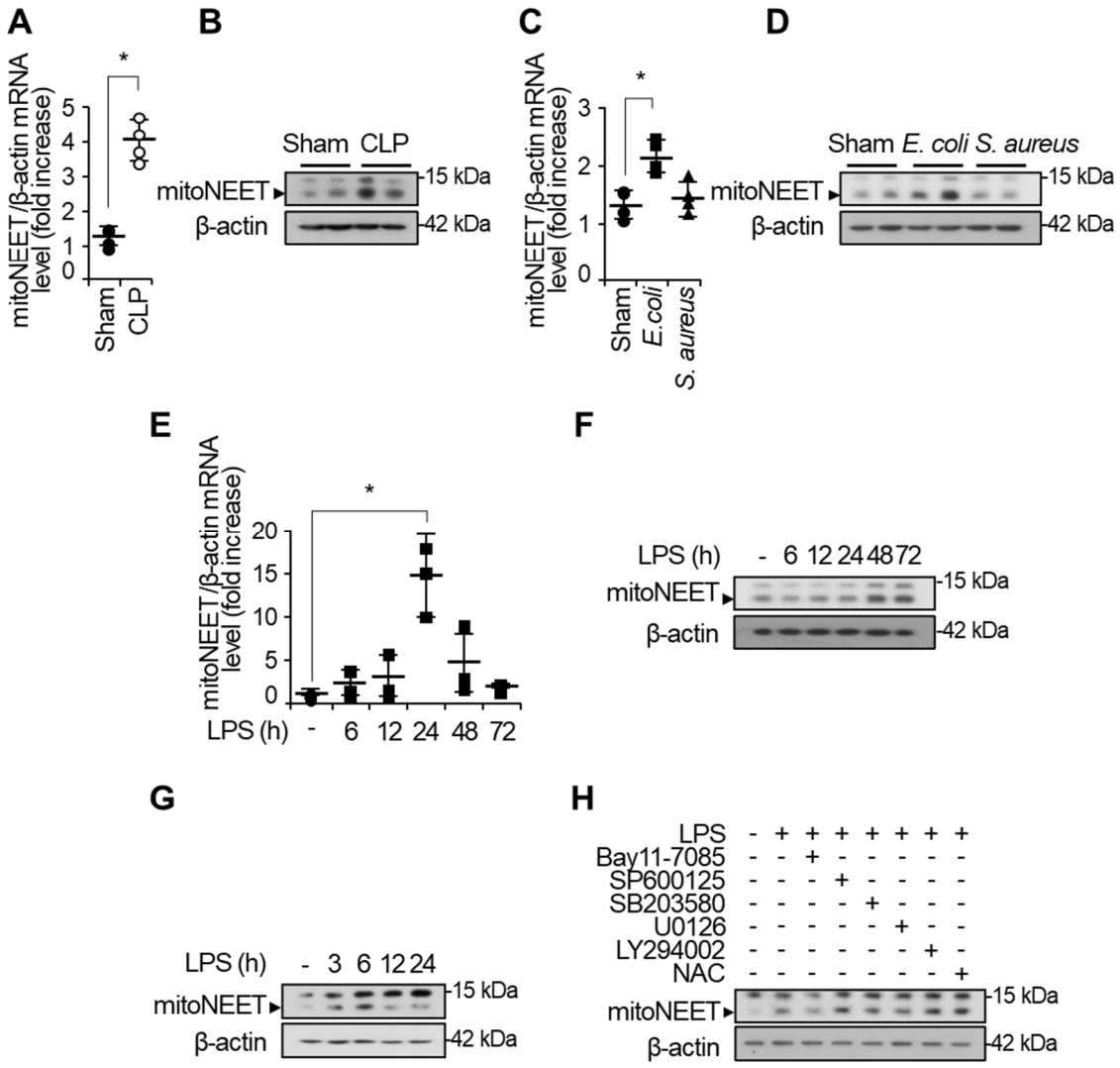
A
B
C
D
E
F
G
H
mitoNEET/β-actin mRNA level (fold increase)
Sham
CLP
Sham CLP
mitoNEET
β-actin
15 kDa
42 kDa
*
E.coli
S. aureus
Sham E. coli S. aureus
LPS (h) - 6 12 24 48 72
LPS (h) - 3 6 12 24
LPS - + + + + + + +
Bay11-7085 - - + - - - - -
SP600125 - - - + - - - -
SB203580 - - - - + - - -
U0126 - - - - - + - -
LY294002 - - - - - - + -
NAC - - - - - - - +

Figure 1. Expression of mitoNEET mRNA and protein increases during microbial sepsis.

Total RNA and protein were extracted from the spleen 48 hours after sham or CLP surgery, and fibrin clot-induced microbial sepsis was triggered by E. coli or S. aureus bacteria (1×10^8 CFU). Expression of mitoNEET mRNA (A, C) and protein (B, D) levels was assessed by quantitative real-time RT-PCR or western blotting. *$P < 0.05$ for sham vs. CLP or fibrin clot-induced microbial sepsis. C57BL/6 mice were injected with LPS (20 mg/kg) or vehicle, and total RNA and protein were extracted from the spleen 6, 12, 24, 48, and 72 hours after administration of vehicle or LPS (100 ng/mL). Total protein was extracted from BMDMs 3, 6, 12, and 24 hours after administration of vehicle or LPS (100 ng/mL). Expression of mitoNEET mRNA (E) and protein (F, G) was assessed by quantitative real-time RT-PCR or western blotting. Total protein was extracted from BMDMs 6 hours after administration of vehicle or LPS (100 ng/mL) plus a signaling inhibitor (5 μM BAY11-7082, 10 μM SP600125, 10 μM SB203580, 10 μM U0126, 10 μM LY2940002, or 20 mM NAC). Expression of mitoNEET protein was assessed by western blotting (H). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for vehicle vs. LPS treatment.

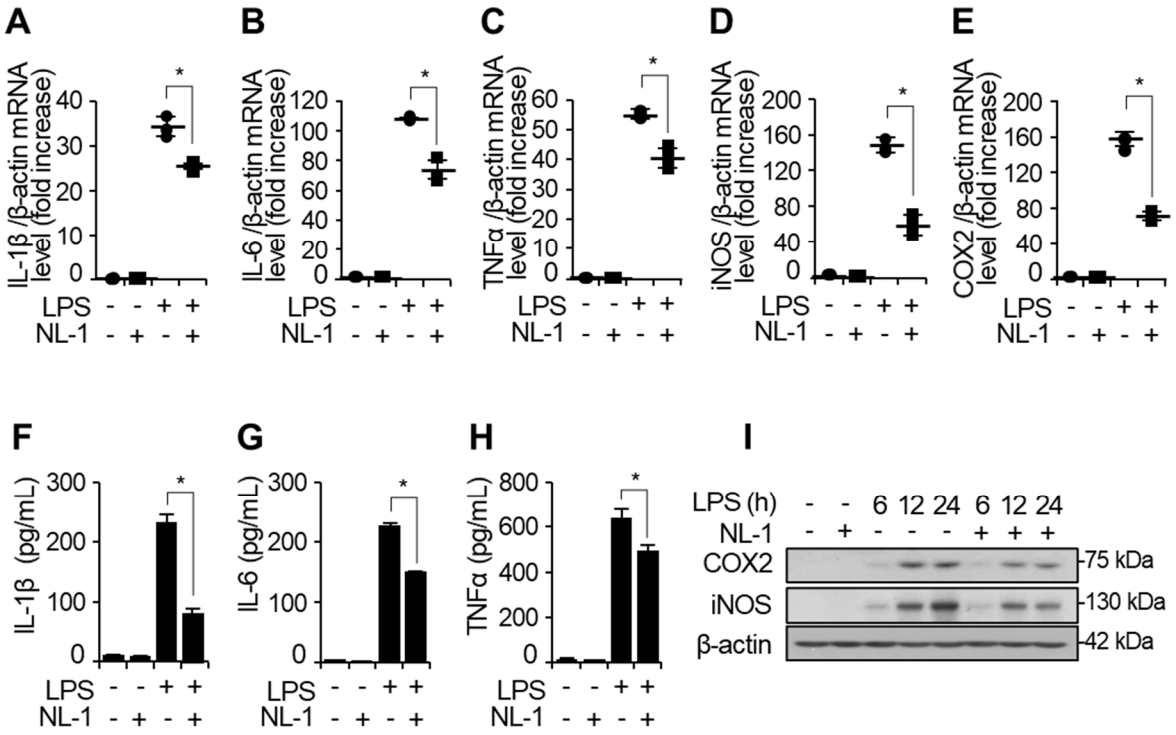

Figure 2. Expression of inflammatory mediators decreases in the presence of the mitoNEET inhibitor NL-1.

Total RNA, cell supernatants, and total protein were harvested from RAW264.7 cells 12 hours (for mRNA) and 24 hours (for protein) after treatment with vehicle, NL-1 (20 μM), LPS (100 ng/mL), or LPS plus NL-1 (20 μM). Levels of mRNA encoding pro-inflammatory mediators IL-1β, IL-6, TNFα, iNOS, and COX2 were measured by quantitative real-time RT-PCR (A–E). Protein levels of pro-inflammatory mediators were analyzed using ELISA or western blotting (F–I). All data are presented as the mean ± SD from three independent experiments. *$P < 0.05$ for LPS vs. LPS plus NL-1.

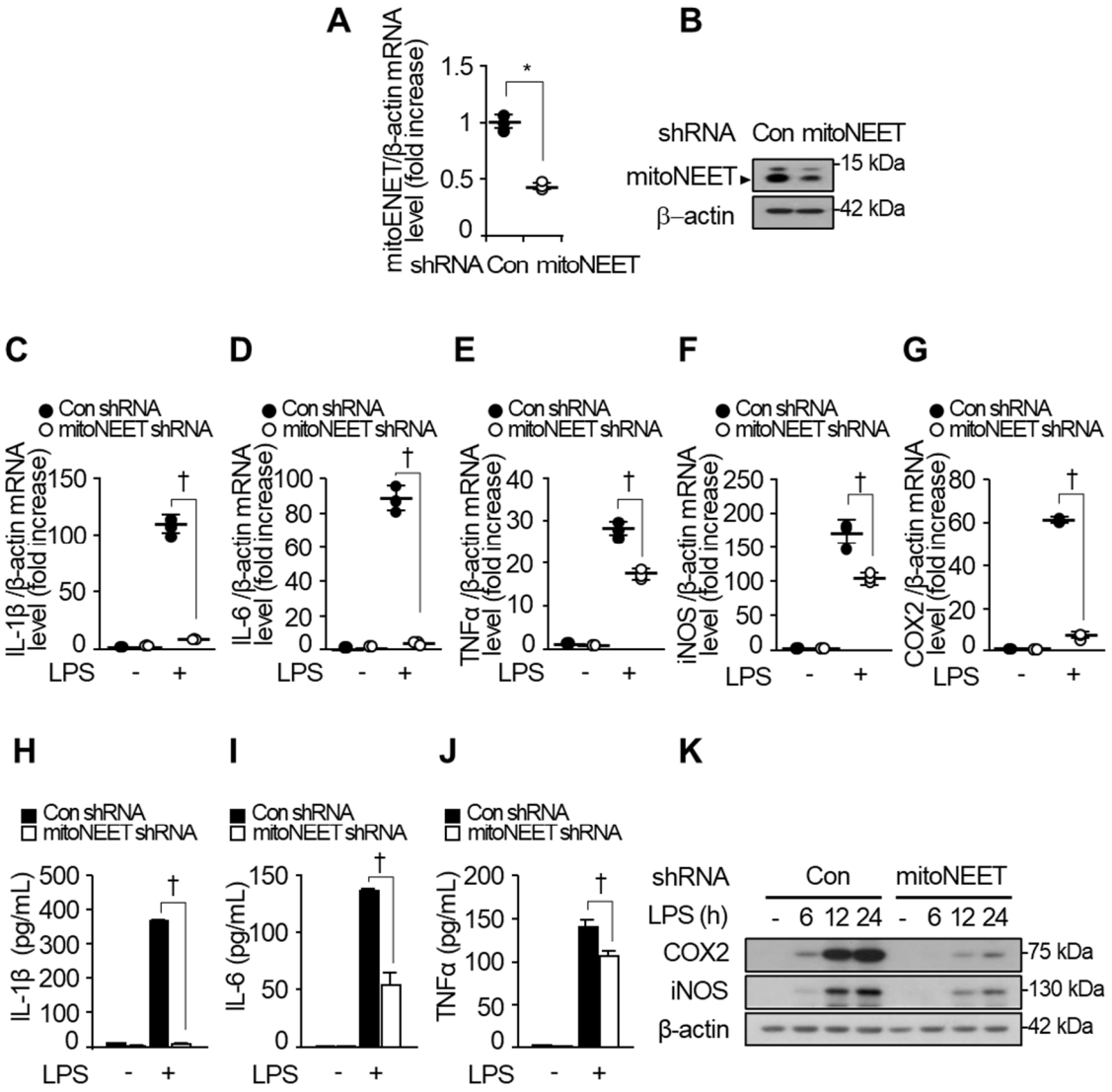
A
mitoENET/β-actin mRNA level (fold increase)
1.5
1
0.5
0
*
shRNA Con mitoNEET
B
shRNA Con mitoNEET
mitoNEET
β–actin
15 kDa
42 kDa
C
D
E
F
G
Con shRNA
mitoNEET shRNA
IL-1β /β-actin mRNA level (fold increase)
IL-6 /β-actin mRNA level (fold increase)
TNFα /β-actin mRNA level (fold increase)
iNOS /β-actin mRNA level (fold increase)
COX2 /β-actin mRNA level (fold increase)
LPS - +
†
H
I
J
K
IL-1β (pg/mL)
IL-6 (pg/mL)
TNFα (pg/mL)
shRNA Con mitoNEET
LPS (h) - 6 12 24 - 6 12 24
COX2
iNOS
β-actin
75 kDa
130 kDa
42 kDa

Figure 3. Expression of mitoNEET shRNA decreases the levels of inflammatory mediators produced by RAW264.7 cells.

RAW264.7 cells were transfected with control shRNA or mitoNEET shRNA and then subjected to RT-PCR or western blotting to verify downregulation of mitoNEET expression (A and B). β-actin was used as a loading control. RAW264.7 cells transfected with control shRNA or mitoNEET shRNA were stimulated with LPS (100 ng/mL) or vehicle. Total RNA, cell supernatants, and total protein were harvested 12 hours (for mRNA) or 24 hours (for protein) later. Expression of mRNA encoding IL-1β, IL-6, TNFα, iNOS, and COX2 was assessed by quantitative real-time RT-PCR (C–G), and protein expression was analyzed by ELISA or western blotting (H–K). All data are expressed as the mean ± SD from three independent experiments. $*P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells. $\dagger P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of LPS.

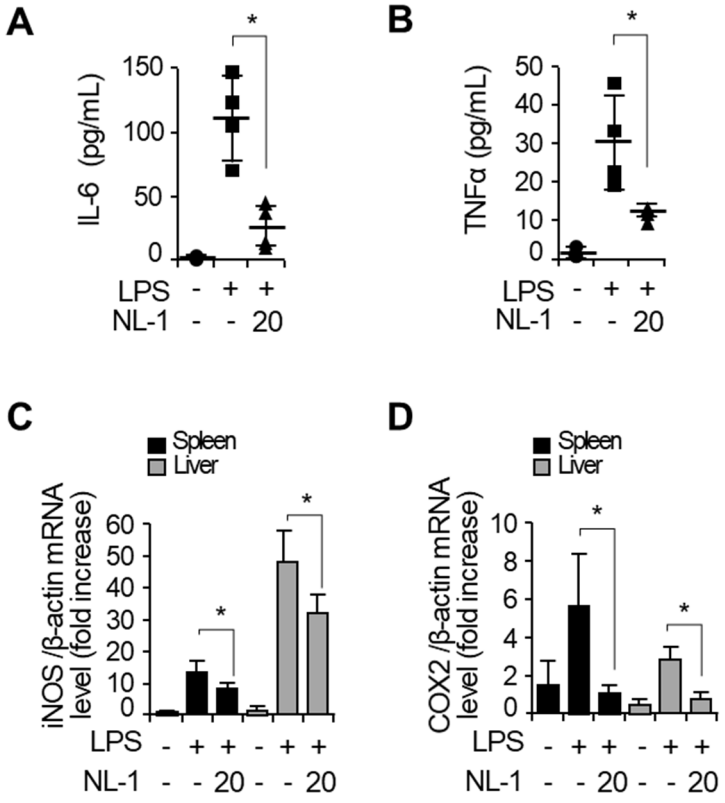

Figure 4. Expression of inflammatory mediators triggered by LPS-induced sepsis was diminished in the presence of a mitoNEET inhibitor.

C57BL/6 mice were injected intraperitoneally with vehicle, LPS (21 mg/kg), or LPS plus NL-1 (20 mg/kg). Blood was collected from the right atrium 48 hours later, and IL-6 and TNFα levels in serum were measured (A and B). Total RNA was harvested from the spleen and liver. Expression of mRNA encoding iNOS and COX2 was assessed in the spleen or liver (C and D) by quantitative real-time RT-PCR. For all real-time PCR analyses, β-actin was used as a control for normalization. All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for LPS vs. LPS plus NL-1 treatment.

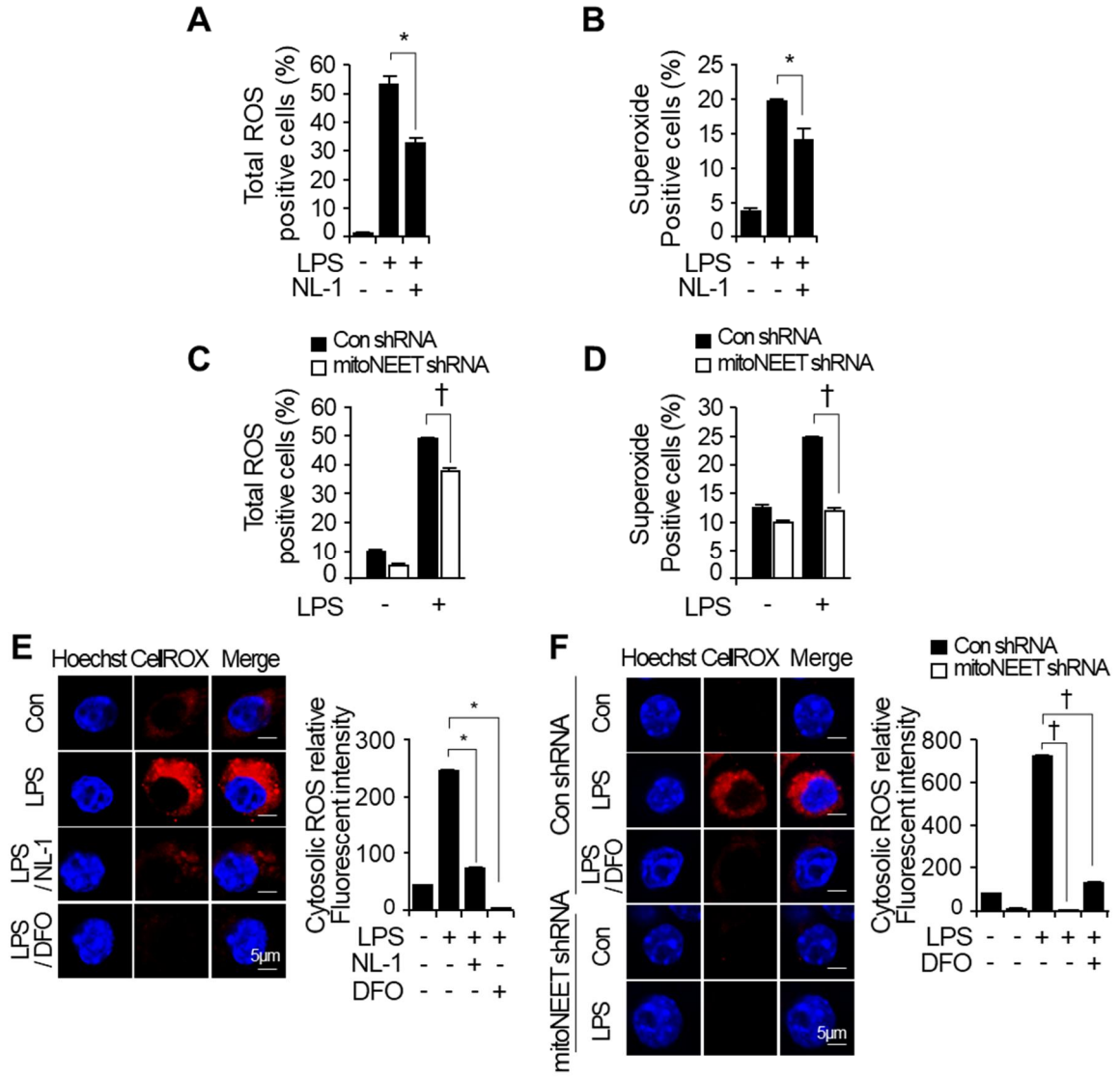
A
Total ROS positive cells (%)
60
50
40
30
20
10
0
*
LPS - + +
NL-1 - - +
B
Superoxide Positive cells (%)
25
20
15
10
5
0
*
LPS - + +
NL-1 - - +
C
Con shRNA
mitoNEET shRNA
Total ROS positive cells (%)
60
50
40
30
20
10
0
†
LPS - +
D
Con shRNA
mitoNEET shRNA
Superoxide Positive cells (%)
30
25
20
15
10
5
0
†
LPS - +
E
Hoechst CellROX Merge
Con
LPS
LPS /NL-1
LPS /DFO
5μm
Cytosolic ROS relative Fluorescent intensity
300
200
100
0
*
*
LPS - + + +
NL-1 - - + -
DFO - - - +
F
Hoechst CellROX Merge
Con shRNA
mitoNEET shRNA
Con
LPS
LPS /DFO
Con
LPS
5μm
Con shRNA
mitoNEET shRNA
Cytosolic ROS relative Fluorescent intensity
800
600
400
200
0
†
†
LPS - - + + +
DFO - - - - +

Figure 5. LPS-induced reactive oxygen species and mitochondrial dysfunction are attenuated by inhibition of mitoNEET.

RAW264.7 cells expressing control shRNA or mitoNEET shRNA were treated with vehicle, LPS (1 μg/mL), or LPS plus NL-1 (20 μM) for 24 hours. Total reactive oxygen species (ROS) and superoxide anions were assayed by flow cytometry using a ROS/Superoxide Detection kit (A–D). All data shown are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for LPS vs. LPS plus NL-1 treatment; †$P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of LPS. *$P < 0.05$ indicates a significant decrease compared with vehicle; †$P < 0.05$ indicates significant decrease compared with control siRNA. RAW264.7 cells expressing control shRNA or mitoNEET shRNA were treated with vehicle or LPS (1 μg/mL) for 12 hours. Cells were then stained with the fluorescent probes Hoechst 33258 (nuclei, blue) and CellROX® Deep Red (total ROS, red). Immunofluorescence images of cells stained with Hoechst 33258 (nuclei, blue) and CellROX® Deep Red (total ROS, red) (E and F). Scale bar: 5 μm. Fluorescence intensity was measured using image analysis software (ImageJ). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for LPS vs. LPS plus NL-1 or DFO. †$P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of LPS or vs. LPS plus DFO.

