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Human umbilical cord mesenchymal stem cell-derived mitochondria (PN-101) attenuate LPS-induced inflammatory responses by inhibiting NFκB signaling pathway

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Inflammation is one of the body's natural responses to injury and illness as part of the healing process. However, persistent inflammation can lead to chronic inflammatory diseases and multi-organ failure. Altered mitochondrial function has been implicated in several acute and chronic inflammatory diseases by inducing an abnormal inflammatory response. Therefore, treating inflammatory diseases by recovering mitochondrial function may be a potential therapeutic approach. Recently, mitochondrial transplantation has been proven to be beneficial in hyperinflammatory animal models. However, it is unclear how mitochondrial transplantation attenuates inflammatory responses induced by external stimuli. Here, we isolated mitochondria from umbilical cord-derived mesenchymal stem cells, referred as to PN-101. We found that PN-101 could significantly reduce LPS-induced mortality in mice. In addition, in phorbol 12-myristate 13-acetate (PMA)-treated THP-1 macrophages, PN-101 attenuated LPS-induced increase production of pro-inflammatory cytokines. Furthermore, the anti-inflammatory effect of PN-101 was mediated by blockade of phosphorylation, nuclear translocation, and trans-activity of NFκB. Taken together, our results demonstrate that PN-101 has therapeutic potential to attenuate pathological inflammatory responses. [BMB Reports 2022; 55(3): 136-141]

INTRODUCTION

Mitochondria are fundamental for the survival of cells and their functions by providing energy in the form of ATP through oxidative phosphorylation. Mitochondria also play critical roles in apoptosis, inflammation, and calcium homeostasis (1). Given their important roles in maintaining cells homeostasis, mitochondrial dysfunction has been implicated in many human diseases including metabolic syndromes, neurodegenerative diseases, cancer, and aging (2, 3). In addition, abnormal inflammatory responses have been linked to increased mitochondrial reactive oxygen species (mtROS), increased amount of extracellular ATP, aberrant calcium flux, and mitochondrial DNA (mtDNA) release, all of which are known to be the result of mitochondrial dysfunction (4).

Lipopolysaccharides (LPS) are known to successfully trigger inflammatory response. They are involved in various inflammatory diseases (5, 6). Binding of LPS to toll-like receptor (TLR)-4 on the surface of macrophages can initiate a phosphorylation cascade in the nuclear factor kappa B (NFκB) signaling pathway. Phosphorylation of inhibitory kappa B (IκB), which binds to NFκB and holds it in the cytoplasm, induces ubiquitin-dependent degradation of IκB which promotes its dissociation from NFκB. Subsequently, phosphorylated NFκB translocates into the nucleus and regulates the expression of various pro-inflammatory genes including tumor necrosis factor alpha (TNF-α) (7, 8). Therefore, attenuating inflammatory responses by inactivating TLR4-mediated NFκB pathway offers a potential therapeutic strategy for various inflammatory diseases.

Recently, transferring healthy mitochondria into cells is becoming an attractive therapeutic strategy for treating mitochondrial dysfunction. Direct application of exogenous mitochondria as a therapeutic agent is referred to as mitochondrial transplantation. The aim of mitochondrial transplantation is to replace intracellular abnormal mitochondria with healthy mitochondria isolated from various cell sources such as mesenchymal stem cells, leading to the recovery of cell function (9-12). Accumulating evidence has confirmed that isolated mitochondria can enter any cell types by a simple co-incubation or brief centrifugation *in vitro* (13-16). They can also become internalized into tissues through local or systemic injection *in vivo* (17-19). These data suggest that mitochondrial transplantation would be a potential therapeutic strategy to treat diseases

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caused by mitochondrial dysfunction.

In the present study, we isolated mitochondria from umbilical cord-derived mesenchymal stem cells (UC-MSCs) named PN-101. We next investigated effects of PN-101 on LPS-induced activation of pro-inflammatory cytokines in PMA-treated human THP-1 macrophages and on survival rates of mice challenged with LPS. Our data showed that administration of PN-101 and mitochondria isolated form human platelets significantly increased survival rates of mice treated with LPS *in vivo* and inhibited inflammatory responses induced by LPS treatment *in vitro*. Furthermore, we found that anti-inflammatory effects of PN-101 were mediated by blockade of the NFκB signaling pathway.

RESULTS

Characterization of PN-101

To study the effect of mitochondrial transplantation on inflammation, we first isolated mitochondria from human UC-MSCs (PN-101) using differential centrifugation protocol. The integrity and activity of PN-101 were assessed by evaluating mitochondrial respiratory chain enzyme activity and citrate synthase because these enzymes could provide energies through the synthesis of ATP in the mitochondrial electron transport chain (20). Results revealed that PN-101 had 3.39 times higher complex I + III activity (0.013 ΔO.D./min) than antimycin A (AA, an inhibitor of complex III of the electron transport chain)-treated PN-101 (AA-PN-101, 0.003 ΔO.D./min) and 24.9 times higher activity than heat-inactivated PN-101 (HI-PN-101, 0.0005 ΔO.D./min) (Fig. 1A). Similarly, complex IV activity of PN-101 was higher (0.006 ΔO.D./min) than that of HI-PN-101 (0.0006 ΔO.D./min) or KCN-treated PN-101 (0.003 ΔO.D./min) where KCN was an inhibitor of complex IV (Fig. 1B). ATP synthase activity of PN-101 was also higher than that of HI-PN-101 (0.43 pmol/μg protein/min and 0.01 pmol/μg protein/min respectively) (Fig. 1C). In addition, citrate synthase activity of PN-101 was 315.8 pmol/μg protein/min, higher than that of HI-PN-101 (10.6 pmol/μg protein/min) (Fig 1D). Taken together, these results indicate that PN-101 cells have better integrity and functions than HI- PN-101 or PN-101 cells treated with inhibitors of the mitochondrial respiratory chain.

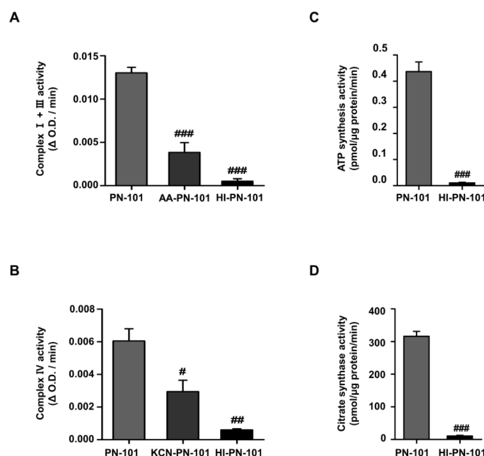

Fig. 1. Characterization of PN-101. A-D. Mitochondria were isolated from human UC-MSC as described in Materials and Methods. To induce mitochondrial damage, PN-101 cells were incubated with 54.6 μM antimycin A or 300 μM KCN (referred to as AA-PN-101 and KCN-PN-101, respectively). To inactivate mitochondria, isolated mitochondria were heated at 90°C for 10 min (referred to as a HI-PN-101). (A) Complex I + III activities of PN-101, AA-PN-101, and HI-PN-101 were analyzed and compared. Antimycin A, an inhibitor of the electron transport chain complex III, partially inhibited complex I + III activity of isolated mitochondria while heat inactivation of isolated mitochondria totally abolished their activity. (B) Comparison of complex IV activities among PN-101, AA-PN-101, and HI-PN-101. KCN, an inhibitor of complex IV, partially suppressed PN-101 complex IV activity while heat inactivation abolished complex IV activity. (C, D) ATP synthase activity (C) and citrate synthase activity (D) were evaluated with PN-101 and HI-PN-101. Each group was replicated four times. Results are presented as mean ± SD and analyzed by one-way analysis of variance. $^{\#}P < 0.05$ compared with PN-101, $^{\#\#}P < 0.01$ compared with PN-101, $^{\#\#\#}P < 0.001$ compared with PN-101.