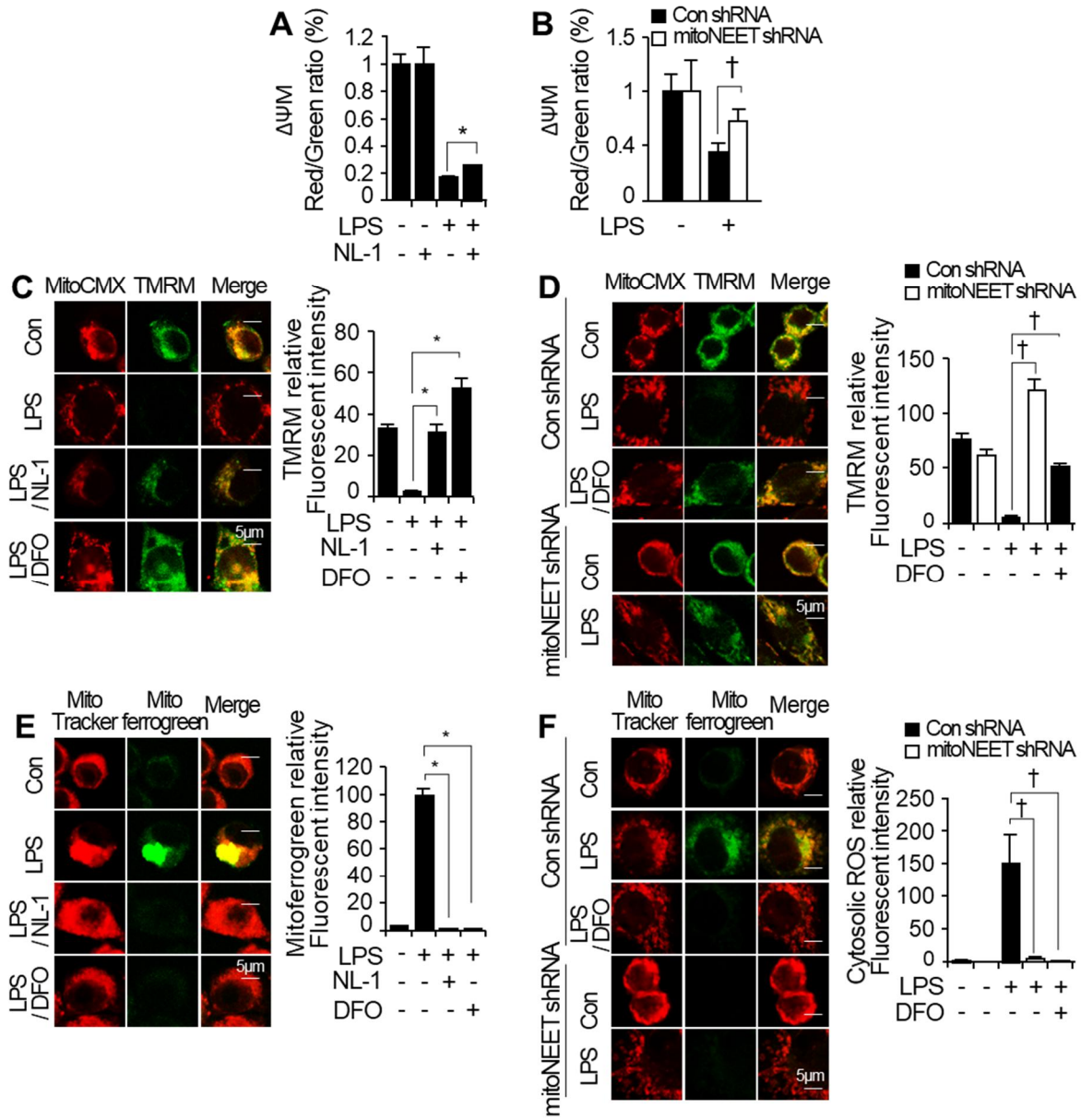
A
ΔΨM Red/Green ratio (%)
1.2
1
0.8
0.6
0.4
0.2
0
*
LPS - - + +
NL-1 - + - +
B
Con shRNA
mitoNEET shRNA
ΔΨM Red/Green ratio (%)
1.5
1
0.4
0
†
LPS - +
C
MitoCMX TMRM Merge
Con
LPS
LPS /NL-1
LPS /DFO
5μm
TMRM relative Fluorescent intensity
80
60
40
20
0
*
*
*
LPS - + + +
NL-1 - - + -
DFO - - - +
D
MitoCMX TMRM Merge
Con shRNA
Con
LPS
LPS /DFO
mitoNEET shRNA
Con
LPS
5μm
Con shRNA
mitoNEET shRNA
TMRM relative Fluorescent intensity
150
100
50
0
†
†
†
LPS - - + + +
DFO - - - - +
E
Mito Tracker
Mito ferrogreen
Merge
Con
LPS
LPS /NL-1
LPS /DFO
5μm
Mitoferrogreen relative Fluorescent intensity
120
100
80
60
40
20
0
*
*
*
LPS - + + +
NL-1 - - + -
DFO - - - +
F
Mito Tracker
Mito ferrogreen
Merge
Con shRNA
Con
LPS
LPS /DFO
mitoNEET shRNA
Con
LPS
5μm
Con shRNA
mitoNEET shRNA
Cytosolic ROS relative Fluorescent intensity
250
200
150
100
50
0
†
†
†
LPS - - + + +
DFO - - - - +

Figure 6. LPS-induced ROS and mitochondrial dysfunction are activated by mitoNEET-mediated iron accumulation.

RAW264.7 cells expressing control shRNA or mitoNEET shRNA were treated with vehicle, LPS (1 μg/mL) or LPS plus NL-1 (20 μM) for 24 hours. The mitochondrial membrane potential (MMP) was measured by flow cytometry using MitoProbe JC-1 (A and B). The histogram shows the ratio of JC-1 polymer (red) to JC-1 monomer (green) fluorescence, which is an index of the MMP. A decrease in the red/green ratio indicates depolarization of the mitochondrial membrane. RAW264.7 cells expressing control shRNA or mitoNEET shRNA were treated with vehicle, LPS (1 μg/mL), LPS plus NL-1 (20 μM), or LPS plus DFO (500 μM) for 12 hours. Cells were then stained with MitoTracker Red CMXRos, DiOC6(3), (red), and TMRM (tetramethylrhodamine, methyl ester, Perchlorate, green). (C and D). Cells were stained with MitoTracker Deep Red (a mitochondrial marker; red) and Mito-FerroGreen (a marker of mitochondrial Fe2+; green) after treatment (E and F). Immunofluorescence images of MitoTracker Red CMXRos, DiOC6(3), (mitochondria, red), TMRM (green), and MitoTracker Deep Red, Mito-FerroGreen (green). Scale bar: 5 μm. Fluorescence intensity was measured using ImageJ. All data are expressed as the mean ± SD from three independent experiments. $*P < 0.05$ for LPS vs. LPS plus NL-1 or DFO. $\dagger P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of LPS or LPS plus DFO.

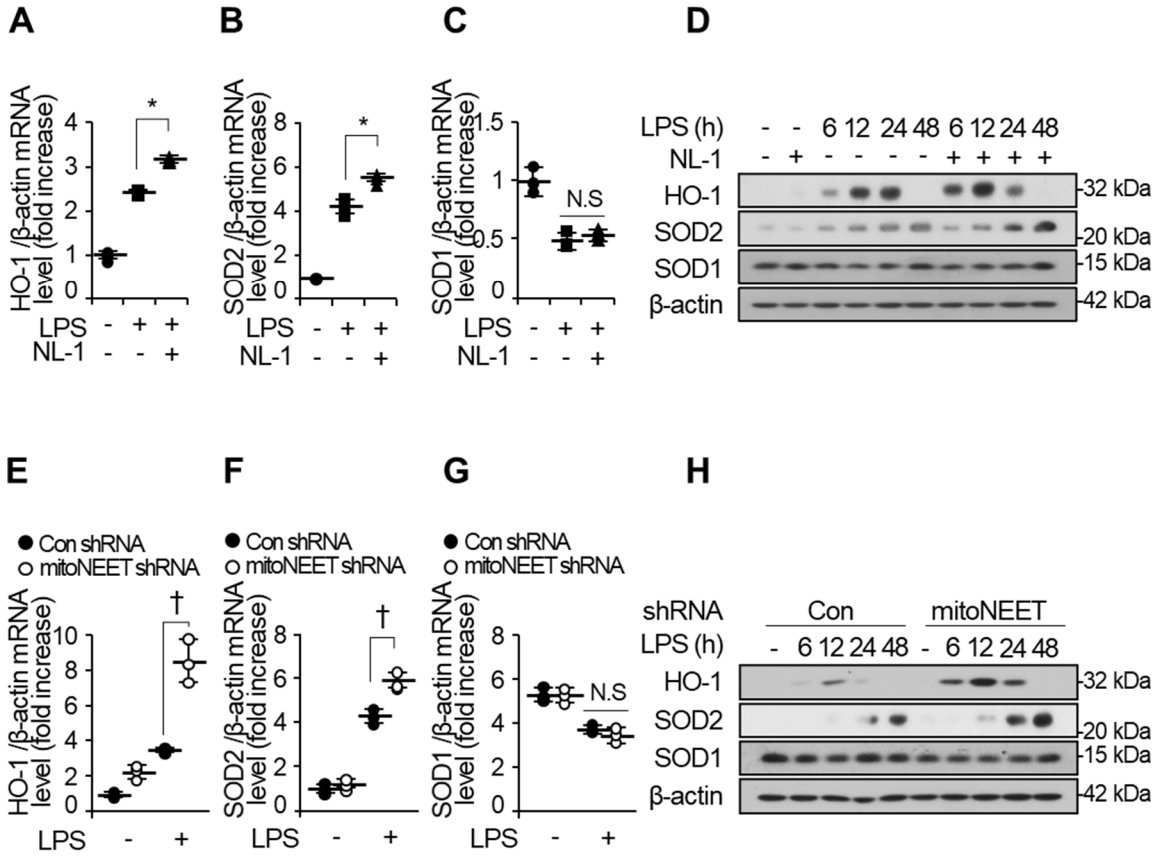

Figure 7. Inhibition of mitoNEET increases expression of antioxidant defense genes.

Control shRNA-expressing or mitoNEET-expressing RAW264.7 cells were treated with vehicle, LPS (1 μg/mL), or LPS plus NL-1 (20 μM). Total RNA and protein were harvested 12 hours later, followed by measurement of mRNA encoding HO-1, SOD2, or SOD1 by quantitative real-time RT-PCR (A–C and E–G). Expression of HO-1, SOD2, and SOD1 protein was detected by western blotting (D and H). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for LPS vs. LPS plus NL-1 treatment. †$P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of LPS. NS, not significant.

Discussion

Sepsis is one of the most serious causes of mortality worldwide. There is increasing evidence that oxidative stress plays a major role in organ dysfunction by driving excessive inflammation [21, 42, 43]. Inflammation-induced ROS production promotes dysfunction of mitochondria, a major site of ROS production, thereby activating oxidative stress and generating a self-feeding cycle [15, 44]. Thus, a therapeutic strategy targeting mitochondrial dysfunction has the potential to break this vicious cycle and prevent progression of oxidative stress and sepsis [21]. Recent studies suggest that iron is an essential component of cellular processes such as mitochondrial energy metabolism; however, mitochondrial iron overload is a major cause of mitochondrial damage and ROS [21, 45, 46]. In this study, we demonstrated that inhibiting mitoNEET, a mitochondrial iron regulator, has a protective effect against inflammatory responses during sepsis. MitoNEET, a mitochondrial protein, plays a key role in energy metabolism, iron regulation, and production of ROS by mitochondria [27, 47]. Our data show that inflammatory stimuli, such as LPS, which induce mitochondrial oxidative damage, trigger production of mitoNEET mRNA and protein in various animal models of and in BMDMs (Fig. 1). Furthermore, the LPS-induced NF-kB signaling pathway is involved in induction of mitoNEET expression during inflammatory conditions (Fig. 1H). These data suggest that mitoNEET could play a key role in energy metabolism as well as in inflammation. Interestingly, expression of inflammatory mediators IL-1β, IL-6, TNFα, iNOS, and COX2 decreased in the presence of a mitoNEET inhibitor, NL-1, or upon expression of mitoNEET shRNA, even in the LPS-induced sepsis model (Figs. 2, 3, and 4). MitoNEET was first identified as a redox-sensitive mitochondrial target of the thiazolidinedione (TZD) pioglitazone [36, 37]. Earlier studies observed that overexpression of mitoNEET inhibits mitochondrial iron transport to the matric and reduces ROS-mediated damage [29]. This led to a reduction in the MMP and accumulation of ROS in mouse adipocytes [30]. Conversely, we found that

ROS levels, MMP, and iron content were reduced in the presence of NL-1 or mitoNEET shRNA under inflammatory conditions (Fig. 5 and 6). Furthermore, expression of the antioxidant enzyme HO-1 and the mitochondrial MnSOD isoform SOD2 was enhanced in the presence of NL-1 or mitoNEET shRNA after LPS administration; however, expression of SOD1, a major cytoplasmic antioxidant enzyme, was not enhanced. The reason for these different results could be because mitoNEET acts differently according to the redox conditions in a cell. The biophysical properties of mitoNEET involve electron and Fe-S cluster transfer [25, 44, 45]. In a reducing environment, mitoNEET is incapable of [2Fe-2S] cluster transfer; thus accumulation of iron in the mitochondria is abrogated by accelerating loss of the [2Fe-2S] cluster [46]. However, only when cells are under oxidative stress does mitoNEET [2Fe-2S] transfer [2Fe-2S] clusters to apo-proteins, and electrons from NADH to oxygen or ubiquinone, in mitochondria [26, 27, 33]. In an oxidizing environment, mitoNEET contributes to oxidative stress and production of superoxide radicals ($O_2^{\bullet -}$) by transferring iron to the mitochondrial matrix and electrons to oxygen through oxidation of NADH (the electron donor). Recent publications demonstrated that pioglitazone stabilizes the 2Fe-2S cluster and inhibits iron transfer from mitoNEET to mitochondria. Pioglitazone, which shows strong preferential binding to mitoNEET in the oxidized state, may therefore act to alleviate stress caused by Fe overload [33]. These data suggest that iron regulation via targeting of mitoNEET rescues ROS production and mitochondrial dysfunction in the oxidized state [40, 47, 48]. Therefore, our results demonstrate that mitoNEET is a possible therapeutic molecule for mitochondrial dysfunction during inflammatory diseases and sepsis.

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PART 2.

The role of PGC-1α for antioxidant activity mediated by inhibition of mitoNEET in inflammatory response.

Abbreviations

AMPK	Adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator-1alpha
NADH	Nicotinamide adenine dinucleotide
MitoNEET	Mitochondrial protein containing NEET sequence
NL-1	NEET Ligand-1
TZD	Thiazolidinediones
PPARγ	Peroxisome proliferator- activated receptor gamma
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-10	Interleukin-10
TNF-α	Tumor necrosis factor-alpha
iNOS	Inducible nitric oxide synthase
COX2	Cyclooxygenase-2
HO-1	Heme oxygenase-1
SOD	Superoxide dismutase
CAT	Catalase
GSH	Glutathione
ETC	Electron transport chain
NF-κB	Nuclear factor kappa B activation

Abstract

Inflammatory disease, such as sepsis is associated with both oxidative stress mediated by mitochondrial dysfunction and glycolysis inhibiting antioxidant capacity. Glycolysis as a source of energy is activated in inflammatory response. Glycolytic ATP (adenosine triphosphate) production inhibit AMPK (adenosine monophosphate-activated protein kinase) activity and expression of PGC-1α (Peroxisome proliferator-activated receptor-gamma coactivator-1alpha). PGC-1α is tightly linked to the anti-ROS system through the production of antioxidant enzymes and plays the role of the induction of mitochondrial biogenesis. Therefore, the activation of PGC-1α through inhibition of glycolysis is an interesting therapeutic target that improved survival rates and protected septic mice from organ oxidative injury and maintained healthy mitochondria. Glycolysis requires NAD+ for use in the production of glycolytic ATP and if NAD+ is not regenerated, glycolysis will stop. Earlier studies demonstrated that in the presence of oxygen, NADH is oxidized to NAD+ through mitochondrial NADH shuttle systems and it is supplied to glycolysis. Recently, increasing evidences suggest that mitoNEET (mitochondrial protein containing NEET sequence) as a novel redox enzyme oxidizing cytosolic NADH to NAD+ may indirectly activate glycolysis and glycolytic ATP production. MitoNEET as a ligand of a thiazolidinedione (TZD) regulate mitochondrial ROS and iron homeostasis. Therefore, mitoNEET has been suggested as a possible molecule of mitochondrial therapeutic target for oxidative stress during damaged mitochondrial disease. However, under oxidizing environment, that inhibition of the electron transfer activity of mitoNEET as a generator of NAD+ activates PGC-1α through regulation of glycolytic ATP has not been studied yet. In this study, we investigated mechanisms of PGC-1α in antioxidant effect mediated by inhibiting mitoNEET during LPS-induced oxidative stress. Levels of PGC-1α protein was increased by inhibition of mitoNEET using inhibitor, or shRNA.

Pharmacological inhibition of mitoNEET with inhibitor, mitoNEET Ligand-1 (NL-1), suppressed LPS-induced ROS and increased antioxidant enzyme when compared with LPS alone in control shRNA cells, but not in PGC-1α shRNA expressing cells. Increase of PGC-1α expression by inhibition of mitoNEET is mediated though AMPK activity increased by prevention of ATP production under inflammatory stimuli. Taken together, these data demonstrated that PGC-1α is a key regulator in antioxidant effect mediated by inhibiting mitoNEET during inflammatory diseases. Therefore, we suggest that targeting mitoNEET is a possible therapeutic strategy bring the effect of energy metabolism control and antioxidant effect through activating PGC-1α in mitochondrial dysfunctional disease.

Introduction

1. Oxidative stress

Oxidative stress is a phenomenon referred to the accumulation of oxygen reactive species by an imbalance between production of oxidants and antioxidants defense (Figure. 1). The superoxide ($O2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$) are oxygen-derived free radicals as the most common ROS. Most ROS are generated as by-products during mitochondrial electron transport and are formed as necessary intermediates of metal catalyzed oxidation reactions. These ROS are necessary for the signaling pathways in biological processes such as cell migration, circadian rhythm, stem cell proliferation, and neurogenesis. In healthy systems, ROS are regulated by the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), and by antioxidants such as glutathione (GSH), vitamin A, ascorbic acid (AA), uric acid, hydroquinone, and vitamin E. When the production of ROS overwhelms the scavenging ability of the defense system, oxidative stress occurs, causing dysfunctions in cell metabolism. Inflammatory disease, such as sepsis is associated with oxidative stress and mitochondrial dysfunction and cause pathophysiological processes [1, 2, 3].

2. Glycolysis

Inflammatory cells rely heavily on glycolysis as a source of energy to fulfill their high energetic and biosynthetic demand rather than OXPHOS-mitochondrial respiration. During the peak of inflammation, immune cells preferentially use glycolysis as a source of energy. Nevertheless, Inflammatory cells still contain functional mitochondria, in which oxidative phosphorylation continues. Thus, the function of mitochondria in inflammation may be the generation of ROS for cells signaling

purpose, but not generation of ATP for energy production. Interestingly, metabolites that accumulate in glycolytic conditions have been described to reinforce the inflammatory response. Therefore, glycolysis pathway is possible to therapeutic targets for inflammation-associated pathologies (Figure 2). Glycolysis occurs in the cytoplasm. it is the process that creates ATP without the use of oxygen but can occur in the presence of oxygen. Even if cells rely primarily on mitochondrial oxidative phosphorylation (OXPHOS) to generate ATP for energy, glycolysis can act as an emergency backup for energy or as the preparation step before oxidative phosphorylation.

2.1. The necessity of NAD+ in Glycolysis

Glycolysis requires NAD+ for use in glycolysis again and if NAD+ is not regenerated, glycolysis will stop. In the presence of oxygen, NADH is oxidized in the mitochondria to regenerate NAD+. The electrons of NADH generated during glycolysis in the cytoplasm be transferred across the mitochondrial membrane through the NADH shuttle systems [6, 7]. The glycerol-3-phosphate (G-3-P) shuttle and malate/aspartate shuttle transfer the electrons from cytosolic NADH to mitochondrial FADH2. Then, the electrons are finally transferred to complex 1 of ETC (electron transport chain). In the glycerol phosphate shuttle, electrons of these mitochondrial NADH dehydrogenases transfer to DHAP and regenerate cytosolic NAD+ at the level of complex I (CI) of ETC [12]. When NADH oxidation by the mitochondria cannot keep pace with glycolysis, glycolysis cannot continue by the insufficient supply of NAD+. Under these conditions, glycolysis is received NAD+ through the enzyme lactate dehydrogenase (LDH) reducing lactic acid from pyruvate. The final product of glycolysis is pyruvate in aerobic settings and lactate as the reduction form of pyruvate by lactate dehydrogenase (LDH) in anaerobic conditions. Lactic acid production occurs always but promotes when glycolysis is going faster than the mitochondria can accommodate the cytosol NADH, regardless in the presence of oxygen [4, 5, 6, 7]. If NADH is reoxidized in a shuttle system, pyruvate can be used for TCA cycle by oxidation to acetyl coenzyme A (acetyl-CoA). Thus, the use of the shuttle systems allows for more ATP to be generated than by anaerobic glycolysis, by both oxidizing the cytoplasmically derived NADH and

TCA cycle derived NADH in ETC (Figure. 3) [11].

2.2. PGC-1α in glycolysis

Glycolytic ATP production inhibit AMPK (adenosine monophosphate-activated protein kinase) activity that opposing the metabolic switch towards glycolysis. AMPK is a master sensor of energy catabolic status. AMPK leads to induction of the expression of PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α), inhibits mTORC1 (mTOR Complex 1), and activates mitophagy by phosphorylating ULK1 (a serine/threonine protein kinase) [8, 9]. PGC-1α play the role of the production of critical antioxidant enzymes and the induction of mitochondrial biogenesis. Thus, AMPK-dependent response regulates metabolism, reprioritizes the expenditure of energy toward the functions necessary for survival, decreases oxidative damage from dysfunctional mitochondria and eventually stabilizes energy balance by mitochondrial biogenesis. Therefore, in glycolysis, inhibition of AMPK resulting in inhibited antioxidant capacity and anti-inflammatory stage [10]. (Figure. 4)

3. The role of PGC-1α

PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α) is key molecule to increase mitochondrial biogenesis and decreases oxidative damage from dysfunctional mitochondria. Thus, PGC-1α expression is important for resistance to oxidative damage increase, neurodegeneration, and apoptotic cell death. In mammalian cells, it has been shown that biogenesis of mitochondria as a main ROS source production is tightly linked to the anti-ROS system [13]. Previously, several studies demonstrated that PGC-1α regulate the expression of genes encoding enzymes involved in the reactive oxygen species (ROS) defense system. Antioxidant enzymes regulated by PGC-1α are catalase and manganese superoxide dismutase (SOD2/MnSOD), peroxiredoxin 3, Heme oxygenase-1 (HO-1). During inflammation, low levels of PGC-1α by inhibition of AMPK by glycolysis downregulate mitochondrial antioxidant gene expression, induce oxidative stress, and promote nuclear factor kappa B activation (NF-κB) [16]. Therefore, several studies suggest that inhibition of glycolysis improved

survival rates and protected septic mice from organ injury through the activation of PGC-1α [14, 15]. PGC-1α acts as an essential node connecting metabolic regulation, redox control, and inflammatory pathways, and it is an interesting therapeutic target that may have significant benefits for a number of metabolic diseases and maintain healthy mitochondria (Figure. 5).