PN-101 improves survival of mice with LPS-induced sepsis

LPS has been used to replicate many clinical features of septic shock including the production of pro-inflammatory cytokines and high mortality (21). To evaluate the effect of mitochondria on LPS-induced death, we examined the survival rate of mice treated with a lethal dose of LPS. LPS-treated mice then received PN-101 as a co-treatment or post-treatment. We observed approximately 95% mortality for mice at 72 h after treatment with LPS. In contrast, survival rates of 100% and 87.5% at 72 h were observed for mice receiving PN-101 as co-treatment and post-treatment, respectively (Fig. 2A). Similarly, while only 7.4% of LPS-treated mice survived, those receiving human

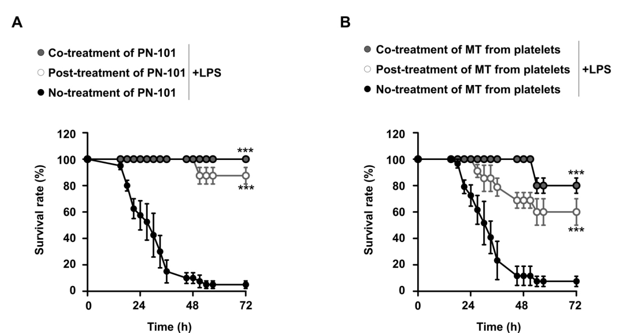

Fig. 2. Effect of PN-101 on survival of LPS challenged mice. (A) Mice were injected intraperitoneally with 7.5 mg/kg of LPS immediately followed by intravenous injection of 10μg of PN-101 (i.e., co-treatment group, n = 10). The same number of PN-101 was injected intravenously at 30 min after LPS injection (i.e., post-treatment, n = 10). (B) Ten μg of mitochondria isolated from human platelets were used for treatment immediately after LPS injection (i.e., co-treatment group, n = 5) or 30 min after LPS injection (i.e., post-treatment group, n = 26). Survival was assessed every 6 h for 3 days. $^{***}P < 0.001$ compared with No-treatment.

platelets derived mitochondria as co-treatment and post-treatment showed survival rates of 80% and 61.5%, respectively (Fig. 2B). Body temperature decreased within 3 h after LPS injection in both PN-101 treated group and untreated group. At 3 h after injection, temperature increased to normal levels in the PN-101 treated group, but not in the untreated group (Supplementary Fig. 1). These data strongly suggest that mitochondrial transplantation can improve survival rates and restore normal body temperatures of LPS-challenged mice with a possible anti-inflammatory effect.

PN-101 suppresses the production of cytokines in THP-1 cells induced by LPS

Cytokines such as TNF-α and IL-6 induced by LPS are important molecules in inflammatory processes (22). To investigate whether mitochondrial transplantation could suppress LPS-induced TNF-α and IL-6 release, we measured TNF-α and IL-6 production by PMA-treated THP-1 derived human macrophages. In the absence of LPS-stimulation, treatment of THP-1 macrophages with vehicle or PN-101 had no impact on TNF-α or IL-6 release. However, LPS-stimulated THP-1 macrophages significantly released TNF-α and IL-6 into the medium. Importantly, treatment of LPS-stimulated THP-1 macrophages with PN-101 significantly reduced levels of TNF-α and IL-6 released into the medium at 4 h and 48 h, respectively (Fig. 3A), suggesting that PN-101 could suppress the production of pro-inflammatory cytokines. Inhibition levels of TNF-α and IL-6 were 71.5% and 32.7%, respectively.

To further explore effects of PN-101 on TNF-α and IL-6 mRNA expression in THP-1 macrophages, quantitative RT-PCR was performed. TNF-α and IL-6 mRNA levels in LPS-treated THP-1 macrophages were found to be significant increased. Pre-treatment of LPS-treated THP-1 macrophages with PN-101 significantly reduced TNF-α ($P < 0.01$, $n = 3$ in each group) and IL-6 ($P < 0.05$, $n = 3$ in each group) mRNAs expression levels (Fig. 3B). These results suggest that PN-101 can suppress TNF-α and IL-6 production at the transcriptional level.

To investigate if PN-101 could directly affect transcriptional

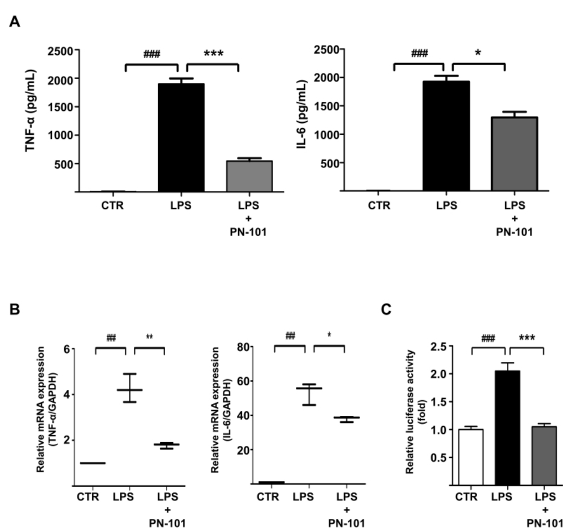

Fig. 3. Effects of PN-101 on LPS-induced TNF-α and IL-6 expression in PMA-treated THP-1 cells. PMA-treated THP-1 macrophages were administered with 1 μg/ml LPS alone or pretreated with PN-101 prior to LPS administration. (A) TNF-α and IL-6 productions were measured at 4 h and 48 h by ELISA. PN-101 attenuated TNF-α and IL-6 production induced by LPS. (B) mRNA expression levels of TNF-α and IL-6 were determined by quantitative real-time RT-PCR. PN-101 reduced expression of TNF-α and IL-6 mRNAs stimulated by LPS. (C) PMA-treated THP-1 macrophages were transfected with a plasmid containing TNF-α promoter driving firefly luciferase. Transfected cells were treated with PN-101 at 1 h prior to LPS stimulation. The basal level of luciferase activity was normalized to 1. All experiments were performed three times independently. Results are presented as mean ± SD. $^{###}P < 0.001$ vs. control group, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. LPS.