4. The role of mitoNEET in glycolysis

mitoNEET, mitochondrial outer membrane protein containing 2Fe-2S cluster was initially identified as a binding target of the type diabetes drug thiazolidinediones, such as pioglitazone [17, 18]. Increasing evidence has shown that mitoNEET is a key regulator of the energy metabolism, iron homeostasis, and production of reactive oxygen species in mitochondria [19, 20, 21]. Especially, recent studies demonstrate another function of mitoNEET that under oxidizing environment, mitoNEET oxidizes NADH in the mitochondrial matrix to regenerate the NAD+ pool, mediating the production of superoxide radicals (O2•-) [22, 23]. Some studies suggest that the electron transfer function of mitoNEET could oxidize NADH generated during glycolysis in cytosol and provide NAD+ to glycolysis. Glycolysis requires NAD+ for use in glycolysis again. NADH generated by glycolysis is oxidized by flavin reductase and FMNH2 is reduced. And then, mitoNEET could facilitate the rapid electron transfer from FMNH2 to oxygen (Figure. 6) [23, 24]. Since NAD+ generated by oxidation of NADH in cytosol is required for glycolysis activity and glycolytic ATP production, mitoNEET as a novel redox enzyme catalyzing electron transfer may indirectly regulate glycolysis by promoting oxidation of cytosolic NADH [17, 24, 25]. These showed that mitoNEET could be a novel regulator of glycolysis. Therefore, these suggest that inhibition of the electron transfer activity of mitoNEET could decrease glycolysis and overall energy metabolism in cells [17]. (Figure. 6).

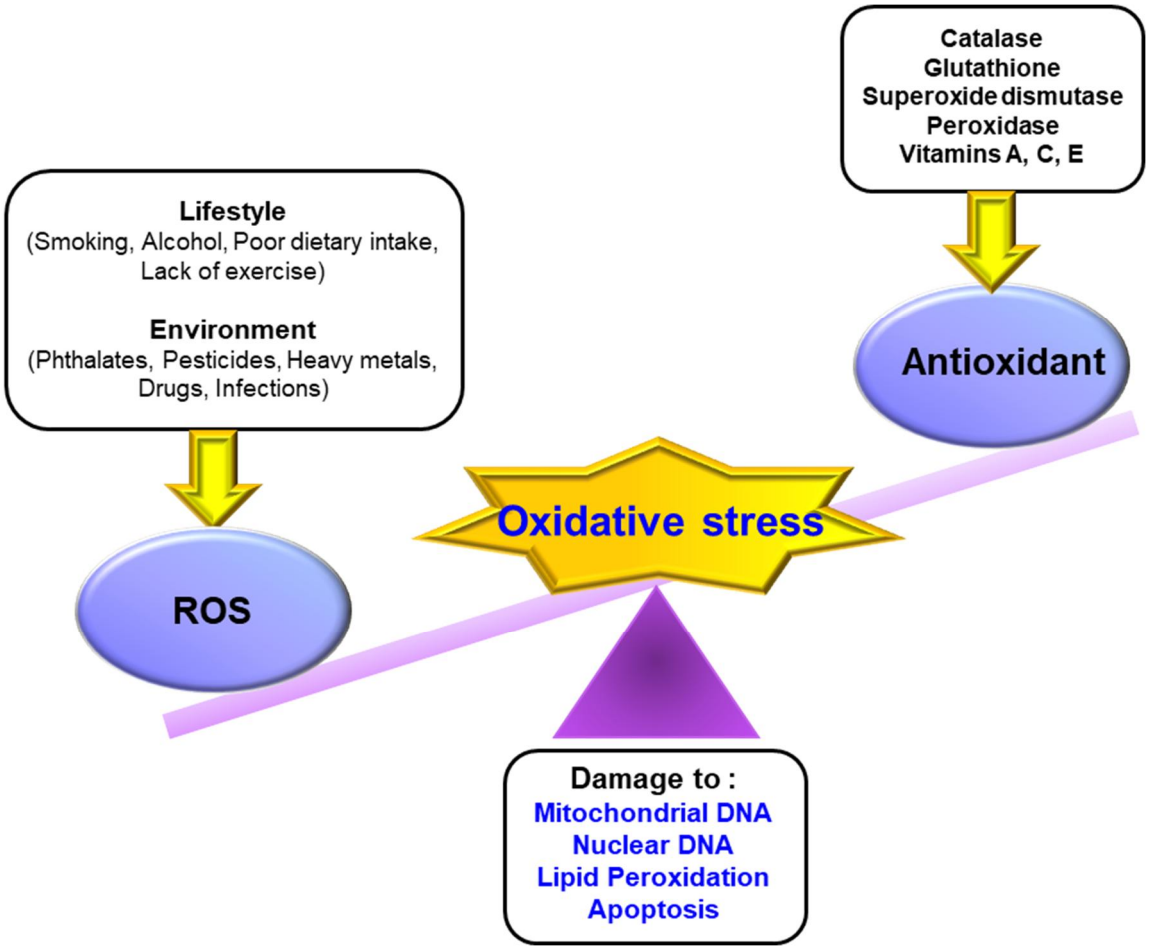

Figure 1. Oxidative stress induced by imbalance between ROS and antioxidants [33].

A healthy individual maintains a proper balance between their ROS generating and scavenging systems. If, however, the balance tilts toward the generation of ROS, excess ROS accumulate, rather than being scavenged, leading to oxidative stress.

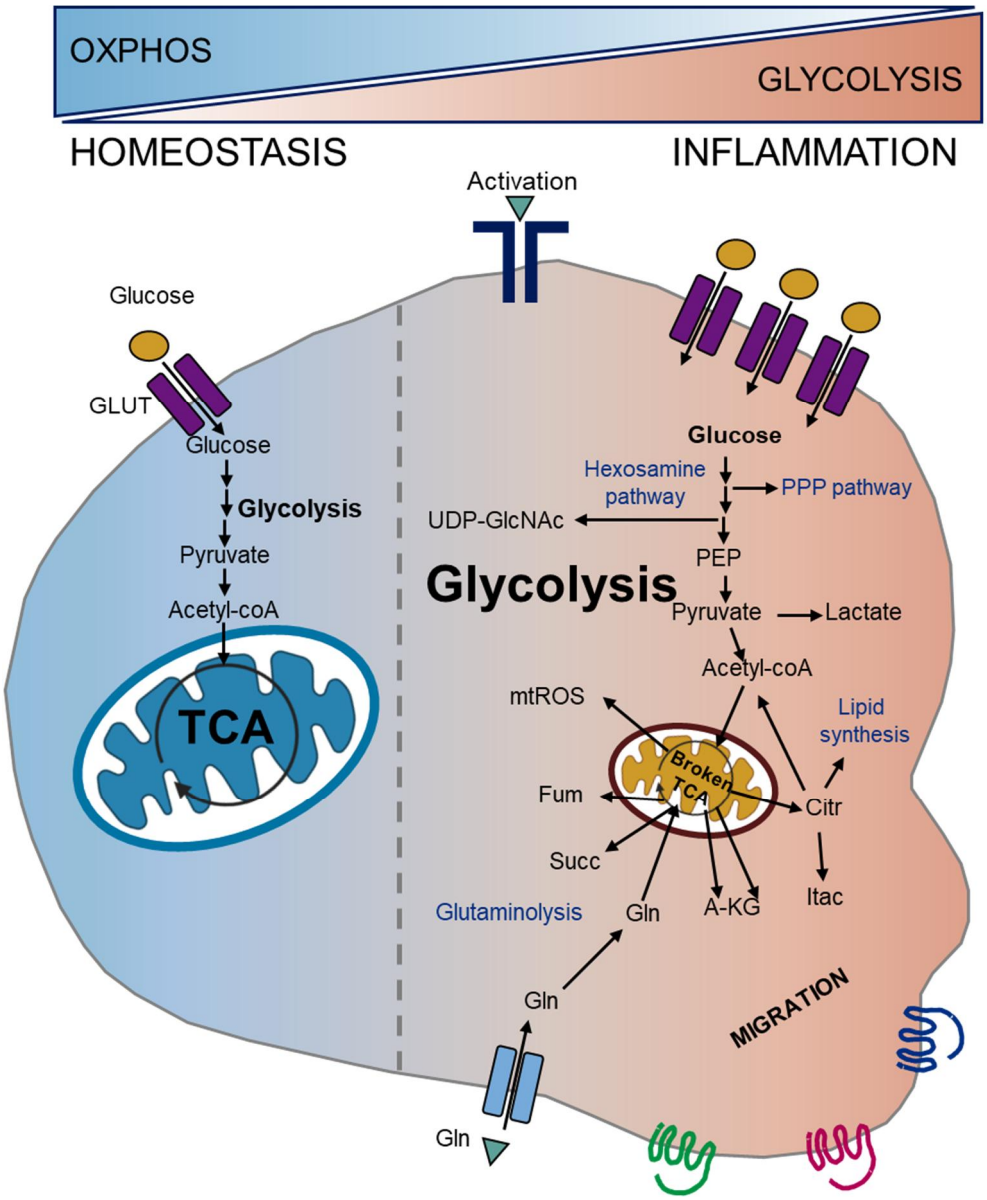

Figure 2. Glycolysis, inflammation, oxidative stress [34].

Glycolysis orchestrates the behaviour of inflammatory cells. Upon inflammation, immune cells are reprogrammed towards glycolysis, enhancing this pathway at the expense of OXPHOS and, in macrophages, disbalancing the TCA cycle. As a consequence, glycolysis and broken TCA cycle released metabolites are accumulated in immune cells promoting the inflammatory response.

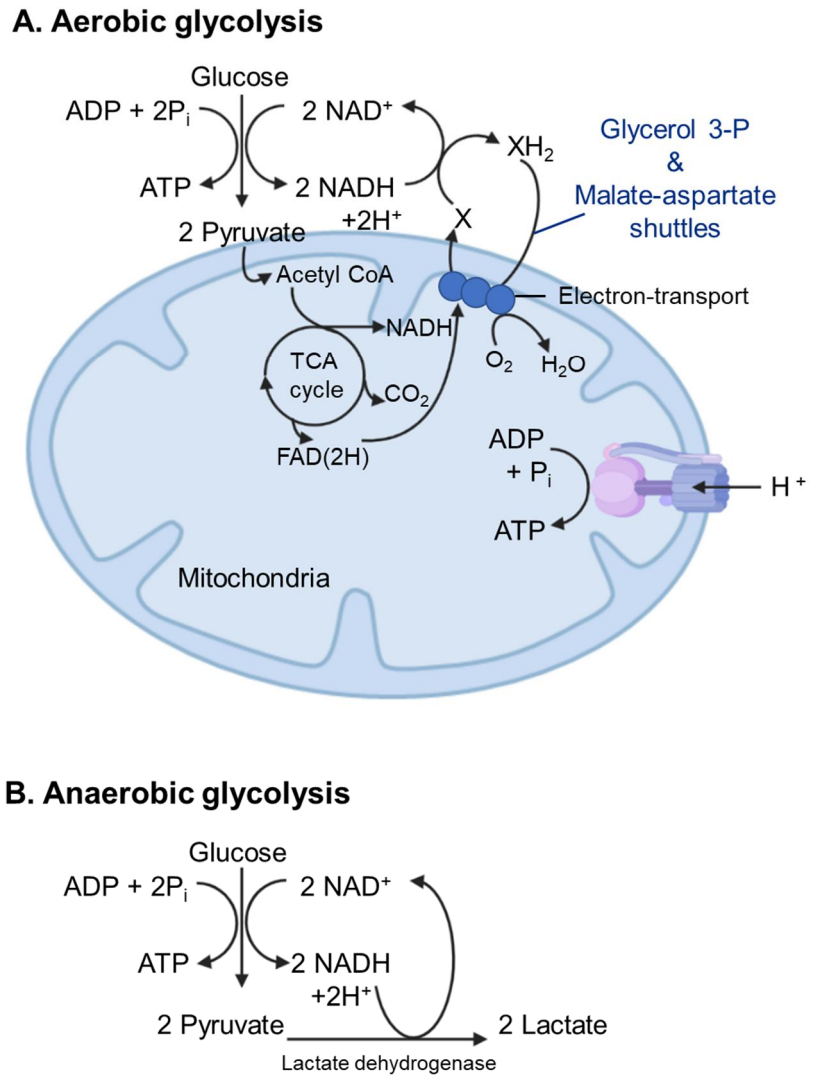

Figure 3. Aerobic glycolysis involving mitochondrial shuttle system and anaerobic glycolysis involving LDH, for supply of NAD+ [11].

The NADH produced from glycolysis must be continuously reoxidized back to NAD+. here are two alternate routes for oxidation of cytosolic NADH. One route is aerobic, involving shuttles that transfer reducing equivalents across the mitochondrial membrane and ultimately to the ETC and oxygen. The other route is anaerobic. The use of the shuttle systems allows for more ATP to be generated than by anaerobic glycolysis, by both oxidizing the cytoplasmically derived NADH in the ETC and by allowing pyruvate to be oxidized completely to CO_2. (Created with BioRender.com)

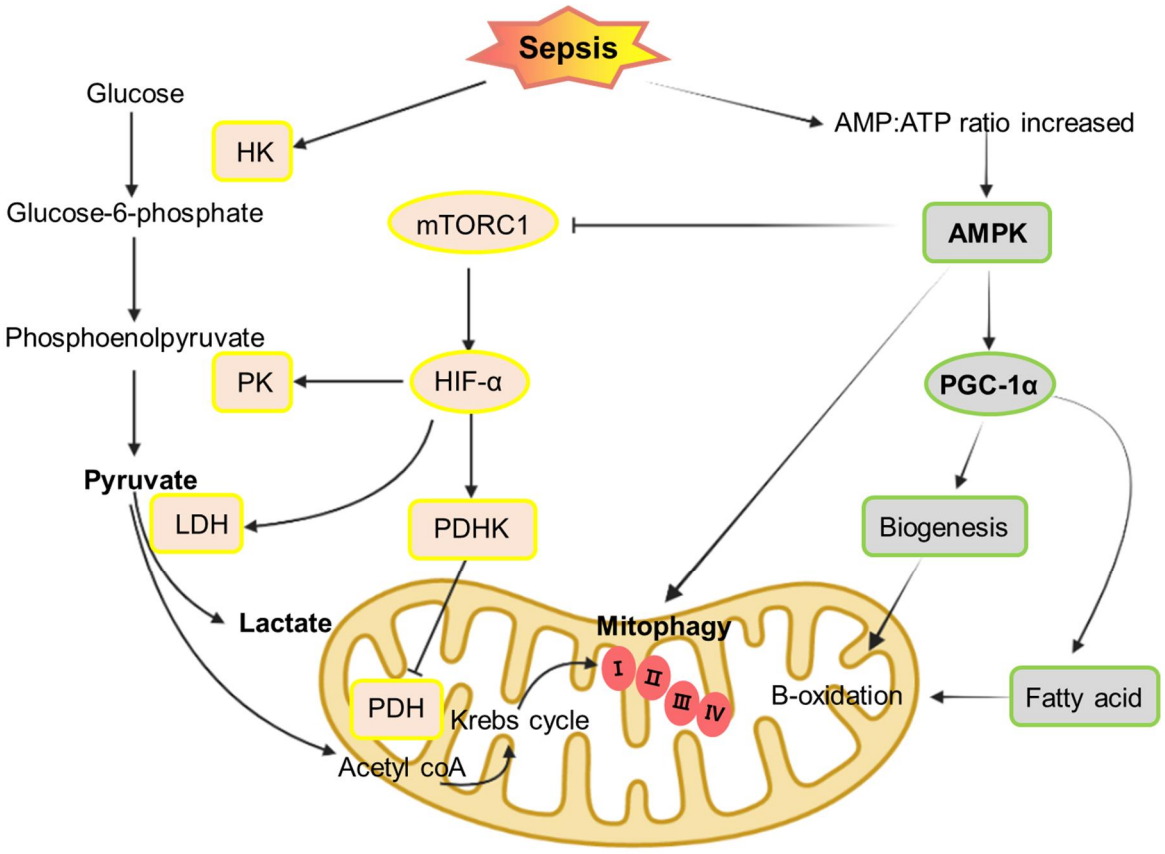

Figure 4. Glycolysis and AMP-activated protein kinase (AMPK) in sepsis [8].

During sepsis, as glycolytic ATP levels increase, adenosine monophosphate–activated protein kinase (AMPK), a master sensor of energy catabolic status, is downregulation. Activation of AMPK can lead to the production of critical antioxidant enzymes and the induction of mitochondrial biogenesis by peroxisome proliferator–activated receptor g coactivator–1a (PGC-1a). (Created with BioRender.com)

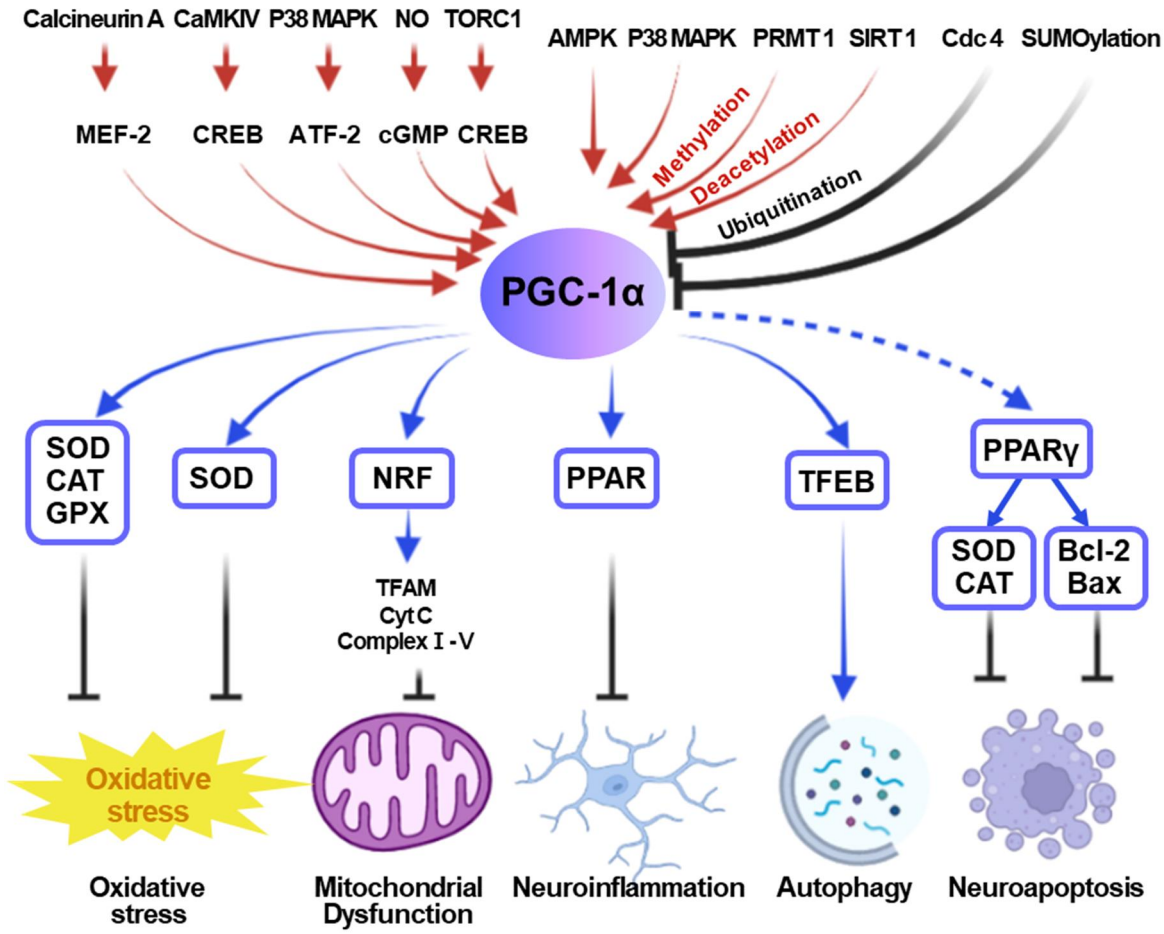

Figure 5. The role of PGC-1α [35, 36].

Calcineurin, calcium-calmodulin-activated kinases (CaMKs), , p38 mitogen-activated protein kinase (p38MAPK), nitric oxide synthase (NOS/cGMP), Target of Rapamycin Complex 1 (TORC1), adenosine-monophosphate-activated kinase (AMPK) have been shown to regulate expression and/or activity of PGC-1a. PGC-1a is a co-activator of transcription factors such asnuclear respiratory factors (NRFs), estrogen-related receptors (ERRs), and PPARs, known to regulate different aspects of energy metabolism including mitochondrial biogenesis, mitochondrial fatty acid oxidation, and antioxidant**.** (Created with BioRender.com)

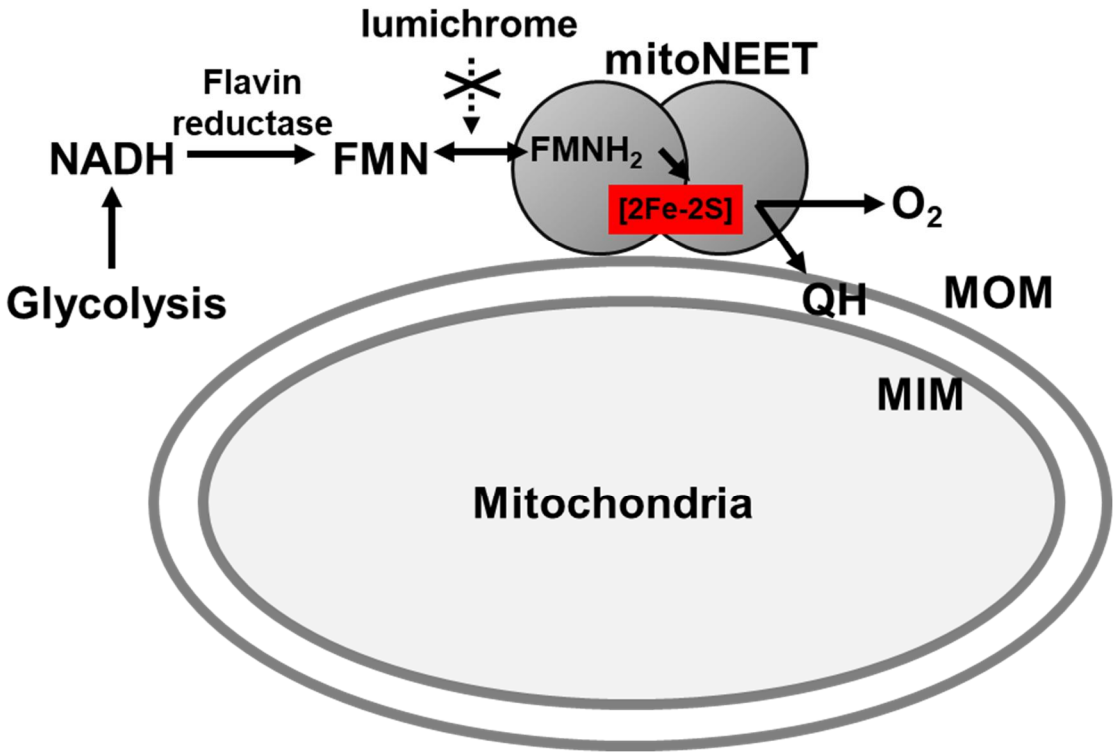

Figure 6 The electron transfer function of mitoNEET in glycolysis [17].