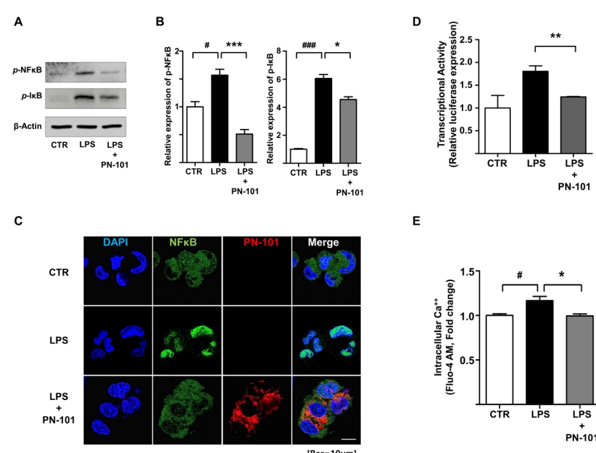

Fig. 4. Effects of PN-101 on NFκB signaling pathway. PMA-treated THP-1 macrophages were pre-treated with PN-101 for 1 h and then exposed to 1 μg/ml LPS. (A) Immunodetection of phosphorylated NFκB and IκB was performed by western blotting. PN-101 inhibited LPS induced phosphorylation of IκB and NFκB. (B) Densitometric analysis of immunodetection results in Fig. 4A was expressed in arbitrary units ($^{\#}P < 0.05$ vs. control, $^{###}P < 0.001$ vs. control, $^{*}P < 0.05$ vs. LPS, $^{***}P < 0.001$ vs. LPS). (C) Localization of NFκB was evaluated by immunocytochemistry and confocal imaging. The image shows that NFκB (green color) is localized in the nucleus of THP-1 cells stimulated with LPS, while it remains mostly in the cytoplasm of unstimulated cells. PN-101 abolished LPS-induced nuclear translocation of NFκB. PN-101 in red and DAPI nuclear staining in blue. Scale bar, 10 μm. The image shown is representative of at least three independent experiments. (D) PMA-treated THP-1 macrophages were transfected with a luciferase gene construct driven by nine copies of NFκB response element upstream of the TK promoter. Transfected cells were pre-treated with PN-101 for 1 h and then exposed to 1 μg/ml LPS. The basal level of luciferase activity was set to be 1 ($^{**}P < 0.01$ vs. LPS). PN-101 decreased NFκB trans- activity. (E) Intracellular calcium concentration was measured with a calcium specific dye Fluo-4 AM. Values are expressed as mean ± SD. $^{\#}P < 0.05$ vs. control group; $^{*}P < 0.05$ vs. LPS).

activation of TNF-α gene, TNF-α promotor activity was analyzed using luciferase reporter gene assay. LPS-stimulation of THP-1 macrophages resulted in a 2-fold increase in TNF-α promoter activity while pre-treatment with PN-101 abolished it (Fig. 3C).

PN-101 inhibits NFκB phosphorylation, nuclear translocation, and trans-activity

NFκB is a key regulator that controls the expression of pro-inflammatory genes in LPS-activated macrophages (23, 24). NFκB activation involves phosphorylation and degradation of IκB, phosphorylation of NFκB, and subsequent translocation of NFκB into the nucleus where it binds to responsive elements in the promoter regions of inflammatory genes triggering gene expression (7). To investigate the effect of PN-101 on NFκB signaling pathway, we first tested its impact on IκB and NFκB phosphorylation by western blot assay and immunocytochemistry. Low levels of phosphorylated IκB and phosphorylated p65 NFκB were found in unstimulated THP-1 macrophages whereas LPS-stimulation increased phosphorylation levels of both IκB and p65 NFκB. Interestingly, treatment with PN-101 significantly reduced phosphorylation levels of IκB and p65 NFκB (Fig. 4A, B). Furthermore, immunocytochemistry confirmed that PN-101 abolished LPS-induced phosphorylation of p65 NFκB in THP-1 macrophages (Supplementary Fig. 2).

Next, we investigated if PN-101 could affect the translocation of p65 NFκB from the cytoplasm to the nucleus in THP-1 macrophages. As shown in Fig. 4C, in the absence of LPS-stimulation, p65 NFκB immunoreactivity was found predominantly in the cytoplasm. After stimulation with LPS, p65 NFκB was exclusively observed in the nucleus. Pre-treatment with PN-101 clearly inhibited LPS-evoked p65 NFκB nuclear translocation.

We then used a reporter gene assay to assess whether PN-101 could attenuate LPS-induced NFκB-mediated transcription. A construct containing luciferase gene driven by nine copies of NFκB response element upstream of the TK promoter was transfected into THP-1 macrophages. As expected, LPS-stimulation resulted in 1.8-fold increase in NFκB luciferase activity. Treatment of LPS-stimulated THP-1 macrophages with PN-101 significantly reduced NFκB luciferase activity to control level (Fig. 4D), indicating that mitochondrial transplantation could attenuate LPS-induced NFκB trans-activity. Taken together, PN-101's anti-inflammatory effects appear to be mediated by blockade of NFκB signaling pathway through inhibition of phosphorylation of IκB and NFκB, blockade of nuclear translocation of NFκB, and decrease of NFκB trans-activity.

PN-101 decreases intracellular free calcium levels

Increase of intracellular calcium concentration ($[Ca^{++}]_i$) can induce nuclear translocation of NFκB by promoting Ca^{++}-dependent phosphorylation of IκB and NFκB (25-27). Because mitochondria are important in cellular calcium homeostasis, we investigated whether PN-101 could regulate cytosol free calcium levels. LPS-treatment of THP-1 macrophages caused approximately a 17% increase of $[Ca^{++}]_i$ compared to the control (Fig. 4E). Surprisingly, PN-101 restored the increase of intracellular calcium level induced by LPS compared to the control (Fig. 4E), suggesting that recovery of altered $[Ca^{++}]_i$ is one of possible mechanisms underlying the anti-inflammatory action of PN-101.

DISCUSSION

The present study demonstrates that mitochondrial transplantation can improve the survival of mice with LPS-induced sepsis and attenuate the expression of LPS-induced inflammatory cytokines including TNF-α and IL-6 by regulating NFκB signaling pathway in THP-1 macrophages. Since mitochondria are essential for maintenance of cellular function and physiology, mitochondrial dysfunction has been implicated in a wide range of human diseases including chronic inflammation and its related diseases (4). Impaired mitochondria contribute to the initiation of pathological inflammatory responses and aggravate inflammatory diseases through elevated ROS generation, increased extracellular ATP, and released mtDNA (4, 28). Therefore, therapies focusing on the recovery of damaged mitochondrial functions are emerging as novel approaches to limit pathological inflammation and ameliorate inflammatory-related diseases. There is accumulating evidence that transplantation of healthy mitochondria into cells with dysfunctional/damaged mitochondria can result in recovery of cell function, suggesting the therapeutic potential of mitochondrial transplantation to treat mitochondrial diseases.

Recently, using an LPS-induced animal model of depression, Wang *et al*. (29) have shown that injecting allogeneic mitochondria isolated from mouse hippocampus can normalize abnormal behavior and decrease neurogenesis, brain-derived neurotrophic factor, and mitochondrial damage. Using a rat cecal slurry model, Hwang *et al*. (30) have reported that transplantation of mitochondria isolated from rat L6 cells can improve survival rate, mitigate mitochondrial dysfunction, and attenuate hyperinflammation. Our study also demonstrated that intravenous administration of mitochondria isolated from human UC-MSCs (PN-101) and those from human platelets could improve survival rates of LPS-challenged mice (Fig. 2). These data support the therapeutic potential of mitochondrial transplantation for inflammatory diseases. However, they fail to address how transplanted mitochondria could attenuate LPS-induced inflammatory responses.

NFκB is a master regulator that can activate the transcription of various pro-inflammatory cytokines and chemokines (31). Under unstimulated conditions, NFκB is inactive. It is found in the cytoplasm as it is bound to its inhibitor IκB. Upon stimulation, IκB is phosphorylated and degraded by the proteasome pathway, allowing NFκB phosphorylation and translocation to the nucleus, where NFκB upregulates the expression of pro-inflammatory and immune genes. Therefore, it is not surprising that blockade of NFκB signaling can attenuate inflammatory responses. Our study clearly showed that PN-101 could inhibit LPS-induced phosphorylation of NFκB and IκB (Fig. 4A, B and Supple-

mentary Fig. 2), subsequently blocking nuclear translocation of NFκB (Fig. 4C). Thus, it is likely that PN-101 can inhibit TNF-α and IL-6 production by suppressing the activation of NFκB signaling.

In macrophages, LPS can elevate intracellular calcium concentration $[Ca^{++}]_i$, resulting in increased NFκB phosphorylation and nuclear translocation (25, 26, 32). In support of this, our study clearly showed that treatment of macrophages with PN-101 abolished LPS-induced $[Ca^{++}]_i$ elevation (Fig. 4E). Mitochondria are important calcium storage organelles. They play a critical role in calcium homeostasis of cells. Mitochondrial transplantation may attenuate LPS-induced hyperinflammation by controlling intracellular calcium concentration. Although our study indicated that mitochondria could exert anti-inflammatory effects by controlling NFκB activation, the exact molecular mechanism(s) linking mitochondria and NFκB signaling is(are) not clarified yet.