MitoNEET is a mitochondrial outer membrane protein that hosts a redox active [2Fe-2S] cluster. mitoNEET, as a novel redox enzyme catalyzing electron transfer from flavin mononucleotide (FMNH2), may effectively promote oxidation of NADH in cytosol with reduction of oxygen or ubiquinone. That may indirectly regulate glycolysis via promoting oxidation of cytosolic NADH.

Results

Inhibition of mitoNEET induces expression of PGC-1α during inflammatory response.

Oxidative stress during inflammatory processes is related with mitochondrial dysfunction. Recent studies demonstrated that mitoNEET, a 2Fe-2S cluster protein, generates ROS from the mitochondria and is a possible drug target for mitochondrial dysfunction [26]. Previously, we reported that inhibition of mitoNEET attenuates oxidative stress and inflammatory mediators by LPS-induced inflammatory response through the regulation of mitochondrial dysfunction and reactive oxygen species (ROS). Peroxisome proliferator activated receptor-gamma coactivator-1alpha (PGC-1α) is a major regulator of the mitochondrial antioxidant defense system and prevents oxidative injury and mitochondrial dysfunction [27, 16]. In this study, we hypothesized that antioxidant activity mediated by inhibiting mitoNEET is associated with PGC-1α during inflammatory response. To identify the relationship with PGC-1α and mitoNEET during inflammation, we harvested total protein at 3, 6, 12, and 24 hours after administration of LPS (100 ng/mL) and assayed expression of PGC-1α protein in bone marrow-derived macrophages (BMDMs) (Fig. 1A). Expression of mitoNEET protein increased significantly at 3, 6 hours compared with vehicle. Also, level of PGC-1α protein began to increase after 6 hours with decrease of mitoNEET protein level. To investigate the role of PGC-1α in inhibition of mitoNEET during inflammatory response, we analyzed expression of PGC-1α mRNA and protein in LPS-stimulated RAW264.7 cells in the presence or absence of mitoNEET inhibitor, mitoNEET Ligand-1 (NL-1), (Fig. 1B and C). We found that NL-1 increased expression of mRNA encoding PGC-1α in cells exposed to LPS (Fig. 1B). This increase was not seen in control cells (treated with LPS alone). In addition, PGC-1α protein level increased, in the presence of NL-1 at 12, 24 hours (Fig. 1C). To investigate whether LPS-induced expression of mitoNEET alter expression of PGC-1α during

inflammatory response, we generated mitoNEET-shRNA or control shRNA-expressing cells. Expression of mRNA encoding PGC-1α increased in mitoNEET shRNA-expressing cells compared with control shRNA-expressing cells (Fig. 1D). This was also the case for protein expression (Fig. 1E). These data indicate that LPS-stimulated expression of mitoNEET is involved in downregulation of expression of PGC-1α. And inhibition of mitoNEET using NL-1 or mitoNEET shRNA in LPS-treated cells promotes expression of PGC-1α.

To investigate whether expression of PGC-1α under LPS alter antioxidant activity mediated by inhibition of mitoNEET, we generated PGC-1α shRNA- or control shRNA- expressing cells. Real-time PCR and Western blot analysis were performed to assess expression of PGC-1α mRNA and protein, respectively (Fig. 2A and B). We stimulated control shRNA- or PGC-1α shRNA- expressing RAW264.7 cells for 24 hours with LPS (1 μg/mL) in the presence or absence of NL-1 and measured total ROS and superoxide levels by flow cytometry. Total ROS and superoxide levels fell in the presence of, NL-1, in control shRNA cells, but not in PGC-1α shRNA expressing cells (Fig. 2C and D). In addition, confocal microscopy clearly demonstrated that LPS-induced cytosolic ROS was suppressed in the presence of mitoNEET inhibitor, NL-1, compared to LPS alone in control shRNA cells, but not in PGC-1α shRNA expressing cells (Fig. 2E). These results demonstrate the PGC-1α is a key molecule of antioxidant activity mediated by inhibition of mitoNEET.

PGC-1α is a key regulator for increase of NL-1-mediated antioxidant defense genes during inflammatory response.

PGC-1 α coordinately regulates several antioxidant genes, including manganese-dependent superoxide dismutase 2 (Mn-SOD (SOD2)), heme oxygenase-1 (HO-1) [22]. During inflammation, mitochondrial antioxidant gene is downregulated with inhibited PGC-1α and oxidative stress is induced [16]. Previously, we reported that inhibition of mitoNEET increased antioxidant enzymes, HO-1 and SOD2 and fells cytosolic ROS against LPS-induced oxidative stress. We hypothesized that PGC-1α is a key regulator of antioxidant activity mediated by inhibiting mitoNEET. To verify effects of PGC-1α for the increase antioxidant gene expression mediated by inhibiting mitoNEET, we examined expression of HO-1 and SOD2 mRNA and protein in LPS-stimulated cells expressing PGC-1α shRNA in the presence or absence of NL-1 (Fig. 3A-3C). NL-1 increased expression of HO-1 and SOD2 mRNA and protein in control shRNA cells, but not in PGC-1α shRNA expressing cells. These results demonstrate that PGC-1α is a key molecule for upregulation of antioxidant enzymes mediated by inhibition of mitoNEET during inflammatory response.

AMPK signal activated by inhibition of mitoNEET mediates expression of PGC-1α under inflammatory stimuli.

In inflammatory response such as sepsis, increased glycolysis inhibits AMPK (AMP-activated protein kinase) activity as an activator of antioxidant gene, PGC-1α, and anti-inflammatory stage. We confirm whether AMPK is a major signal of antioxidant effect mediated by inhibition of mitoNEET during inflammatory stimuli, we stimulated RAW264.7 cells with LPS (100ng/mL) and measured expression of phosphate-AMPKα in the presence or absence of NL-1. NL-1 increased expression of phosphate-AMPKα protein when compared with LPS alone (Fig. 4A). Consistent with this, phosphate-AMPKα protein level in cells expressing mitoNEET shRNA were higher than those in control shRNA-expressing cells (Fig. 4C).

Early studies suggested that under oxidizing environment, mitoNEET as a generator of NAD+ contribute to glycolysis and glycolytic ATP production [17, 22]. Glycolytic ATP production inhibit AMPK (AMP-activated protein kinase) activity inducing the expression of PGC-1α (peroxisome proliferator-activated receptor γ coactivator-1α) [23]. We hypothesized that activation of AMPK mediated by inhibition of mitoNEET is induced through diminished LPS-induced glycolytic ATP production. Cells were treated with LPS in the presence or absence of NL-1 for 3 hours. Next, Intracellular ATP levels detecting were assayed using ATP Assay Kit and ATP levels fell in the presence of NL-1 (Fig. 4B). Furthermore, to investigate whether downregulation of mitoNEET regulates intracellular ATP levels, we stimulated control shRNA- or mitoNEET shRNA- expressing RAW264.7 cells for 3 hours with LPS (100ng/mL). Intracellular ATP levels were diminished in LPS-treated cells expressing mitoNEET shRNA (Fig. 4D). These data suggest that inhibition of mitoNEET as a generator of NAD+ activates phosphate AMPKα through the inhibition of LPS-induced glycolytic ATP synthesis.

To confirm the signaling pathway involved in regulating expression of PGC-1α in LPS-stimulated cells in presence NL-1, we used specific inhibitors Compound C (APMK inhibitor).

RAW264.7 cells were treated with vehicle, LPS (100 ng/mL), or LPS plus NL-1 (20 μM) or LPS plus NL-1 plus Compound C (20 μM) for 6, 12 hours. Compound C (APMK inhibitor) blocked NL-1-mediated PGC-1α upregulation in LPS-treated cells (Fig. 4E). Also, under LPS stimuli, upregulated PGC-1α in cells expressing mitoNEET shRNA was blocked by Compound C (APMK inhibitor) (Fig. 4F). These results demonstrate that activation of PGC-1α mediated by inhibiting mitoNEET is induced by phosphate AMPKα through inhibition of LPS-induced glycolytic ATP synthesis.

Antioxidant activity of PGC-1α mediated by inhibiting mitoNEET during LPS-stimuli attenuate inflammatory mediators.

Recent studies show that dysregulation of PGC-1α alters redox homeostasis in cells and exacerbates inflammatory response. Thus, PGC-1α acts as an essential node connecting metabolic regulation, redox control, and inflammatory pathways [16]. We analyzed whether antioxidant activity of PGC-1α mediated by inhibiting mitoNEET leads to attenuate against inflammatory response in LPS-treated RAW264.7 cells. We stimulated control shRNA- or PGC-1α shRNA- expressing RAW264.7 cells for 24 hours with LPS (100 ng/mL) in the presence or absence of NL-1. We found that NL-1 reduced expression of mRNA encoding pro-inflammatory cytokines IL-1β, IL-6, and TNFα (Fig. 5A-C) and mRNA encoding inflammatory mediators, iNOS, COX2 (Fig. 5G, H) in cells exposed to LPS in control shRNA-expressing cells, but not in PGC-1α shRNA-expressing cells. This was also the case for protein expression. Protein levels of pro-inflammatory mediators were analyzed using ELISA (Fig. 5D-F) or western blotting (Fig. 5I). These results demonstrate that antioxidant activity of PGC-1α mediated by inhibiting mitoNEET using NL-1 or mitoNEET shRNA is connected with anti-inflammatory activity.

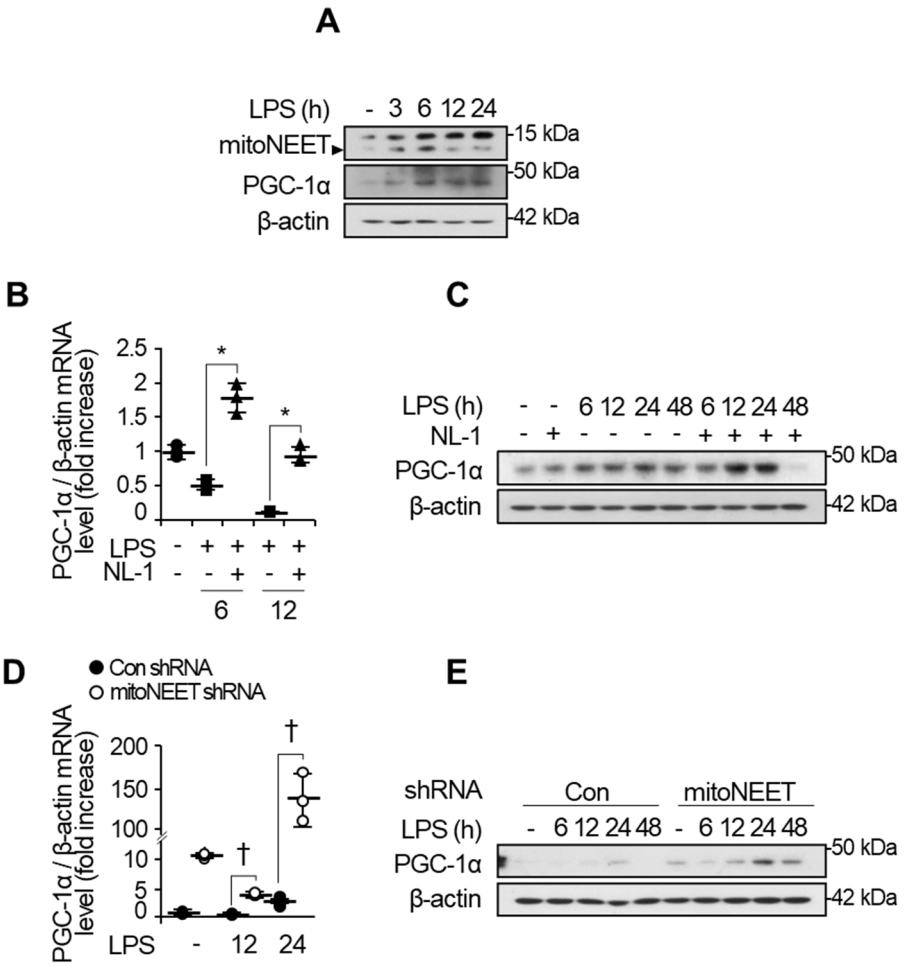

Figure 1. Expression of PGC-1α mRNA and protein enhances by inhibition of mitoNEET in LPS-treated cells.

Total protein was extracted from BMDMs at 3, 6, 12, and 24 hours after treated with vehicle or LPS (100 ng/mL). Expression of mitoNEET, PGC-1α protein were assessed by western blotting (A). Total RNA and total protein were harvested at 6, 12 and 24 hours for mRNA and at 6, 12, 24 and 48 hours for protein after treated with vehicle, NL-1 (20 μM), LPS (100 ng/mL), or LPS plus NL-1 (20 μM). Levels of PGC-1α mRNA were measured by quantitative real-time RT-PCR (B). Protein levels of PGC-1α were analyzed using Western blotting (C). RAW264.7 cells transfected with control shRNA or mitoNEET shRNA were stimulated with LPS (100ng/mL) or vehicle. Total RNA and total protein were

harvested at 6, 12 and 24 hours for mRNA and at 6, 12, 24 and 48 hours for protein after treated with vehicle, LPS (100 ng/mL). Levels of PGC-1α mRNA were measured by quantitative real-time RT-PCR (D). Protein levels of PGC-1α were analyzed using Western blotting (E). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for LPS vs. LPS plus NL-1 treatment. †$P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of LPS.

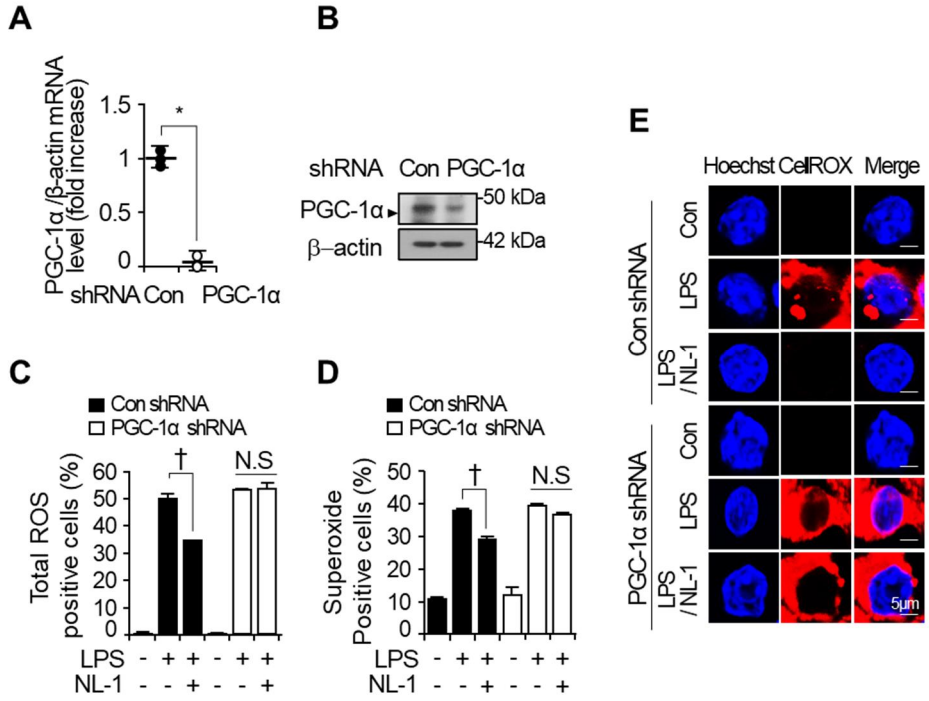

Figure 2. PGC-1α is a key regulator of NL-1-mediated antioxidant activity in LPS-induced oxidative stress.

RAW264.7 cells were transfected with control shRNA or PGC-1α shRNA and then subjected to RT-PCR or western blotting to verify downregulation of PGC-1α expression (A and B). β-actin was used as a loading control. RAW264.7 cells transfected with control shRNA or PGC-1α shRNA were stimulated with vehicle, LPS (1 μg/mL), NL-1 (20 μM), or LPS plus NL-1 (20 μM) for 24 hours. Total reactive oxygen species (ROS) and superoxide anions were assayed by flow cytometry using a ROS/Superoxide Detection kit (C, D). Control shRNA, or PGC-1α shRNA expressing cells were treated with vehicle or LPS (1 μg/mL), LPS plus NL-1 (20 μM) for 24 hours. Cells were then stained with the fluorescent probes Hoechst 33258 (nuclei, blue) and CellROX® Deep Red (total ROS, red). Immunofluorescence images of cells stained with Hoechst 33258 (nuclei, blue) and CellROX® Deep Red (total ROS, red) (E). Scale bar: 5 μm. All data are expressed as the mean ± SD from three independent experiments. *$*P < 0.05$* for control shRNA-expressing cells vs. PGC-1α shRNA-expressing cells. *$†P < 0.05$* for LPS vs. LPS plus NL-1 in control shRNA-expressing cells. NS, not significant.

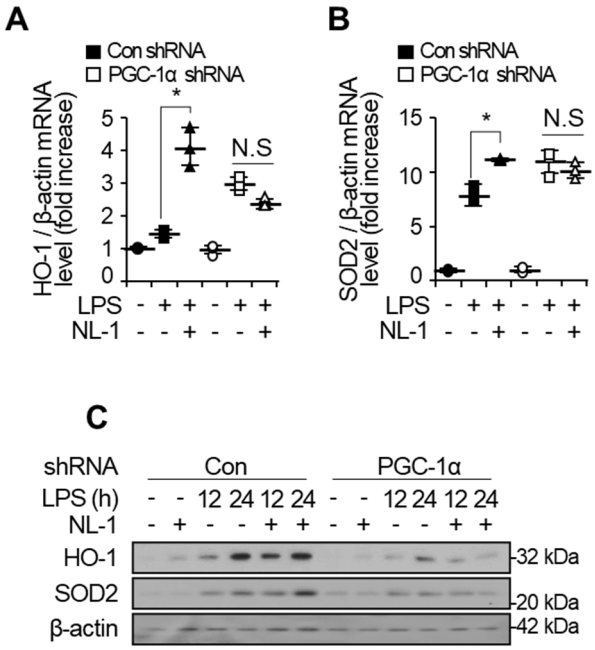

Figure 3. PGC-1α activated by NL-1 mediates expression of antioxidant defense genes.

Control shRNA, or PGC-1α shRNA expressing cells were treated with vehicle, LPS (1 μg/mL), LPS plus NL-1 (20 μM). Total RNA and protein were harvested and mRNA levels of HO-1, SOD2 were analyzed by quantitative real-time RT-PCR 12 hours after treatment (**A, B)**. Protein levels of HO-1, SOD2, were detected using Western blotting (**C**). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for LPS vs. LPS plus NL-1 treatment in control shRNA-expressing cells. NS, not significant.

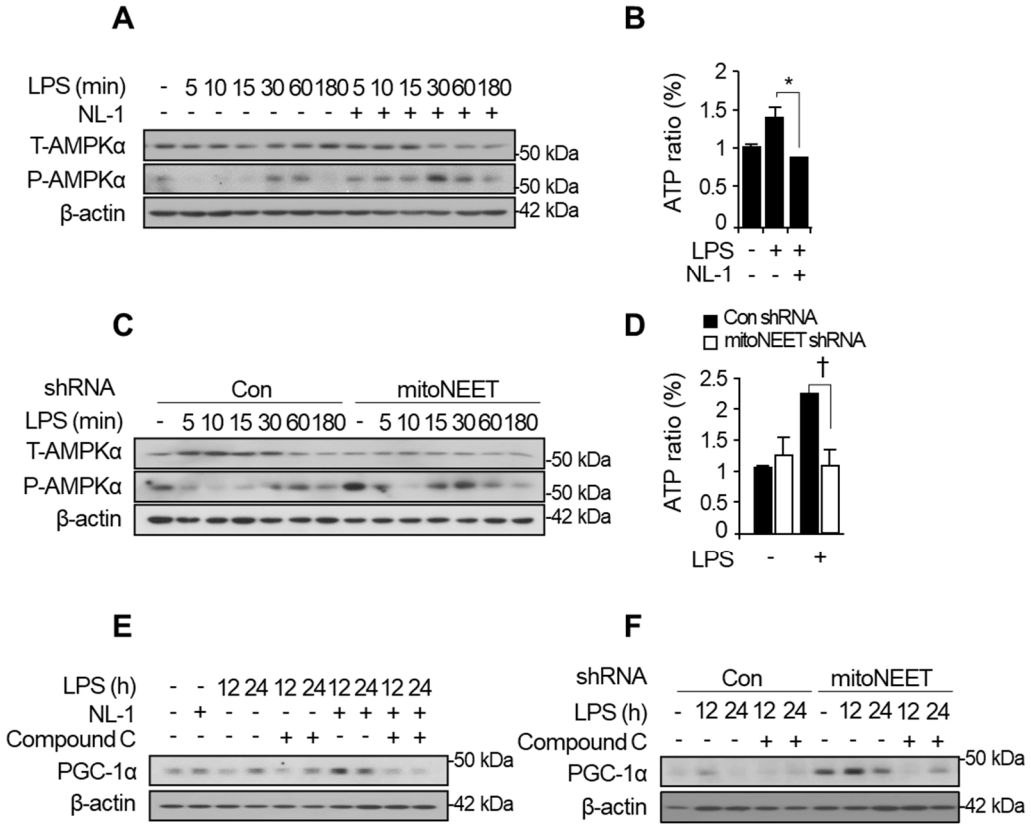

Figure 4. Activation of PGC-1α by NL-1 is mediated by phosphate AMPKα.

RAW264.7 cells, control shRNA-expressing or mitoNEET shRNA-expressing cells were treated with vehicle, LPS (100 ng/mL) or LPS plus NL-1 (20 μM) (A-D). Total protein was harvested, followed by measurement of protein level of Total- or phosphate-AMPKα by western blotting. β-actin was used as an internal loading control (A and C). Intracellular ATP levels were detected by ATP Assay Kit at 1 hours (B and D). RAW264.7 cells, control shRNA-expressing or mitoNEET shRNA-expressing cells were treated with vehicle, NL-1 (20 μM), LPS (100 ng/mL) or LPS plus NL-1 (20 μM), LPS plus Compound C (20 μM) and LPS plus NL-1 (20 μM) plus Compound C (20 μM) for 6, 12 hours (E and F). Expression of PGC-1α protein were analyzed using western blotting (E, F). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for LPS vs. LPS plus NL-1 treatment. †$P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of LPS.

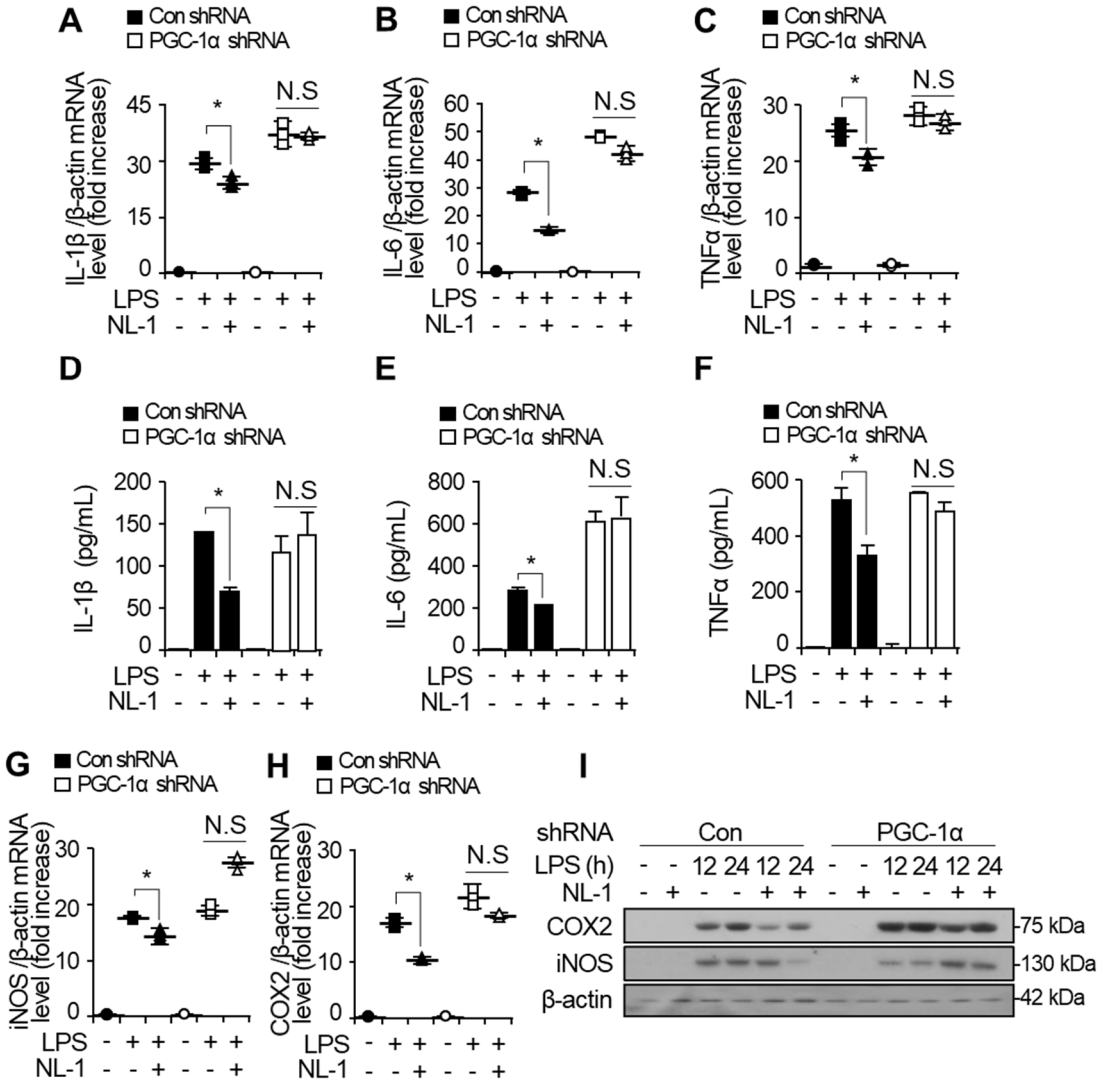

Figure 5. PGC-1α is a key regulator of anti-inflammatory activity mediated by NL-1.

Control shRNA-expressing or PGC-1α shRNA-expressing cells were treated with vehicle, LPS (100 ng/mL) or NL-1 (20 μM), LPS plus NL-1 (20 μM). Total RNA, cell supernatants, and total protein were harvested. Expression of mRNA encoding IL-1β, IL-6, TNFα (A-C), and iNOS, COX2 (G, H) was assessed by quantitative real-time RT-PCR at 12 hours and protein expression was analyzed by ELISA at 24 hours or Western blotting at 12, 24 hours (D-F, I). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for LPS vs. LPS plus NL-1 treatment in control shRNA-expressing cells. NS, not significant.

Discussion

Inflammatory disease, such as sepsis, and oxidative stress are closely related and tightly linked pathophysiological processes [28, 2]. Increasing evidence suggest that therapeutic targeting mitochondrial dysfunction for regulation of oxidative stress deserves further investigation [29, 30]. Mitochondrial function is particularly susceptible to oxidative damage, leading to altered mitochondrial biogenesis, energy metabolism and promoted oxidative stress [29]. Previously, we showed that mitoNEET as a ligand of a thiazolidinedione (TZD) is a possible molecule as mitochondrial therapeutic target for mitochondrial dysfunction and oxidative stress during inflammatory diseases and sepsis. Recent studies show that peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α is a regulator connecting oxidative stress and mitochondrial metabolism with inflammatory response and metabolic syndrome [31]. And earlier study provided that TZDs normalize mitochondrial ROS production by induction of MnSOD and the promotion of mitochondrial biogenesis by activating PGC-1α [32]. In this study, we have demonstrated that PGC-1α is a major molecule in antioxidant effect mediated by inhibiting mitoNEET against oxidative stress during inflammatory response. Our data show that inflammatory stimuli such as LPS, which induce mitochondrial oxidative damage, trigger production of PGC-1α in BMDMs. In addition, inhibition of mitoNEET using NL-1 or mitoNEET shRNA promoted PGC-1α expression in LPS-treated cells (Fig. 1). Furthermore, LPS-induced ROS levels inducing mitochondrial oxidative damage was suppressed in the presence of NL-1 compared with LPS alone in control shRNA cells, but not in PGC-1α shRNA expressing cells (Fig. 2). These data suggest that PGC-1α could play a key role in antioxidant effect mediated by inhibiting mitoNEET. Previously, we reported that antioxidant enzymes are upregulated by inhibition of mitoNEET during LPS stimuli. Expression of antioxidant enzymes, HO-1 and the mitochondrial MnSOD isoform SOD2

were activated in the presence of NL-1 compared to LPS alone in control shRNA cells, but not in PGC-1α shRNA expressing cells. These results demonstrate the PGC-1α is a key mediator in the increase of antioxidant enzymes mediated by inhibiting mitoNEET during inflammatory response. Recently, increasing evidence suggests that mitoNEET as a novel redox enzyme may indirectly regulate glycolysis and glycolytic ATP production by promoting oxidation of cytosolic NADH under oxidizing environment. During inflammatory response, Glycolytic ATP production inhibit AMPK (AMP-activated protein kinase) activity and expression of PGC-1α. Here, we showed that mitoNEET as a generator of NAD+ promotes ATP production in inflammatory response and that ATP decreased by inhibition of mitoNEET could lead to AMPK activity and increase of PGC-1α (Fig. 4). Our results demonstrated that the relationship of mitoNEET and PGC-1α work out the opposite way and is related with the effects of mitoNEET on glycolysis during inflammatory response. In addition, we showed that antioxidant activity of PGC-1α mediated by inhibiting mitoNEET during inflammatory stimuli could attenuate inflammatory mediators (Fig. 5). Taken together, our results demonstrate that PGC-1α is a major molecule in NL-1 mediated antioxidant effect during inflammatory response. Therefore, targeting mitoNEET is a possible therapeutic strategy bring maintained healthy mitochondria and antioxidant effect through activating PGC-1α through energy metabolism control in mitochondrial dysfunctional disease.

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PART 3.

The role of mitoNEET under CCCP-induced Pink-Parkin mitophagy

Abbreviations

CCCP	Carbonyl Cyanide m-Chlorophenylhydrazine
MQC	Mitochondrial quality control
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator-1alpha
MitoNEET	Mitochondrial protein containing NEET sequence
NL-1	NEET Ligand-1
TZD	Thiazolidinediones
AMPK	AMP-activated protein kinase
Ulk1	Unc-51 like autophagy activating kinase 1
PINK1	PTEN-induced putative kinase 1
SOD	Superoxide dismutase
ATP	Adenosine triphosphate
Pink1	PTEN- induced kinase 1
Parkin	Cytosolic E3 ubiquitin ligase
ATG	Autophagy-related
IMJ	Intermitochondrial junctions
ROS	Reactive oxygen species
MOM	Mitochondrial outer membrane
Caspase-9	Cysteine-aspartic proteases-9
Caspase-3	Cysteine-aspartic proteases-3
PARP-1	Poly [ADP-ribose] polymerase 1

Abstract

MitoNEET, a mitochondrial outer membrane protein containing the Asn-Glu-Glu-Thr (NEET) sequence, controls the formation of intermitochondrial junctions and confers autophagy resistance. Moreover, mitoNEET as a mitochondrial substrate undergoes ubiquitination by activated Parkin in the initiation of mitophagy. Therefore, mitoNEET is linked to the regulation of autophagy and mitophagy. Mitophagy is a selective removal process of damaged or unnecessary mitochondria, which is crucial to sustaining mitochondrial quality control. In numerous human diseases, the accumulation of damaged mitochondria by impaired mitophagy has been observed. The therapeutic strategy involving the targeting of mitoNEET as a mitophagy-enhancing mediator requires further research. Herein, we confirmed that mitophagy is indeed activated by mitoNEET inhibition. CCCP (carbonyl cyanide m-chlorophenyl hydrazone), which leads to mitochondrial depolarization, induces mitochondrial dysfunction and superoxide production. This, in turn, contributes to the induction of mitophagy. mitoNEET protein levels were initially increased before the increase in LC3- protein level following CCCP treatment. Pharmacological inhibition of mitoNEET using mitoNEET Ligand-1 (NL-1) promoted accumulation of PTEN-induced putative kinase 1 (Pink1) and Parkin, which are mitophagy-associated proteins, as well as activation of mitochondria–lysosome crosstalk, in comparison to CCCP alone. Inhibition of mitoNEET using NL-1, or mitoNEET shRNA transfected into RAW264.7 cells, abrogated CCCP-induced ROS and mitochondrial cell death, which demonstrated by confocal microscopy and flow cytometry or lactate dehydrogenase (LDH)-release assay; additionally, it activated the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and superoxide dismutase (SOD2), regulators of oxidative metabolism, detected using western blotting. In particular, the increase in PGC-1α, which is a major regulator of mitochondrial biogenesis, promotes

mitochondrial quality control. These data indicated that mitoNEET may be employed as a therapeutic target in numerous human diseases to enhance mitophagy and protect cells by maintaining a network of healthy mitochondria.

Introduction

1. Mitochondrial quality control (MQC)

Mitochondria are highly dynamic organelles that continuously undergo fusion and fission in highly regulated manners. That mitochondrial quality control (MQC) maintains the stability and integrity of mitochondrial structure and function and operates through the coordination of various processes (biogenesis, dynamics- Fusion / Fission, and mitophagy) (Figure. 1). These activities affect the number and morphology of mitochondria in the cell and that plays critical roles for diverse mitochondrial activity such as energy production, metabolism, intracellular signaling, and apoptosis. Therefore, MQC is an important for maintaining mechanism for cells to survive from mitochondrial damage [1, 2, 3]. Intracellular quality control of mitochondria consists of biogenesis, fusion, fission, and mitophagy (mitochondrial degradation). Mitochondrial fission is the process dividing mitochondria and is necessary for the dissociation of damaged and dysfunctional mitochondria. Mitochondria fission requires Drp1 (dynamin-related protein 1) recruitment from cytoplasm to mitochondrial outer membrane. Damaged and dysfunctional mitochondria deleterious to the cell are degraded by mitophagy. Mitophagy degrades mitochondria via a process the selective autophagy of mitochondria [4, 5]. Mitochondrial biogenesis involves the synthesis of new mitochondrial DNA (mtDNA), protein, and mitochondrial membrane. Especially, peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) play a critical role in the transcription of genes required for mitochondrial biogenesis. Mitochondrial fusion joins two mitochondria together and exchange mtDNA, proteins, and metabolites. Fusion mediators is large dynamin-related GTPase proteins Mfn1, Mfn2 and OPA1 [5, 6].

2. Autophagy

Autophagy is a highly conserved pathway of cleaning out damaged cells in order to regenerate healthier cells as a self-degradative process. Autophagy can be activated as an adaptive cellular stress response to environmental stimuli such as hypoxia, nutrient starvation, and different cancer drugs and therefore considered as a major cytoprotective process. Because autophagy breaks down macromolecules, it is considered a 'catabolic' process [7, 8]. Autophagy relies on the formation of autophagosomes as a double-membrane vesicles, leading to the degradation of cargo, such as damaged organelles, protein aggregates. The major steps of autophagy are: initiation, elongation and autophagosome formation, fusion, and autolysosome formation as fusion with lysosome for the degradation and recycling of autophagosome constituents (Figure. 2). Autophagy is initiated by the ULK1 complex. Autophagosome elongation and formation involves ubiquitin-like conjugation systems, such as the light chain (LC-3) of microtubule-associated protein 1 (MAP-1) and the Atg12 systems. The autophagy process involves more than 13 autophagy related (ATG) proteins. The autophagosome fuses with a lysosome to form an autolysosome, which degrades macromolecules into their basic components (amino acids, fatty acids, and nucleotides) [8, 9].

3. Mitophagy

Mitophagy is an autophagic process of selective removal of damaged or unnecessary mitochondria using autophagic machinery [10, 11]. Mitophagy plays to control metabolic homeostasis and maintain mitochondrial quality control and function [10]. These roles are critical to prevent developing aging-related dysfunctions and subsequent molecular events, such as oxidative stress, that lead to disease development [10, 11]. Increasing evidence supports that impaired mitophagy and mitochondrial dysfunction are closely related with a variety of human disease. Mitophagy impairments have been proposed to lead to accumulation of damaged mitochondria and defective organelle [11, 12] and contribute to age-related neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases, metabolic diseases, vascular complications of diabetes, myocardial injury, muscle dystrophy, and liver disease, among others [13]. Many studies tried to the identification of new regulatory genes and

mechanisms of mitophagy. The initial stage of mitophagy process is stimulated by kinase such as 5′-AMP-activated protein kinase (AMPK), unc-51 like autophagy activating kinase 1 (Ulk1), PTEN-induced putative kinase 1 (Pink1). These promote each other and stimulate a downstream regulator of mitophagy [14]. After then, Pink1-Parkin-mediated mitophagy or receptor-mediated mitophagy or adaptor proteins mediated mitophagy is triggered. Especially, polyubiquitination of various OMM proteins by parkin induces the recognition of several adaptor molecules and light chain 3 (LC3) and autophagosomal formation (Figure. 3) [15]. Discover of mitophagy enhancing process is facilitate the elimination of irreversibly damaged mitochondria and necessary for therapeutic strategy of several diseases. However, the regulator and molecular mechanism of mitophagy is yet clear.

4. mitoNEET function related with mitophagy

Currently, mitoNEET, a mitochondrial outer membrane protein exposed at the cytosolic surface of mitochondria, has been suggested to be a key component regulating IMJ morphology by acting as a tether to link two adjacent mitochondria. mitoNEET controls the formation of intermitochondrial junctions (IMJ) and mitochondrial network morphology (Figure. 4) [16]. mitoNEET structurally tethers adjacent mitochondria without actually fusing. Several studies showed that deletion of mitoNEET alters the integrity of inter-mitochondrial junctions and exogenous H2O2 application leads to fragmentation by inhibiting mitoNEET (Figure. 5) [17, 18]. Some studies suggested that the redox activity of the mitoNEET iron-sulfur clusters could potentially be involved as a molecular switch in response to oxidative stress to change the conformation of mitoNEET and hence tethering properties [41]. Early studies have shown that mitoNEET has been involved in the regulation of autophagy signaling prevents autophagy [19, 20]. In cells with reduced expression of mitoNEET, proteins involved in autophagy accumulates and electron microscopy also revealed autophagosome accumulation [19, 21]. In addition, mitoNEET in with integral mitochondrial proteins in mitochondrial outer membrane during mitophagy process undergo ubiquitination. After, the ubiquitin on the mitochondrial substrate is phosphorylated by PINK1 and then integral mitochondrial proteins containing mitoNEET are significantly reduced in

response to E3 ubiquitin protein ligase (Parkin) recruitment during early stage of mitophagy process (Figure. 6) [22, 23]. These studies demonstrated that mitoNEET is a direct substrate of Parkin to recruit autophagy adaptors and the removal of mitoNEET is necessary to initiate mitophagy process [23,24]. However, that the targeting of mitoNEET works as an enhancer of mitophagy has not been studied yet. Herein, we showed that inhibiting the expression or activity of mitoNEET promotes not only accumulation of mitophagy-linked proteins but also the activation of the crosstalk between lysosomes and mitochondria during carbonyl cyanide m-chlorophenyl hydrazone (CCCP)-induced mitochondrial damage. Thus, mitoNEET play the conclusive role in mitophagy regulation and the elimination of damaged mitochondria, which has been clearly demonstrated in the results of the current study. Therefore, we hypothesized that mitoNEET is a targeted molecular candidate that eliminates irreversibly damaged mitochondria and maintains mitochondrial quality control in the treatment of several diseases.

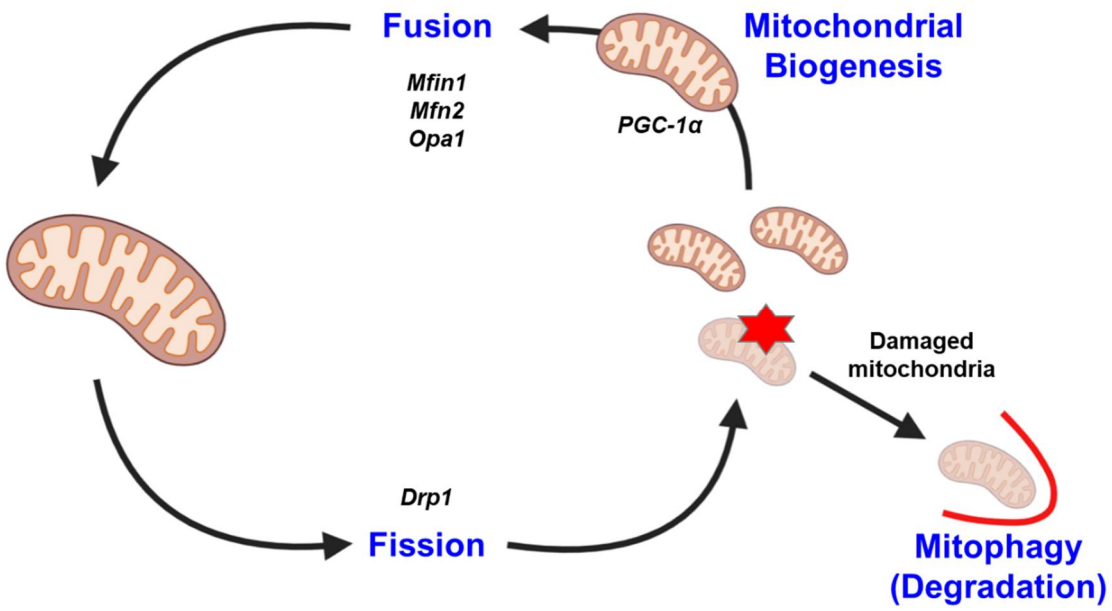

Figure 1. Schematic representation of quality control of mitochondria [44].

Intracellular quality control of mitochondria consists of mitochondrial fission and fusion, mitochondrial biogenesis, and mitophagy which coordinately control mitochondrial morphology, quantity, quality, turnover, and inheritance. Damaged and dysfunctional mitochondria undergo mitochondrial fission and then removed by mitophagy. Mitochondrial biogenesis involves the synthesis of new mtDNA, protein, and membrane. Mitochondrial fusion involves the physical merging of two originally distinct mitochondria. (Created with BioRender.com)

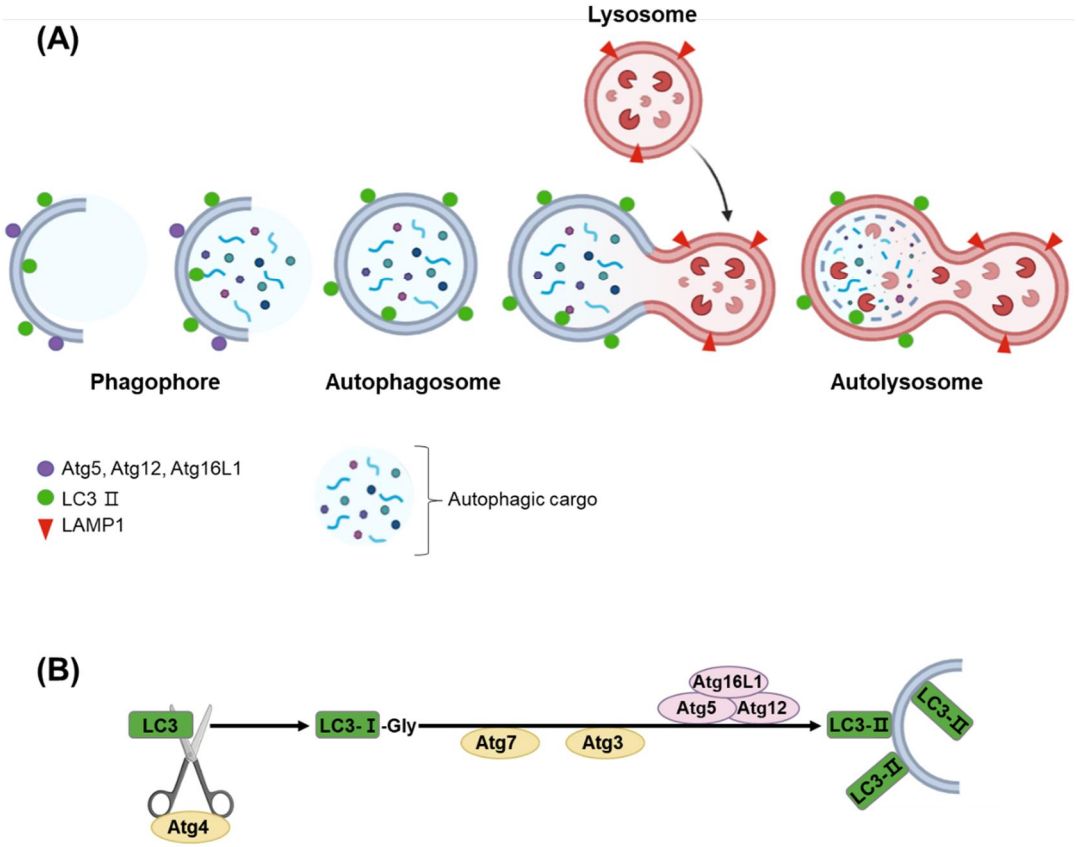

Figure 2. Process of autophagy [45, 46].

(a) *Schematic of autophagosome formation and degradation*: Within the cytoplasm, double-membraned autophagic precursors containing a ATG12–ATG5–ATG16L1 proteins complex enables the conjugation of LC3-II to the membranes. As the phagophore enlarges forming autophagosome, the ATG complex dissociates from the outer membrane, whereas LC3-II remains associated. Autophagosomes fuse with the lysosomes and their contents are degraded. (Created with BioRender.com)

(b) *Lipidation of LC3-II*: LC3 are conjugated to the lipid PE in autophagosome membranes. The cysteine protease ATG4 cleaves pro-LC3 and forms cleaved LC3 (LC3-I). The conjugation of LC3-I to PE in membranes to form LC3-II is mediated by a complex of ATG as an E3-like ligase. ATG8/LC3 proteins might also support the expansion and closure of autophagosomal membranes and are crucial for autophagosome–lysosome fusion and inner autophagosomal membrane degradation.