Recently, it has been shown that metabolic reprogramming can affect inflammatory responses of immune cells (33, 34). Mitochondrial TCA cycle-derived metabolites including succinate, itaconate, fumarate, α-ketoglutarate, and acetyl-CoA, have been implicated in the regulation of inflammatory response and cytokine production. For example, succinate acts as a pro-inflammatory metabolite (35) and itaconate and fumarate show anti-inflammatory actions in macrophages (36, 37). Since mitochondria are important organelles for metabolite production, mitochondrial transplantation could cause intracellular metabolic changes and modulate inflammation in diverse ways. Thus, whether mitochondrial transplantation is capable of inducing metabolic reprogramming warrants further investigation.

MATERIALS AND METHODS

Cell culture and treatments

Human monocyte THP-1 cells were cultured in RPMI medium (Welgene, Korea) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (penicillin and streptomycin) and 0.05 mM of mercaptoethanol. Human umbilical cord-derived mesenchymal stem cells (UC-MSC) were obtained by primary culture of umbilical cord from a healthy pregnant woman with informed consent. The study was approved by Public Institutional Review Board (IRB) designated by Ministry of Health and Welfare, Korea (IRB No. P01-202002-31-008). UC-MSC were cultured in Minimum Essential Medium Eagle Alpha Modification (α-MEM; Hyclone, USA) supplemented with 10% FBS and 10 ng/ml basic fibroblast growth factor (FGF-2; CHA Meditech Co, Korea).

Isolation of mitochondria from UC-MSC and platelet

UC-MSC were used at passage 7 for mitochondria preparation. Cells were harvested from culture flasks, depressurized in SHE buffer [0.25 M Sucrose, 20 mM HEPES (pH 7.4), 2 mM EGTA, 0.1% defatted bovine serum albumin (BSA)] using nitrogen cavitation (Parr Instrument, USA) (38), and then centrifuged at 2,000 × g for 10 min at 4°C to remove cellular debris and nuclei. The supernatant was then centrifuged at 12,000 × *g* for 15 min at 4°C to pellet the mitochondria. The pellet was washed twice by suspension in 500 μl SHE buffer followed by centrifugation at 20,000 × *g* for 10 min at 4°C. The final pellet was resuspended in 100 μl suspending buffer and kept on ice until use. Isolated mitochondria were quantified by determining protein concentrations using a bicinchoninic acid (BCA) assay.

LPS-induced sepsis mouse model and mitochondria injection

The animal maintenance and treatments were carried out in accordance with the Animal Care and Use guidelines of INVIVO (IV-RA-17-2008-32), Korea. Male BALB/C mice (8 weeks old, Samtako bio korea Co., Ltd, Korea) were randomly assigned to three groups: LPS only, LPS + PN-101 (co-treatment), and LPS + PN-101 (post-treatment). For LPS intoxication, mice received an injection of 7.5 mg/kg LPS intraperitoneally (i.p.). Ten μg of PN-101 and mitochondria isolated from platelets were injected intravenously immediately (co-treatment) or 30 min (post-treatment) after LPS injection. Body temperature was measured for 3 days. Following injection, mice were individually housed in groups of five with ad libitum food and water for 3 days.

Intracellular free-calcium determination

Intracellular free-calcium levels were measured after incubating the cells with 4 μM Fluo-4 AM (Molecular Probes; Sigma) for 30 min at 37°C. Cells were then washed with PBS and counterstained with 1 μM Hoechst 33342 for 10 min, and fluorescence intensities were determined at 485/528 nm. The Fluo-4 AM intensity was normalized to the Hoechst 33432 staining. All data are presented as percent of control.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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