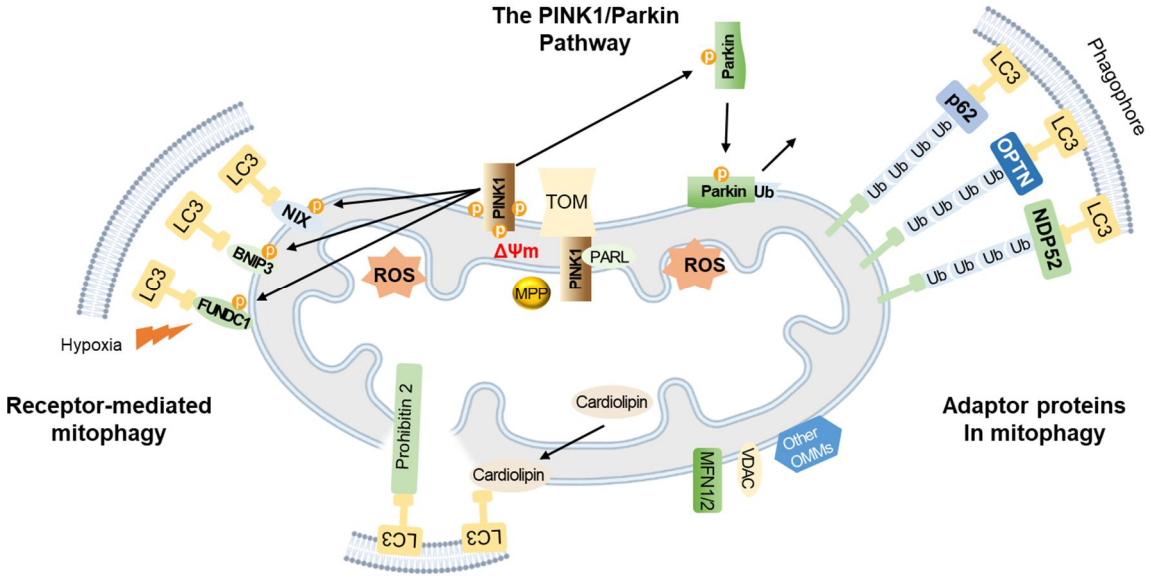

Figure 3. Mechanistic insights into mitophagy process [15].

Dysfunctional mitochondria redirect PTEN-induced kinase 1 (Pink1) to the outer mitochondrial membrane (OMM). Pink-1 recruits Parkin through a series of modifications, such as phosphorylation of both Parkin and ubiquitin. In turn, Parkin, an E3 ubiquitin ligase, triggers the polyubiquitination of various OMM proteins. Ubiquitinated membrane proteins are selectively recognized by several adaptor molecules, including p62, optineurin (OPTN), and NDP52, promoting their recognition by LC3 and autophagosomal formation. Receptor-mediated mitophagy relies on various mitochondrial outer membrane proteins BNIP3, NIX (BNIP3L), and FUNDC1. (Created with BioRender.com)

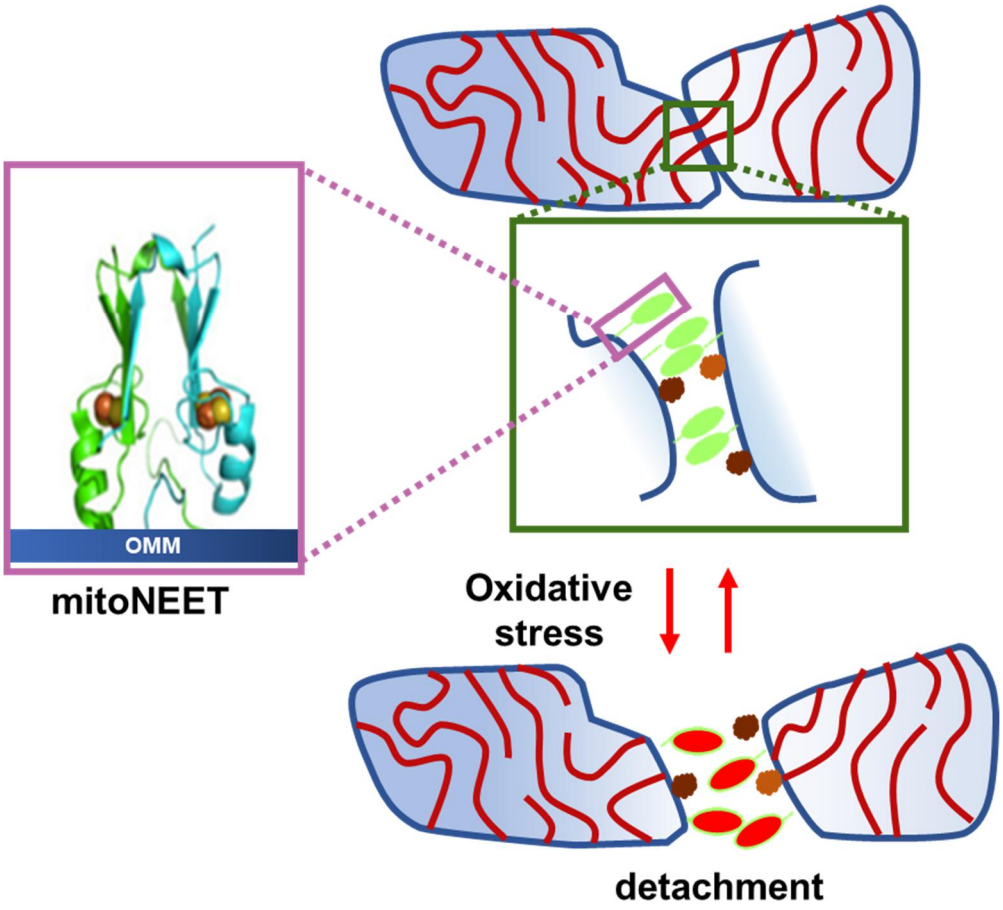

Figure 4. mitoNEET as a protein involved in mitochondrial fusion [41].

MitoNEET (depicted by the green shape) has been proposed to be a key molecule regulating intermitochondrial junctions (IMJ) morphology by acting as a tether to link two adjacent mitochondria. The cartoon speculates that the redox activity of the mitoNEET [2Fe-2S] clusters could potentially be involved as a molecular switch in response to oxidative stress to change the conformation of mitoNEET (now shown as red/green shape) and hence tethering properties.

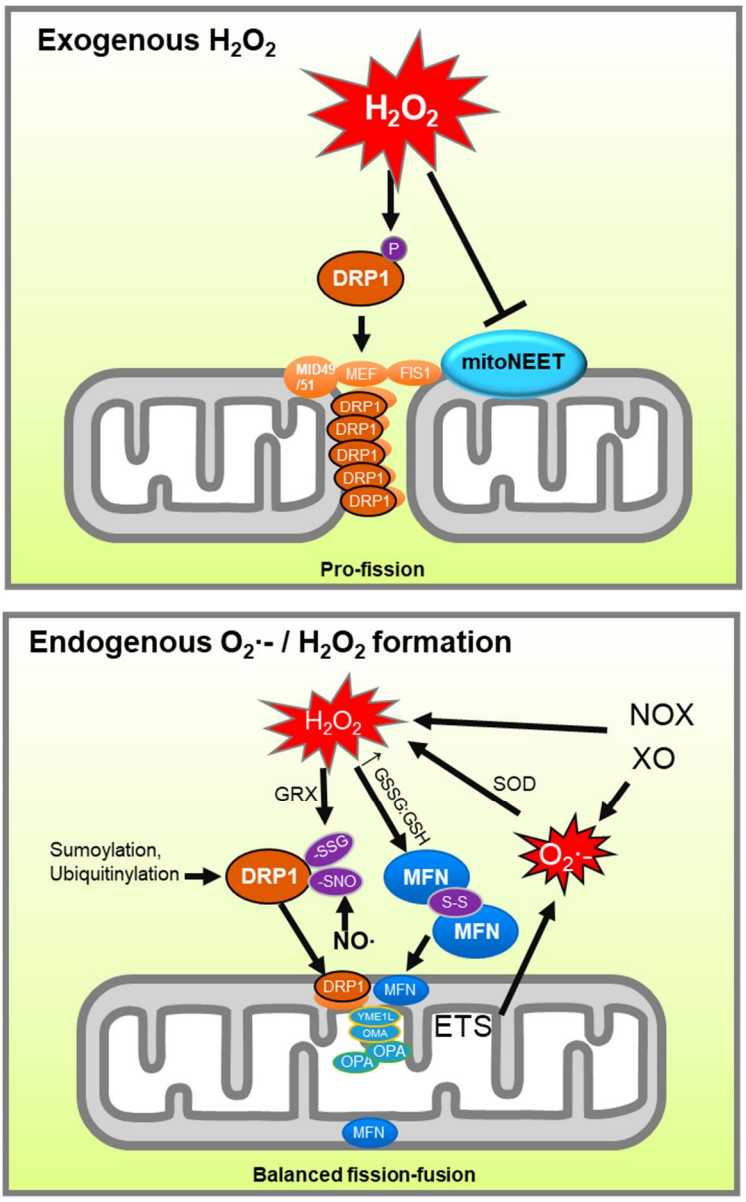

Figure 5. Exogenous H2O2 leads to fragmentation via a mitoNEET dependent mechanism [18].

Intermitochondrial junctions (IMJ) are formed by mitoNEET, based on its amino acid sequence), which structurally tethers adjacent mitochondria without actually fusing. Exogenous H2O2 application induces to fragmentation through the activation of DRP1 via a mitoNEET dependent mechanism.

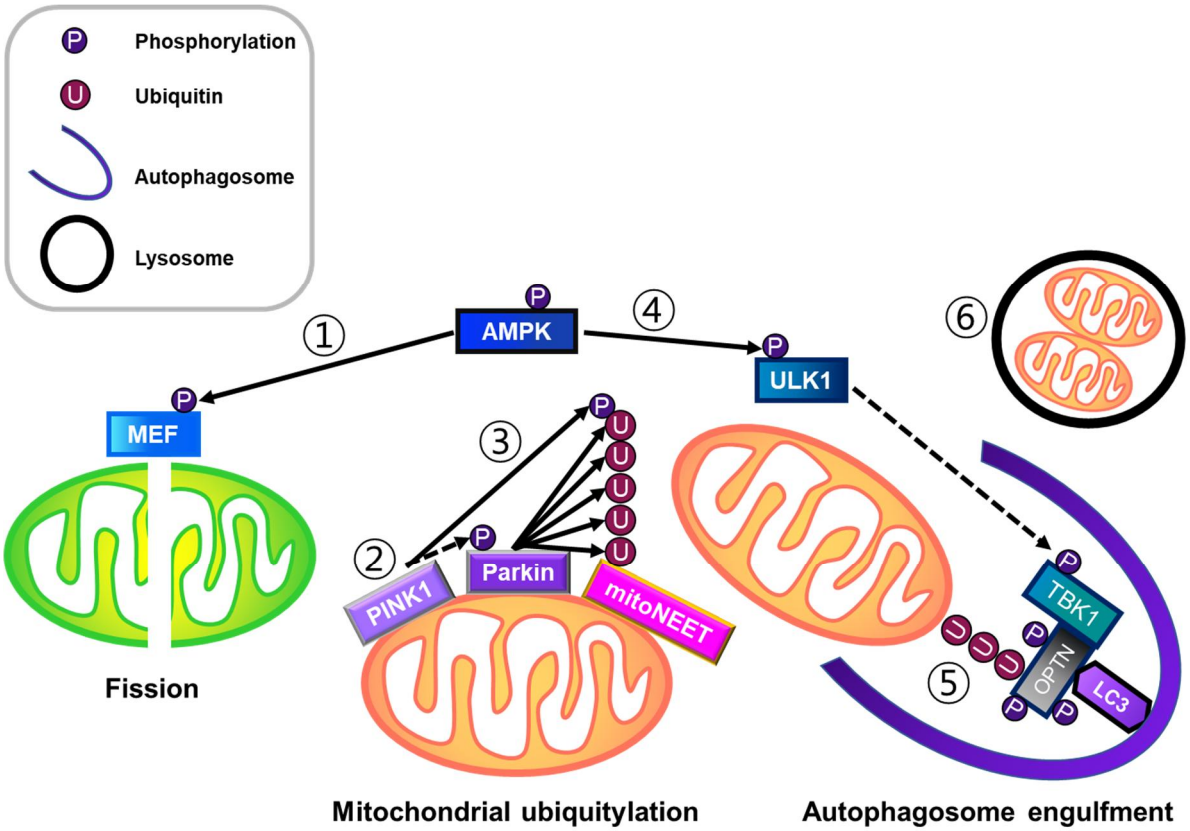

Figure 6. mitoNEET are significantly reduced in response to E3 ubiquitin protein ligase (Parkin) [24].

(1) AMP-activated protein kinase (AMPK) activation initially promotes mitochondrial fission via direct phosphorylation of mitochondrial fission factor (MFF). This allows the separation of healthy and depolarized mitochondria. In depolarized mitochondria, (2) Pink1 accumulates on OMM and phosphorylates both ubiquitin (Ub) and Parkin. (3) This is suggested to promote the recruitment of Parkin, an E3 ubiquitin ligase, to the OMM. Parkin then ubiquitylates OMM proteins including mitoNEET. Meanwhile, (4) AMPK activation leads to TBK1 phosphorylation possibly via ULK1 (5) TANK-binding kinase 1 (TBK1) activation enhances the binding capacity of autophagy receptors to ubiquitylated mitochondria. (6) Subsequently, the autophagosome fuses with the lysosome for mitochondrial degradation

Results

CCCP-mediated mitochondrial depolarization induced mitoNEET expression in macrophages.

Mitochondrial depolarization due to environmental stress induces mitophagy-mediated clearance of damaged mitochondria [25]. However, defective mitophagy leads to disease development [13]. Early studies demonstrated that mitoNEET, an outer mitochondrial membrane protein, is involved in the formation of intermitochondrial junctions and is associated with autophagy or mitophagy [16, 19, 23]. In the present study, we hypothesized that mitoNEET contributes to faulty mitophagy in diseases of mitochondrial dysfunction. To elucidate the role of mitoNEET in mitophagy, we assayed mitoNEET expression during mitophagy. We induced mitophagy using CCCP, resulting in mitochondrial depolarization. We harvested total protein at 0.5, 1, 3, 6, 9, and 12 hours after CCCP administration in RAW264.7 cells (Fig. 1A). mitoNEET protein expression was significantly increased at 1 and 3 hours prior to the conversion of LC3-I to LC3- protein. Additionally, LC3- protein levels began to increase 6 hours following CCCP treatment, with decreased mitoNEET protein levels. These data indicated that mitoNEET is involved in the initial stages of autophagy. To investigate the role of mitoNEET in autophagy signaling, we analyzed the expression of autophagosomal marker LC3- (microtubule associated protein 1 light chain 3-) and autophagy adaptor protein p62/SQSTM1 in mitoNEET inhibition by inhibitor NL-1. We found that NL-1 enhanced the expression levels of LC3- and p62/SQSTM1 (p62) protein in cells exposed to CCCP (Fig. 1B), followed by a rapid decrease at 12 hours. To investigate whether CCCP-induced expression of mitoNEET alters autophagy responses, we generated mitoNEET shRNA- or control shRNA-expressing cells. Expression levels of LC3- and p62/SQSTM1 (p62) protein in cells expressing mitoNEET shRNA were higher than those in control

shRNA-expressing cells (Fig. 1C), and then decreased rapidly at 12 hours. These results demonstrated that the elimination of mitoNEET is necessary for autophagy activation, because of its inhibitory effect. Moreover, the possible effects of mitoNEET inhibition by NL-1 or mitoNEET shRNA expression could activate the CCCP-induced autophagy response. To verify that the inhibition of mitoNEET induces mitophagy, we assessed mitophagy using mitochondrial probes Mitochondrial marker deep red and LysoTracker Green, which allow visualization of colocalization of lysosomes and mitochondria by confocal microscopy (Fig. 1D, E). Confocal microscopy clearly demonstrated that NL-1 or mitoNEET shRNA increased CCCP-induced colocalization of mitochondria and lysosomes when compared with CCCP alone. Taken together, these data indicated that inhibiting mitoNEET in RAW264.7 cells induces recruitment of autophagosomes and the selective degradation of mitochondria during CCCP-induced mitochondrial depolarization.

Inhibition of mitoNEET activated Pink1–Parkin-mediated mitophagy during CCCP-induced mitochondrial depolarization.

Prior studies demonstrated that CCCP induces protein kinase (AMPK) activation and promotes mitochondrial fission, manifest as heightened Pink1 and Parkin E3 ligase activity toward mitoNEET [24]. However, whether targeting of mitoNEET plays a role as an activator of Pink1–Parkin-mediated mitophagy remains unknown. To confirm that CCCP induces AMPK and mitophagy-associated molecules Pink1 (PTEN-induced kinase 1) and Parkin (cytosolic E3 ubiquitin ligase), we examined protein levels using western blot analysis in CCCP-treated RAW264.7 cells at 0.5, 1, 3, 6, 9, and 12 hours (Fig. 2A). Phosphate AMPKα protein expression level was significantly increased in the early stages compared with the corresponding expression level in the vehicle, followed by a rapid decrease. In addition, Pink1 protein levels began to increase 1 hours following CCCP administration, and there was a corresponding sequential increase in Parkin expression. We showed that inhibition of mitoNEET using NL-1 or mitoNEET shRNA augments autophagy marker expression and colocalization of lysosomes and mitochondria following CCCP treatment (Fig. 1). To verify whether mitoNEET inhibition activates CCCP-induced Pink1–Parkin-mediated mitophagy, we examined the expression levels of phosphate AMPKα, Pink1, and Parkin protein in CCCP-stimulated RAW264.7 cells in the presence or absence of NL-1 (Fig. 2B). We found that NL-1 enhanced the expression levels of phosphate AMPKα, Pink1, and Parkin proteins. Consistent with this, AMPKα, Pink1, and Parkin protein levels in cells expressing mitoNEET shRNA were higher than the corresponding levels in control shRNA-expressing cells (Fig. 2C). These results demonstrated that mitoNEET inhibition by NL-1 or mitoNEET shRNA could activate mitophagy through the upregulation of mitophagy-associated genes in the Pink1–Parkin pathway during CCCP-induced mitochondrial depolarization. Proton ionophore CCCP is a strong inducer of AMPK through inhibition of ATP (adenosine triphosphate) synthesis [26]. Additionally, mitoNEET function is associated with complex 3, ATP synthase, pyruvate dehydrogenase, enzymes involved in β-oxidation of fatty acids [27]. We hypothesized that activated phosphate AMPKα

in mitoNEET inhibition is generated by suppressing ATP synthesis during CCCP treatment, and assessed intracellular ATP levels using an ATP Assay Kit. We found that NL-1 reduced the intracellular ATP level in cells exposed to CCCP for 3 hours (Fig. 2D). Consistent with this, intracellular ATP levels in cells expressing mitoNEET shRNA were decreased in comparison with the corresponding levels in control shRNA-expressing cells in the presence of CCCP (Fig. 2E). These data indicated that inhibiting mitoNEET in RAW264.7 cells promotes CCCP-mediated inhibition of ATP synthesis and enhances the phosphate AMPKα level. This leads to stimulation of Pink1–Parkin during CCCP-induced mitochondrial damage. Taken together, targeting mitoNEET stimulates Pink1–Parkin, which acts as a downstream regulator of mitophagy, via phosphate AMPKα during CCCP-induced mitochondrial damage.

Inhibition of mitoNEET attenuated CCCP-induced mitochondrial ROS.

CCCP as a mitochondrial oxidative phosphorylation uncoupler causes superoxide production through mitochondrial depolarization [28, 29]. It has been postulated that mitochondrial dysfunction-induced reactive oxygen species (ROS), such as superoxide, govern the pathogenesis of various diseases [30]. Mitophagy activated in response to mitochondrial depolarization decreases ROS generation through the removal of damaged mitochondria [42]. We analyzed whether mitophagy enhanced by inhibiting mitoNEET attenuates CCCP-induced mitochondrial ROS. Cells were treated with CCCP in the presence or absence of NL-1 for 9 hours and superoxide levels were assayed by flow cytometry using a ROS/Superoxide Detection kit. The CCCP-induced superoxide level decreased in the presence of NL-1 (Fig. 3A). Additionally, mitoSOX levels were assessed by mitoSOX mitochondrial superoxide indicator. The CCCP-induced mitoSOX level decreased following mitoNEET inhibition by NL-1 (Fig. 3B). Furthermore, to investigate whether downregulation of mitoNEET regulates CCCP-induced mitochondrial ROS, we stimulated control shRNA- or mitoNEET shRNA-expressing RAW264.7 cells for 9 hours with CCCP (70 μM) and measured superoxide levels by flow cytometry using an ROS/Superoxide Detection kit. Superoxide levels fell in CCCP-treated cells expressing mitoNEET shRNA (Fig. 3C). Consistent with this, CCCP-induced mitoSOX decreased in mitoNEET shRNA-expressing cells (Fig. 3D). In addition, confocal microscopy clearly demonstrated that NL-1 or mitoNEET shRNA suppressed CCCP-induced cytosolic ROS when compared with CCCP alone (Fig. 3E, F).

To confirm the antioxidant effect of NL-1, during CCCP-induced ROS production, we examined the expression of PGC-1α, as well as the mitochondrial MnSOD isoform SOD2 mRNA, in the presence or absence of NL-1 (Fig. 4A, B). We found that NL-1 enhanced the mRNA and protein expression levels of PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha) and SOD2 (superoxide dismutase activity) in RAW264.7 cells. This was also the case for protein expression (Fig. 4C). Consistent with this, PGC-1α, SOD2 mRNA (Fig. 4D, E) and protein (Fig. 4F)

levels in cell expressing mitoNEET shRNA were higher than those in control shRNA-expressing cells. These results demonstrated that NL-1 or mitoNEET shRNA may attenuate CCCP-induced mitochondrial ROS production by upregulating expression of antioxidant defense genes with enhanced mitophagy. Furthermore, PGC-1α, as a major regulator of mitochondrial biogenesis, maintains a network of healthy mitochondria through balance of the generation of new organelles and the removal of damaged mitochondria [31, 32, 33]. These data indicated that by targeting mitoNEET, it is possible to sustain mitochondrial quality by maintaining a network of healthy mitochondria during the enhancement of mitophagy following CCCP treatment.

Inhibition of mitoNEET hampered CCCP-induced mitochondrial apoptosis.

Mitochondria play key roles in activating apoptosis in mammalian cells [34]. Mitochondrial depolarization by CCCP induces ROS-mediated cell death [29]. We showed that CCCP-induced mitochondrial ROS was attenuated by mitoNEET inhibition (Fig. 3). We assessed the effects of mitoNEET inhibition during CCCP-induced cell death using the annexin V-FITC/PI assay (Fig. 5A, B). Cells were treated with CCCP (25 μM) in the presence or absence of NL-1 for 12 hours. Next, Annexin V–FITC / propidium iodide-PE positive cells (apoptosis) were assayed by flow cytometry using an Annexin V (AV)/Propidium Iodide (PI) Apoptosis Detection Kit. Inhibition of mitoNEET rescued CCCP-induced apoptosis (Fig. 5A). CCCP-induced apoptosis was also rescued by mitoNEET shRNA (Fig. 5B). Moreover, mitoNEET inhibition by NL-1 or mitoNEET shRNA resulted in a decreased level of lactate dehydrogenase (LDH), the release of which serves as a cell-death marker (Fig. 5C, D). We treated cells with CCCP and then performed a cell-viability assay. Cell viability was evaluated by the MTT assay using the Ez-Cytox Cell viability Assay Kit. CCCP-induced cell death was rescued by mitoNEET inhibition by NL-1 or mitoNEET shRNA (Fig. 5E, F). These data indicated that inhibiting mitoNEET bestows a protective effect against CCCP-induced apoptosis through the attenuation of mitochondrial ROS during mitochondrial damage. CCCP directly interferes with mitochondrial function and induces apoptosis [35]. We hypothesized that cell death regulated by mitoNEET inhibition during CCCP-induced mitochondrial damage is mitochondrial apoptosis. We analyzed whether inhibiting mitoNEET protects RAW264.7 cells against CCCP-induced mitochondrial apoptosis. Cells were treated with CCCP (10 μM) in the presence or absence of NL-1 for 12, 24, and 36 hours. Next, levels of mitochondrial apoptosis marker protein were detected using western blotting. CCCP-induced cleavage of caspase-9 (cysteine-aspartic proteases-9), caspase-3 (cysteine-aspartic proteases-3), and PARP-1 (Poly [ADP-ribose] polymerase 1) proteins was decreased in the presence of NL-1 in RAW264.7 cells (Fig. 5G). Consistent with this, cleavage of caspase-9, caspase-3, and PARP-1 proteins was also decreased in CCCP-treated cells expressing mitoNEET shRNA (Fig. 5H). Taken together,

these data demonstrated that mitoNEET serves as a key molecule to mediate mitochondrial cell death through the regulation of mitochondrial quality following CCCP treatment.

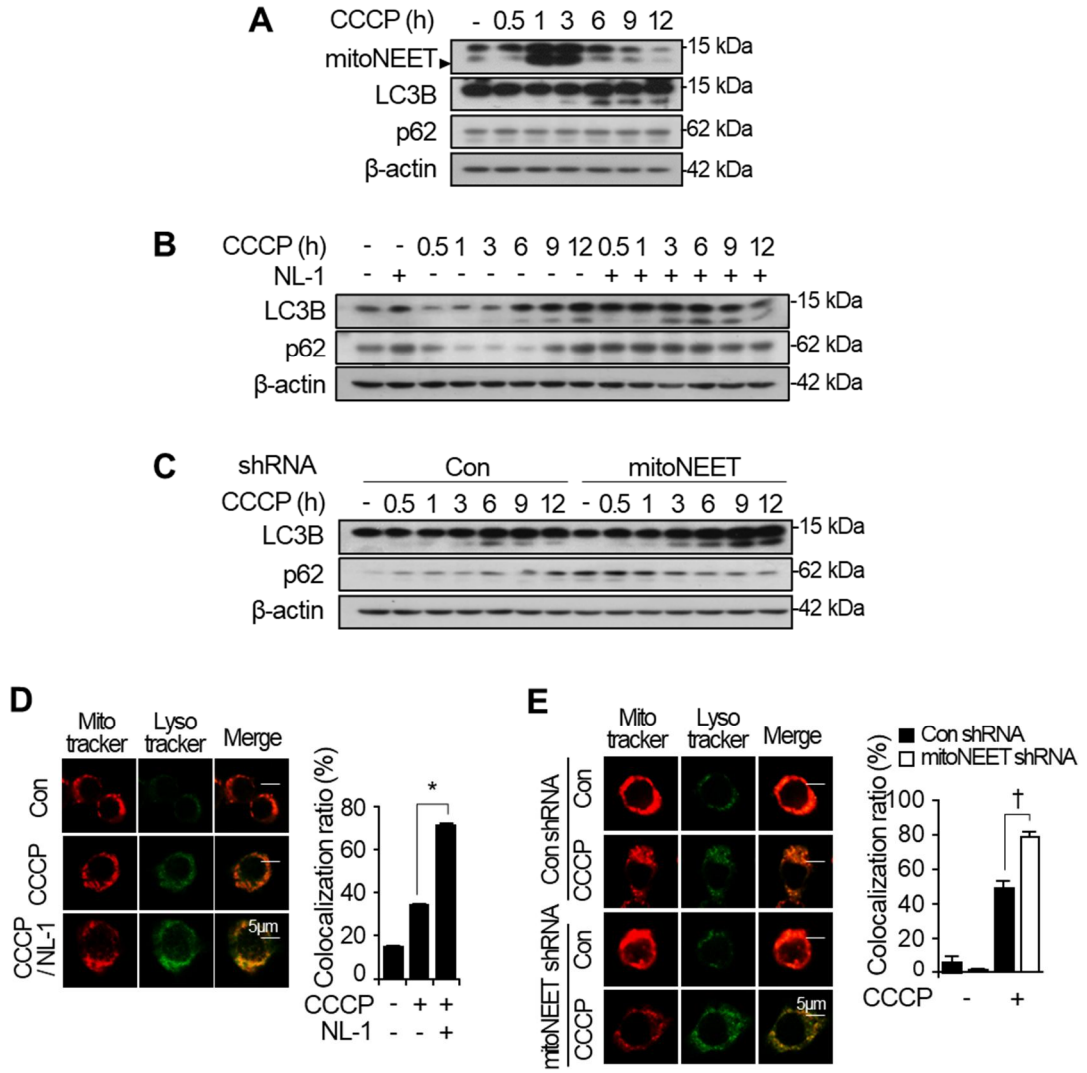

Figure 1. Inhibition of mitoNEET increases CCCP-induced mitophagy.

Total protein was extracted from RAW264.7 cells after administration of vehicle or CCCP (25 μM) for variable time points (A). Expression levels of mitoNEET and LC3-□, p62 proteins were assessed by western blotting. RAW264.7 cells were treated with CCCP or CCCP plus NL-1 (20 μM) for variable time points (B). RAW264.7 cells transfected with control shRNA or mitoNEET shRNA were stimulated with CCCP (25 μM) or vehicle for variable time points (C). Expression levels of

autophagy-related proteins, LC3- and p62 were analyzed by western blot (B, C). RAW264.7 cells (D) and control shRNA- or mitoNEET shRNA-expressing RAW264.7 cells (E) were treated with vehicle, CCCP (25 μM) or CCCP plus NL-1 (20 μM) for 6 hours. Cells were then stained with LysoTracker (green fluorescence) and MitoTracker (red fluorescence) and were analyzed by confocal fluorescence microscopy (D, E). Scale bar: 5 μm. Fluorescence intensity was measured using Image J. All data are expressed as the mean ± SD from three independent experiments. $*P < 0.05$ for CCCP vs. CCCP plus NL-1. $\dagger P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of CCCP.

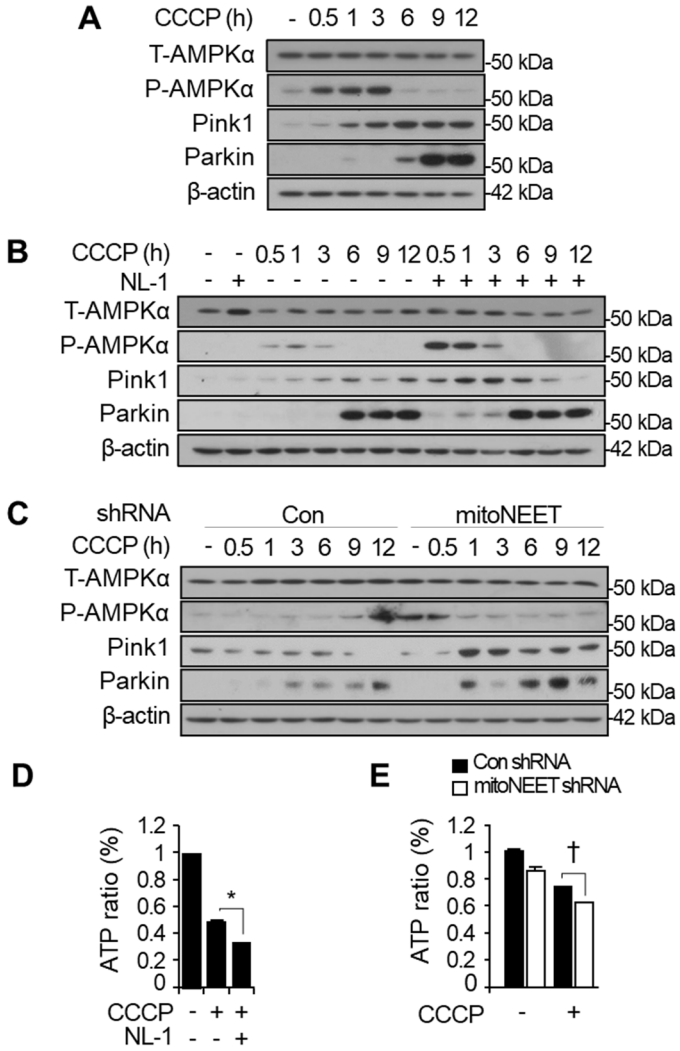

Figure 2. Inibition of mitoNEET increases CCCP-induced Pink1–Parkin-mediated mitophagy.

RAW264.7 cells were treated with vehicle, CCCP (25 μM) for variable time points (A). RAW264.7 cells were treated with CCCP or CCCP plus NL-1 (20 μM) for variable time points (B, D). RAW264.7 cells expressing-control shRNA- or mitoNEET shRNA were stimulated with vehicle or CCCP for variable time points (C, E). Total protein was harvested and the expression levels of mitophagy related proteins, Total- or phosphate-AMPKα and Pink1, Parkin were analyzed by western blotting, β-actin was used as a loading control (A-C). Intracellular ATP levels were detected by ATP Assay Kit at 3 hours (D, E). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for CCCP vs. CCCP plus NL-1. †$P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of CCCP.

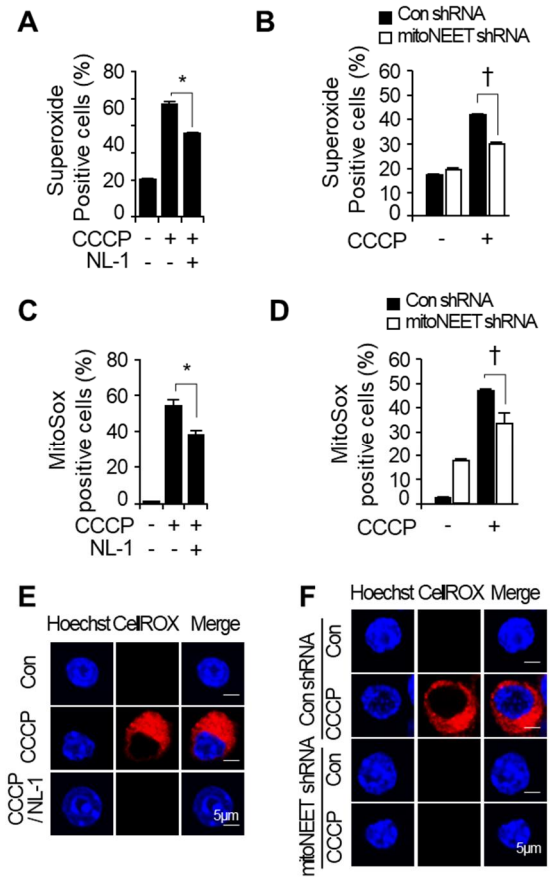

Figure 3. Inhibition of mitoNEET attenuates CCCP-induced mitochondrial reactive oxygen species and enhances expression of antioxidant defense genes.

RAW264.7 cells were treated with vehicle, CCCP (70 μM) or CCCP plus NL-1 (20 μM) for 9 hours (A, C and E). RAW264.7 cells expressing control shRNA or mitoNEET shRNA were treated with vehicle or CCCP (70 μM) for 9 hours (B, D and F). Superoxide (A, B) and MitoSox (C, D) were measured by flow cytometry using the fluorescent probes superoxide detection reagent (orange) and mitoSOX mitochondrial superoxide indicator (red), respectively. All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for CCCP vs. CCCP plus NL-1. †$P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of CCCP. Cytosolic ROS (E, F) was assayed at 6 hours after administration of vehicle, CCCP (25 μM), or CCCP plus NL-1 (20 μM) for confocal fluorescence microscopy using the fluorescent probes and Hoechst 33258 (nuclei, blue) and CellROX® Deep Red (total ROS, red). Scale bar: 5 μm.

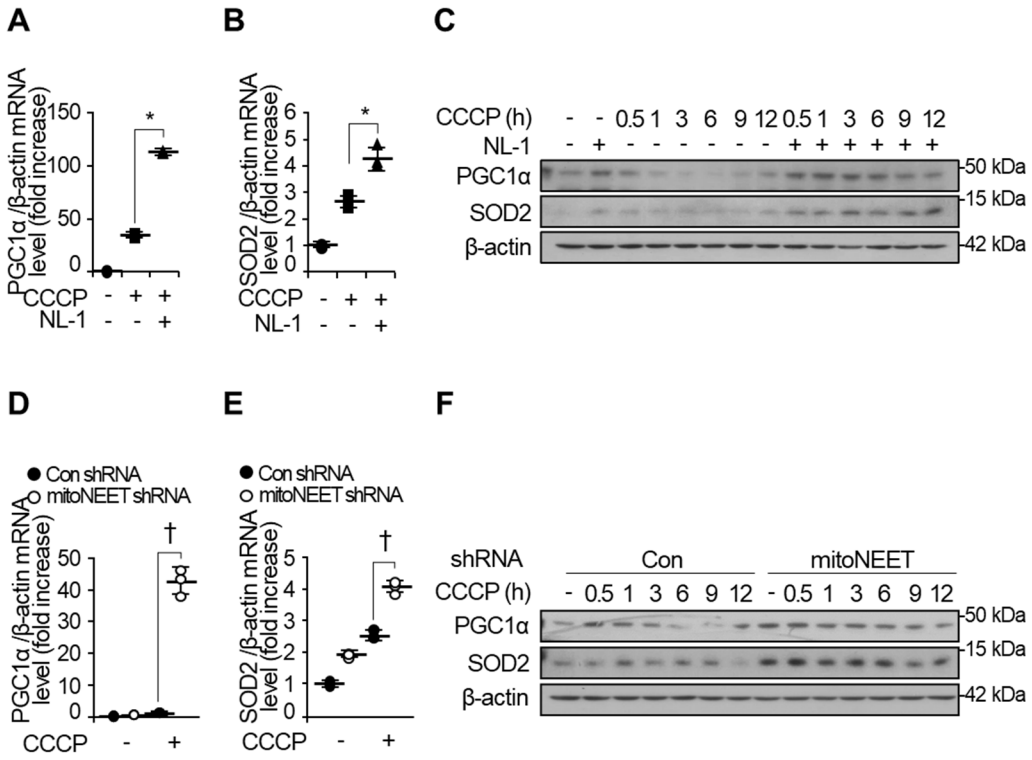

Figure 4. Inhibition of mitoNEET hinders CCCP-induced mitochondrial apoptosis.

RAW264.7 cells, Control shRNA-expressing or mitoNEET-expressing RAW264.7 cells were treated with vehicle, CCCP (25 μM), or CCCP plus NL-1 (20 μM). Total RNA and protein were harvested 12 hours later, followed by measurement of mRNA encoding PGC-1α, SOD2 by quantitative real-time RT-PCR (A, B and D, E). Expression levels of PGC-1α and SOD2 protein were detected by western blotting (C and F). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for CCCP vs. CCCP plus NL-1. †$P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of CCCP.

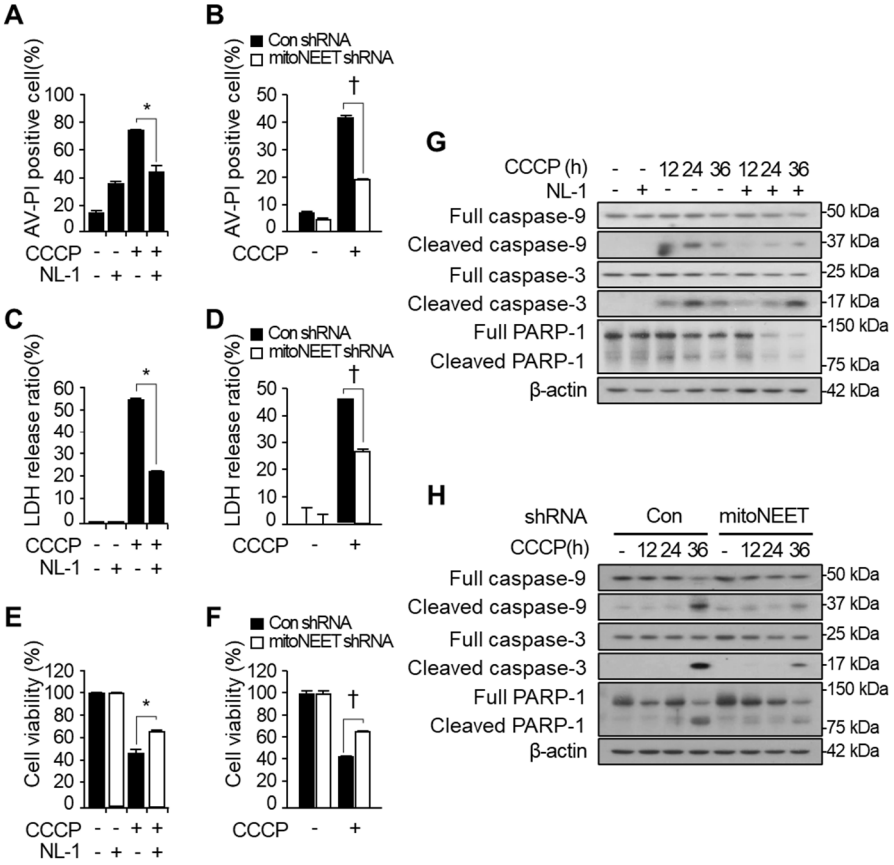

Figure 5. Inhibition of mitoNEET hinders CCCP-induced mitochondrial apoptosis.

RAW264.7 cells, control shRNA-expressing or mitoNEET-expressing RAW264.7 cells were treated with vehicle, CCCP (25 μM), or CCCP plus NL-1 (20 μM). Cells were then stained with Annexin V-FITC and PI and detected by flow cytometry at 12 hours (A, B). The LDH release was analyzed by the LDH-release assay at 12 hours (C, D). Cell viability was analyzed using Ez-Cytox Cell viability Assay Kit at 6 hours (E, F). All data are expressed as the mean ± SD from three independent experiments. *$P < 0.05$ for CCCP vs. CCCP plus NL-1. †$P < 0.05$ for control shRNA-expressing cells vs. mitoNEET shRNA-expressing cells in the presence of CCCP. Total protein was harvested 12, 24 and 36 hours after administration of vehicle, CCCP (10 μM) or CCCP plus NL-1 (20 μM) in RAW264.7 cells and control shRNA-expressing or mitoNEET-expressing RAW264.7 cells. Expression levels of mitochondrial apoptosis related proteins, cleaved-caspase-9, -caspase-3, and -PARP-1, were detected by western blotting, β-actin was used as an internal loading control (G, H).

Discussion

Selective autophagy of mitochondria, known as mitophagy, is an important mitochondrial quality control mechanism that eliminates damaged mitochondria [36]. The pathogenesis of several human diseases is associated with impaired mitophagy [37]. In current research, defective mitophagy is emerging as a potential therapeutic target for the treatment of human diseases (11, 38). Early studies suggested that mitoNEET, a mitochondrial outer membrane protein, plays a role in the intermitochondrial junction and in the regulation of autophagy or mitophagy [17, 19, 23]. In the present study, we proposed that targeting mitoNEET may serve as a potential enhancer of mitophagy in mitochondrial dysfunctional diseases. Our data showed that CCCP influences mitophagy by reducing the mitochondrial membrane potential. It also induces mitoNEET protein levels prior to the elevation in the levels of LC3- and p62, which are autophagy-related proteins, in RAW264.7 cells (Fig. 1). These data demonstrated that mitoNEET is operative in the early stage of the autophagic response. Prior studies reported that mitoNEET is significantly reduced in response to E3 ubiquitin protein ligase (Parkin) recruitment in CCCP-induced mitophagy [24]. However, whether the targeting of mitoNEET plays a role as a mitophagy activator has not been investigated yet. Interestingly, our data showed that mitoNEET inhibition by NL-1 or mitoNEET shRNA augments autophagy-related proteins, in addition to the colocalization of mitochondria and lysosomes (Fig. 1). Furthermore, mitophagy-associated protein levels of phosphate AMPKα, Pink1, and Parkin were also enhanced by mitoNEET inhibition (Fig. 2). Therefore, our results showed that inhibiting mitoNEET, which acts as a regulator of the intermitochondrial junction, could enhance Pink1–Parkin-mediated mitophagy. Recent studies have reported that mitoNEET is associated with mitochondrial dysfunction in disease progression. Mitochondrial dysfunction could induce mitophagy as a feedback mechanism to remove damaged

mitochondria. A prior study advanced an opinion that mitoNEET is a mitophagy-activating molecule because of the observation of accumulating autophagosomes caused by mitoNEET-mediated mitochondrial dysfunction [39] However, in several human diseases, inefficient mitophagy results in the accumulation of dysfunctional mitochondria and mitophagosome due to the decreased fusion with lysosomes and consequent failed mitochondrial quality control [11, 43]. In our study, we showed that LC3- protein levels began to rise with the attenuation of mitoNEET protein levels, which were initially increased. In addition, mitoNEET inhibition promoted not only accumulation of mitophagy-linked proteins but also the activation of mitochondria–lysosome crosstalk. These findings demonstrated that mitoNEET-mediated dysfunctional mitochondria generation under stress conditions needs to be suppressed for the enhancement and initiation of mitophagy. This would consequently mediate clearance of damaged mitochondria, thereby surmounting impaired mitophagy in multiple human diseases. Furthermore, inhibition of mitoNEET using NL-1 or mitoNEET shRNA attenuated CCCP-induced ROS or superoxide (Fig. 3) and increased PGC-1α and SOD2 mRNA and protein levels (Fig. 4). Particularly, as PGC-1α is a major regulator of mitochondrial biogenesis, elevated PGC-1α ensures the removal of damaged and/or superfluous mitochondria, as well as the balanced generation of new organelles [31, 32, 40]. CCCP-induced ROS and disequilibrium between mitochondrial biogenesis and selective autophagy cause deterioration of cellular function and cell death [32]. Our data showed that CCCP-induced cell death recovered in the presence of a mitoNEET inhibitor, NL-1, or upon expression of mitoNEET shRNA (Fig. 5). These data indicated that targeting mitoNEET exerts a protective effect against cell stress through mitochondrial quality control with elevated mitophagy. Therefore, our results delineated a new paradigm for the role of mitoNEET in the mitophagic process and demonstrated that mitoNEET is a possible therapeutic target for the elimination of irreversibly damaged mitochondria and maintaining mitochondrial quality control in numerous diseases.

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Material and Method

Cell culture

RAW264.7 cells were cultured in Dulbecco Modified Eagle Medium (Life Technologies, Grand Island, NY, USA), 5% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin under an atmosphere of 95% air and 5% CO2 at 37 °C. Bone marrow-derived macrophages (BMDMs) from C57BL/6 mice were isolated and differentiated as described previously [35]. Briefly, bone marrow cells (3 × 107 cells) were cultured in macrophage differentiation medium with GM-CSF at 37 °C for 7 days. The adherent macrophages were detached from culture dishes by treatment with 5% EDTA in PBS, followed by scraping with a sterile cell scraper. The resuspended cells were then directly seeded on cell culture plates for other experiments.

Construction of mitoNEET shRNA-expressing cells

The mitoNEET shRNA and the nonspecific control shRNA (Sigma-Aldrich, St Louis, MO) were transfected into RAW264.7 cells using transfection reagents (Promega, Madison, WI, USA) according to the manufacturer's protocol. The sequences of mouse mitoNEET shRNA were as follow: 5'-CCG GCG TAG GAC CTC TGA TCA TCA ACT CGA GTT GAT GAT CAG AGG TCC TAC GTT TTT TG-3'. The expression of mitoNEET and □-actin in stable cells was measured.

Reagent

mitoNEET Inhibitor, NL-1, was purchased from (Merck Millipore, Billerica, MA, USA, 475825). Deferoxamine mesylate salt (DFO) was purchased from (Sigma-Aldrich, St Louis, MO, D9533). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was purchased from (Sigma-Aldrich, St Louis,

MO, C2759). Lipopolysaccharides from Escherichia coli O26:B6 was purchased from (Sigma-Aldrich, St Louis, MO, L2654).

Animal experiments

Animal care and use for all experiments were approved from the animal facilities at University of Ulsan (SWC-14-012). C57BL/6 mice were purchased from ORIENT BIO Inc (Busan, Korea). CLP-induced polymicrobial sepsis and fibrin clot experiment were performed as previously described [50]. Using sterile conditions, the fibrin clot containing *E. coli* or *S. aureus* (1×10^8 CFU) was placed within the peritoneal cavity of C57BL/6 mice. Lipopolysaccharides from *E. coli* 0127:B8 – purified by phenol extraction was purchased from (Sigma-Aldrich, St Louis, MO, L3129) for LPS-induced sepsis. mitoNEET inhibitor, NL-1 (20 mg/kg), was administrated to C57BL/6 mice 12 hours before LPS (20 mg/kg) injection (pre-administration).

Western immunoblotting and enzyme-linked immunosorbent assay

Cell extracts were harvested using RIPA buffer (Tris/Cl (pH 7.6); 100 mmole/L, EDTA; 5 mmole/L, NaCl; 50 mmole/L, b-glycerophosphate; 50 mmole/L, NaF; 50 mmole/L, Na3VO4; 0.1 mmole/L, NP-40; 0.5%, Sodium deoxycholate; 0.5%) with 1×Complete™ protease inhibitor Cocktail (Roche Applied Science, Mannheim, Germany). Protein concentrations of cell lysates were determined using Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) and were resolved by SDS-polyacrylamide gels. Proteins were transferred on Pure PVDF membranes. Membranes were blocked for 2 hours at room temperature with a 5% nonfat milk solution in TBST buffer (20 mM Tris–HCl, pH 7.4, 500 mM NaCl, 0.1% Tween20). The blots were incubated with antibody an anti-CISD1 (1:2000) (Protein Tech, 16006-1-AP), an anti-β-actin (1:5000) (Sigma-Aldrich, St Louis, MO, A5441), an anti-phospho-IkBα (1:1000) (Santa Cruz Biotechnology, sc-8404), an anti-IkBα (1:1000) (Santa Cruz Biotechnology, sc-847) and an anti-COX2 (1:1000) (Cayman chemical, 160106), an anti-iNOS (1:1000) (Santa Cruz Biotechnology, sc-650) and an anti-HO-1 (1:3000) (Enzo Life Sciences, ADI-SPA-896), an anti-SOD2

(1:3000) (Santa Cruz Biotechnology, sc-30080), an anti-SOD1 (1:1000) (Santa Cruz Biotechnology, sc-11407) and anti-NLRP3/NALP3 (1:1000) (Adipogen, AG-20B-0014-C100), an-anti-P2X7R (1:3000) (Alomone Labs, APR-004), an anti-LC3- (1:2000) (Sigma-Aldrich, L7543), an anti-p62/SQSTM1 (1:1000) (Sigma-Aldrich, P0067) and an anti-AMPKα (23A3) (1:1000) (Cell signaling technology, #2603), an anti-Phospho-AMPKα (Thr172) (1:1000) (Cell signaling technology, 2535S) and an anti-PINK1 (1:1000) (Proteintech, 23274-1-AP), an anti-Parkin (H-300) (1:1000) (Santa Cruz Biotechnology, sc-30130) and an anti-Caspase-9 (1:1000) (Cell signaling technology, #9504), an anti-Cleaved Caspase-9 (Asp353) (1:1000) (Cell signaling technology, #9509) and an anti-Caspase-3 (1:1000) (Cell signaling technology, #9662), an anti-Cleaved Caspase-3 (Asp175) (1:1000) (Cell signaling technology, #9664) and an anti-PARP-1 (H-250) (1:1000) (Santa Cruz Biotechnology, sc-7150) in TBST overnight at room temperature. The blots were incubated with an anti-secondary antibody (1:5000) in TBST, immunoblots were detected by SuperSignal® West Pico Chemiluminescent Substrate (Pierce) and visualized after exposure to X-ray film.

Mouse IL-1β (R&D systems, Minneapolis, MN, USA, DY401), IL-6 (R&D systems, Minneapolis, MN, USA, DY406), and TNFα (R&D systems, Minneapolis, MN, USA, DY410) were measured from cell culture supernatant of BMDMs and RAW264.7 cells using enzyme-linked immunosorbent assay (ELISA).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated TRIzol reagent (Invitrogen, Life technologies, Carlsbad, CA), Reverse transcription was performed using SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was conducted using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Triplicate samples per condition were analyzed on an Applied Biosystems StepOnePlusTM Real-Time PCR System using absolute quantification settings. The primers sequences were as follows: mouse mitoNEET (forward: 5'-CAA GGC TAT GGT GAA TCT TCA G-3' and reverse: 5'-GTG CCA TTC TAC GTA AAT CAG-3'), mouse β-actin (forward: 5'-GAT CTG GCA CCA CAC

CTT CT-3' and reverse: 5'-GGG GTG TTG AAG GTC TCA AA-3'). Mouse IL-1β (forward: 5'-TTG ACG GAC CCC AAA AGA TG-3' and reverse: 5'-AGA AGG TGC TCA TGT CCT CA-3'), Mouse IL-6 (forward: 5'-GAG GAT ACC ACT CCC AAC AGA CC-3' and reverse: 5'-AAG TGC ATC ATC GTT GTT CAT ACA-3'), Mouse TNFα (forward: 5'- GCC TCT TCT CAT TCC TGC TTG-3' and reverse: 5'-CTG ATG AGA GGG AGG CCA TT-3'), Mouse IL-6 (forward: 5'-GAG GAT ACC ACT CCC AAC AGA CC-3' and reverse: 5'- AAG TGC ATC ATC GTT GTT CAT ACA-3'), mouse COX2 (forward: 5′-CAA GGG AGT CTG GAA CAT TG-3′ and reverse: 5′-ACC CAG GTC CTC GCT TAT GA-3′), mouse iNOS (forward: 5′-AAC GGA GAA CGT TGG ATT TG-3′ and reverse: 5′-CAG CAC AAG GGG TTT TCT TC-3′), mouse HO-1 (forward: 5′-CGC CTT CCT GCT CAA CAT T-3′ and reverse: 5′-TGT GTT CCT CTG TCA GCA TCA C-3′), mouse SOD2 (forward: 5′- ATG GTG GGG GAC ATA TT-3′ and reverse: 5′-GAA CCT TGG ACT CCC ACA GA -3′), Mouse PGC-1α (forward:5′-CCG AGA ATT CAT GGA GCA-3′ and reverse: 5′-TTT CTG TGG GTT TGG TGT-3′). Amplification of cDNA started with 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minutes at 60 °C.

ATP Measurements.

Total ATP levels were monitored using an ATP Colorimetric/Fluorometric Assay Kit (K354) as per the manufacturer's instructions (BioVision, Milpitas, CA, USA). The cells (1×10^6) were lysed by quickly piptetting up and down a few times in 100 µL of ATP assay buffer and then centrifuged under ice-cold conditions at 15,000g for 2 min to pellet the insoluble materials. The supernatant was collected and 2–50 µL of this supernatant was added to a 96-well plate, with the final volume topped up to 50 µL/well with ATP assay buffer. ATP reaction mix was made (ATPassay buffer 44 µL, ATP probe 2 µL, ATP converter 2 µL and developer mix 2 µL), and 50 µL of this reaction mix was added to each well containing a test sample. Then the plate was incubated at room temperature for 30 min in the dark, and the OD was measured at 570 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometry for total reactive oxygen species (ROS) and superoxide measurement

Cultured RAW264.7 were seeded 1.5 × 10^5 cells/well in duplicate on 12-well plates. Next day, cells were treated with vehicle, LPS, LPS plus NL-1, or LPS plus DFO (desferoxamine) or CCCP or CCCP plus NL-1. Then, cells were washed with PBS wash collected by centrifugation with 5% FBS containing PBS. Remove supernatant from cells and carefully wash cells with 1X Wash Buffer. Samples were then centrifuged at 1000 rpm for 3 min and the pellets were resuspended in 500 µL of ROS/Superoxide Detection Solution 2X (ROS-ID® Total ROS/Superoxide Detection Kit, Enzo Life Sciences, Farmingdale, NY, USA, ENZ-51010). Cells were incubated for 30 min at 37 °C in the dark. Measurements were performed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer.

Flow cytometry for mitoSOX red mitochondrial superoxide

Cultured RAW264.7 were seeded 1.5 × 10^5 cells/well in duplicate on 12-well plates. Next day, cells were treated with CCCP (70 µM) or CCCP plus NL-1 (20 µM) for the indicated times. After for 9 hours, cells were incubated with 5 µM Mitosox red (MitoSOX™ Red Mitochondrial Superoxide Indicator, for live-cell imaging, (Invitrogen, Life Technologies, Grand Island, NY, M36008) for 30 min at 37 °C in the dark. After 30 minutes of loading, samples were the centrifuged at 1000 rpm for 3 minutes and the pellets were resuspended in 500 µL of 5% FBS containing PBS. Measurements were performed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer.

Confocal microscopy

RAW264.7 cells were seeded at 1 × 10^5 cells per well on coverslips in 24-well plates and treated with reagents. After reagent treatment, media was removed by washing with PBS and cells were incubated with serum free media containing 5 µM Cell ROX red (CellROX® Deep Red Reagent for oxidative stress detection, Invitrogen, Life Technologies, Carlsbad, CA , C10422) or 200 nM Mitotracker red CMXRos (Invitrogen, Life Technologies, Carlsbad, CA, M7512) and 500 nM TMRM

(tetramethylrhodamine, methyl ester, Perchlorate, Invitrogen, Life Technologies, Carlsbad, CA, T668) or 100 nM Mitotracker red (Mitochondrial marker deep red, Invitrogen, Life Technologies, Carlsbad, CA, M22426) or 75nM LysoTracker Green (LysoTracker™ Green DND-26, Invitrogen, Life Technologies, Carlsbad, CA, L7526) and 5 μM Mito-FerroGreen (Dojindo Laboratories, Kumamoto, Japan, M489) and for 30 minutes at 37 °C in the dark. And then, cells fixed for 20 minutes in 4 % formaldehyde, rinsed 3 times in PBS. A nuclear counterstaining was made with a solution of 1 μg/mL Hoechst 33258 stain for 5 minutes and mounting on a slide Fluorescence Mounting Medium (DAKO North America Inc, Carpinteria, CA, United States, S3023). Olympus FV1000 MPE microscope was used to acquire images.

Annexin V (AV)/Propidium Iodide (PI) staining assay

Cell death was analyzed by PI staining with flow cytometry. Cultured RAW264.7 were seeded 1×10^5 cells/well in duplicate on 12-well plates. Next day, cells were treated with CCCP (25 μM) or CCCP plus NL-1 (20 μM) for the indicated times. After for 12 hours, the cells were harvested, washed with phosphate-buffered saline (PBS), and stained with the Annexin V (AV)/Propidium Iodide (PI) Apoptosis Detection Kit (Becton Dickinson, San Jose, CA, USA, 556547). Measurements were performed on a FACS Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer.

Lactate dehydrogenase (LDH)-release assay

Cultured RAW264.7 were seeded 1×10^5 cells/well in duplicate on 12-well plates. Next day, cells were treated with CCCP (25 μM) or CCCP plus NL-1 (20 μM) for the indicated times. After reagent treatment, In the high control wells, 10 μL cell lysis solution (LDH-Cytotoxicity Colorimetric Assay kit II, BioVision, 155 S Milpitas Boulevard, Milpitas, CA 95035, USA, #K313-500) was added, and the plate was shaken for 1 min. Quantitative analysis was performed on the cell culture supernatant (20 μL/well). For each well, 100 μL of LDH Reaction Mix (LDH Assay Buffer : WST substrate Mix = 50 : 1) was added. Absorbance was then measured at 450 nm using a SpectraMax M2 microplate reader (Molecular

Devices, Sunnyvale, CA, USA) to calculate LDH release percentage.

Cell viability assay

Cell viability was determined by the MTS assay using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, G3581). Cells were seeded at 1×10^4 cells per well in 96-well plates. After reagent treatment, 20 μL of MTS solution was added to each well. Plates were incubated for an additional 2~4 hours at 37°C. Absorbance at 490 nm was then measured using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) to calculate the cell survival percentages.

Statistical analysis

All results were confirmed in at least three independent experiments; data from one representative experiment are shown. Quantitative data are shown as means ± standard deviation and significance of statistical analysis was determined with two-tailed, unpaired Student's *t*-test. *P*-values <0.05 were considered significant.

미토콘드리아 기능 장애에서 mitoNEET의 역할과 작용 기전 연구

이승희

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초록

미토콘드리아(Mitochondria)는 세포의 동력원 역할을 하는 소기관으로서, 미토콘드리아 기능의 완전성은 세포 생활의 기본이며 신체의 거의 모든 장기에 영향을 미친다. 미토콘드리아는 산화적 인산화(OXHOS)를 통해 에너지 대사 및 활성 산소 종(ROS) 생산에 역할을 하고, 다양한 생합성 경로와 세포사멸 경로를 조정한다. 따라서, 미토콘드리아 기능 장애는 암, 노화, 당뇨병 및 심혈관 질환과 같은 인간 병리와 관련이 있고, 미토콘드리아 유래 산화 스트레스는 패혈증에 의한 장기부전 및 신경퇴행성 질환을 유발한다. 미토콘드리아는 증식(biogenesis), 융합(fusion), 분열(fission), 미토파지(mitophagy) 와 같은 미토콘드리아 품질 관리 (mitochondrial quality control)를 통해 조절되어 미토콘드리아 손상으로부터 보호된다. 하지만, 많은 미토콘드리아 질환에서 미토파지의 손상과 미토콘드리아의 기능 장애의 축적이 나타나고, 이것은 질병의 악화와 사망으로 이어진다. 따라서, 많은 연구가 미토콘드리아 기능 장애를 표적으로 하는 치료적 접근에 초점을 맞추고 있다. 최근, 미토콘드리아 단백질인 mitoNEET가 미토콘드리아 치료의 새로운 약물 표적으로 떠오르고 있다. MitoNEET는 당뇨병 치료제로서, PPAR-γ의 작용자인 TZD (thiazolidinedione) 약물에 결합되면서 발견되었으며, PPAR-γ 만으로 설명할 수 없는 TZD약물의 유익한 효과를 납득하게 하는 연구결과가 나타나고 있다. 미토콘드리아 외막에서 세포질을 향해 있는 MitoNEET는 철-황산 클러스터를 함유하는 단백질로서, 미토콘드리아 철 함량 (iron)과 활성 산소 종(ROS)의 강력한 조절자이고 미토콘드리아 네트워크 형태의 제어와 미토파지 작용에 관여한다. 본 연구에서는 미토콘드리아 기능장애에서 mitoNEET의 역할과 기전을 규명하고 손상된 미토콘드리아로 인한 질환에서 mitoNEET가 치료적 표적으로 가능한지 알아보고자 하였다.

첫째, 패혈증과 같은 염증성 질환에서 mitoNEET가 미토콘드리아 기능 장애와 산

화 스트레스에 기여함을 확인하고자 하였다. LPS에 의한 염증 자극은 골수 유래 대식세포(BMDM)나 패혈증 동물 모델에서 mitoNEET의 발현을 mRNA와 단백질 수준에서 증가시킴을 확인하였다. 염증 상태동안 mitoNEET의 발현은 LPS에 의한 NF-κB 신호 전달 경로에 의해 유도되었다. 우리는 염증 반응에서 mitoNEET의 역할을 확인하기 위해 PPAR-γ의 친화력이 제거된 TZD인 NL-1과 shRNA를 형질주입 시킨 대식세포를 이용하였다. 흥미롭게도, LPS로 자극된 대식세포나 패혈증 모델에서 염증 매개체인 IL-1β, IL-6, TNFα, iNOS, COX2의 발현이 mitoNEET 억제제인 NL-1과 mitoNEET shRNA를 통한 mitoNEET의 억제에 의해서 감소되는 것을 RT-PCR, ELISA, Western blotting을 통해 확인하였다. 또한, 대식세포에서 LPS로 유도된 ROS와 미토콘드리아 탈분극, 미토콘드리아 내부에 축적된 철이 mitoNEET의 억제에 의해서 회복되는 것을 Flow cytometry, confocal microscopy를 통해서 확인하였다. 이와 함께, LPS처리에서 mitoNEET의 억제는 항산화 효소인 HO-1과 SOD2의 발현을 mRNA와 단백질 수준을 증가시켰다. 위의 결과들은 LPS로 유도되는 염증반응에서 발현되는 mitoNEET가 미토콘드리아 기능장애와 산화스트레스에 역할을 가지고, 패혈증에서 나타나는 과도한 염증 반응의 가속화에 기여할 수 있음을 보여준다.

둘째, 염증반응 동안 mitoNEET의 억제로 매개되는 항산화 활성에 PGC-1α가 관여함을 확인하고자 하였다. PGC-1α는 미토콘드리아의 생합성과 항산화 효소인 HO-1과 SOD2의 주요 조절자이다. 미토콘드리아 산화 손상을 유도하는 LPS의 염증 자극이 골수유래-대식세포(BMDM)에서 mitoNEET의 발현을 증가시켰고, 증가하였던 mitoNEET가 감소될 때 PGC-1α가 유도되는 것을 단백질 수준에서 확인하였다. 흥미롭게도, LPS를 처리한 대식세포에서, mitoNEET 억제제인 NL-1과 mitoNEET shRNA를 통한 mitoNEET의 억제는 mRNA와 단백질 수준에서 PGC-1α 발현을 크게 증가시켰다. LPS로 유도되는 산화적 스트레스가 NL-1에 의해 억제될 때, mitoNEET의 억제에 의해 활성화되는 PGC-1α가 항산화 활성에 역할을 하는지 확인하기 위해, PGC-1α shRNA 시스템을 이용하였다. NL-1은 LPS로 유도되는 ROS를 감소시켰지만, PGC-1α shRNA를 발현하는 세포에서는 이러한 현상이 나타나지 않는 것을 Flow cytometry, confocal microscopy를 통해 확인하였다. 뿐만 아니라, LPS가 처리된 대식세포에서 NL-1은 항산화 효소인 HO-1과 SOD2를 증가시켰지만, PGC-1α shRNA를 발현하는 세포에서는 이러한 현상이 나타나지 않는 것을 mRNA와 단백질 수준에서 확인하였다. PGC-1α는 염증반응동안 활성화된 해당과정(Glycolysis)에 따른 ATP에 의해 억제되어 있다. 최근, 산화환원 효소로서 mitoNEET의 전자전달 작용이 해당과정을 간접적으로 활성화시킬 수 있다는 것이 제시되었다. 우리는 mitoNEET에 의한

PGC-1α의 발현 조절이 해당과정의 ATP와 AMPK 신호와 연관 되어있는 것을 확인하였다. mitoNEET의 억제는 ATP의 감소를 유도하고, 이에 따라 AMPK신호 활성 및 PGC-1α의 증가로 이어질 수 있음을 나타냈다. 게다가 mitoNEET조절에 의한 PGC-1α의 항산화 작용은 염증 매개체를 약화시킬 수 있는 것을 RT-PCR과 ELISA, Western blotting을 통해 확인할 수 있었다. 위의 결과들은 PGC-1α가 NL-1 매개 항산화 효과의 주요 조절자임을 나타낸다. 그러므로, 패혈증에서 mitoNEET를 표적으로 하는 치료적 접근은 PGC-1α의 활성화를 통해 산화적 손상으로부터 장기의 보호와 건강한 미토콘드리아 유지의 효과를 가져올 수 있다.

셋째, 미토콘드리아 간의 접합에 관여하는 분자인 mitoNEET의 표적화가 미토콘드리아 기능 장애 질환에서 미토파지 (mitophagy)의 증강 인자로 가능한지 확인하고자 하였다. CCCP는 미토콘드리아 탈분극을 통해 미토콘드리아의 손상을 유발함으로써 간접적인 미토파지 유도제로 알려진다. 대식세포에서 CCCP의 처리가, 자가포식 (autophagy)의 마커로 알려진 LC3-Ⅱ와 p62의 단백질을 상승시키기 전에, mitoNEET의 발현을 증가시키는 것을 단백질 수준에서 확인하였다. CCCP가 처리된 대식세포에서 NL-1과 mitoNEET shRNA를 이용한 mitoNEET의 억제는 자가포식의 마커로 알려진 LC3-Ⅱ와 p62 발현을 더 촉진시켰고, 미토파지의 마커로 알려진 AMPK, Pink1, Parkin의 발현을 크게 증가시켰다. 뿐만 아니라, mitoNEET억제가 CCCP로 유도되는 미토콘드리아와 리소좀의 결합을 더 활성화시키는 것을 confocal microscopy를 통해 확인하였다. NL-1과 mitoNEET shRNA를 이용한 mitoNEET의 억제가 CCCP에 의한 미토콘드리아 ROS와 세포의 사멸을 약화시키는 것을 flow cytometry를 통해 나타냈다. 이와 함께, 산화 대사 조절인자인 PGC-1α 및 SOD2의 발현이 활성화되는 것을 mRNA, 단백질 수준에서 확인하였다. 위의 결과들은 CCCP에 의한 미토콘드리아 기능장애에서 mitoNEET의 억제가 미토파지 강화제로서 가능하고, 미토콘드리아 기능장애로부터 세포를 보호할 수 있는 것을 보여준다.

따라서, 본 연구는 산화 스트레스 상황에서 다르게 작용하는 mitoNEET의 기능을 처음으로 규명하여, 패혈증과 같은 염증성 질환에서 mitoNEET 기능에 대한 이해에 크게 기여하고 mitoNEET와 관련된 일부 논란을 해결하는데 도움이 된다. 또한, 우리 연구에서 미토파지의 새로운 증강인자로서 mitoNEET의 표적에 대한 새로운 패러다임을 제안하였다. 위의 연구는 미토콘드리아 기능장애와 손상된 미토파지로 인한 질환에서 mitoNEET가 치료적 표적으로서 가능함을 나타내어, 질환의 치료제 개발에 도움이 될 것으로 생각된다.