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Full paper

Imeglimin attenuates NLRP3 inflammasome activation by restoring mitochondrial functions in macrophages

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ABSTRACT

Imeglimin is a novel oral antidiabetic drug for treating type 2 diabetes. However, the effect of imeglimin on NLRP3 inflammasome activation has not been investigated yet. Here, we aimed to investigate whether imeglimin reduces LPS-induced NLRP3 inflammasome activation in THP-1 macrophages and examine the associated underlying mechanisms. We analyzed the mRNA and protein expression levels of NLRP3 inflammasome components and IL-1β secretion. Additionally, reactive oxygen species (ROS) generation, mitochondrial membrane potential, and mitochondrial permeability transition pore (mPTP) opening were measured by flow cytometry. Imeglimin inhibited NLRP3 inflammasome-mediated IL-1β production in LPS-stimulated THP-1-derived macrophages. In addition, imeglimin reduced LPS-induced mitochondrial ROS production and mitogen-activated protein kinase phosphorylation. Furthermore, imeglimin restored the mitochondrial function by modulating mitochondrial membrane depolarization and mPTP opening. We demonstrated for the first time that imeglimin reduces LPS-induced NLRP3 inflammasome activation by inhibiting mPTP opening in THP-1 macrophages. These results suggest that imeglimin could be a promising new anti-inflammatory agent for treating diabetic complications.

1. Introduction

Imeglimin is the first in a novel tetrahydrotriazine-containing class of oral glucose-lowering agents called "glimins".[1] This drug is a small molecule synthesized from metformin as a precursor via a single step chemical reaction.[2] Multiple clinical studies, including three phase III clinical trials, have shown that imeglimin has significant glucose lowering effects and good safety and tolerability profiles as monotherapy or add-on therapy.[3–5] Based on these findings, imeglimin was first approved as a treatment for type 2 diabetes in Japan in 2021.[6] Imeglimin has a unique mode of action compared to that of other classes of antidiabetic agents. Imeglimin improves glycemic control by affecting the pancreas, skeletal muscle, and liver. It improves insulin secretion and sensitivity, which are the main pathogenesis of type 2 diabetes.[7] Previous animal studies showed that imeglimin increases glucose-dependent insulin secretion, preserves beta-cell function, glucose uptake in skeletal muscle, and decreases hepatic gluconeogenesis.[8–12]

Beyond glycemic control, imeglimin was shown to protect against endothelial cell death induced by oxidative stress and left ventricle dysfunction in a metabolic syndrome-related cardiomyopathy animal model.[13,14] Imeglimin also improved colonic hyperpermeability and visceral hypersensitivity in models of irritable bowel syndrome.[15] This drug improves mitochondrial function by reducing reactive oxygen species (ROS) production and delaying mitochondrial permeability transition pore (mPTP) opening in oxidative stress conditions.[1] However, the complete pharmacological properties of imeglimin and the molecular mechanisms underlying its therapeutic effect have not been fully clarified.

Inflammatory diseases are a group of conditions caused by overactive immune responses, leading to tissue damage and chronic inflammation. The pathogenesis of inflammatory diseases involves both innate and adaptive immunity, and macrophages play a pivotal role in regulating inflammation. The Nod-like receptor family pyrin domain

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containing 3 (NLRP3) inflammasome is the most studied inflammasome; it plays an important role in the production of proinflammatory cytokines that contribute to the onset and progression of various inflammatory diseases, such as diabetes and atherosclerosis.[16,17] Therefore, a better understanding of the relationship between the pathogenesis of inflammatory diseases and the NLRP3 inflammasome is important for developing novel and effective therapeutic strategies for these diseases. As innate immune sensor complexes, inflammasomes recognize proinflammatory stimuli, including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). In response to PAMPs and DAMPS, NLRP3 is activated and recruits pro-caspase-1 through the adapter protein apoptosis-associated speck--like protein containing a caspase-1 recruitment domain (ASC). After assembly, the NLRP3 inflammasome complex activates caspase-1, which then induces the maturation of proinflammatory cytokines, such as IL-1β and IL-18, and triggers a series of inflammatory responses.[17] Emerging evidence suggests a close association between mitochondrial events and the activation of the NLRP3 inflammasome. Mitochondrial damage, characterized by mitochondrial ROS production, can trigger the activation of the NLRP3 inflammasome.[18] Subsequent investigations have further demonstrated that reducing mitochondrial ROS production effectively inhibits NLRP3 inflammasome activation.[19,20] Therefore, preserving mitochondrial health emerges as a promising strategy for regulating NLRP3 inflammasome activation.[21]

Currently, no information is available on the effects of imeglimin on NLRP3 inflammasome-mediated inflammation in macrophages. Therefore, we aimed to investigate the effects of imeglimin on LPS-induced NLRP3 inflammasome activation and to explore the mechanisms underlying THP-1 macrophage responses.

2. Materials and methods

2.1. Cell culture and treatment

The THP-1 human leukemia monocytic cell line (American Type Culture Collection, Manassas, VA, USA) was cultured at densities ranging from 5×10^5 to 8×10^5 cells/mL in RPMI 1640 medium (Corning, Manassas, VA, USA). The medium was supplemented with 10% fetal bovine serum (Corning, Woodland, CA, USA), 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 1% penicillin/streptomycin, and maintained at 37 °C with 5% CO2.

To promote macrophage differentiation, THP-1 cells were seeded into 6-well culture plates and exposed to 100 ng/mL phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, Saint Louis, MO, USA) for 48 h. Following attachment to the culture plate surface, THP-1 cells were washed with PBS. THP-1 macrophages were pretreated with or without imeglimin for 24 h and exposed to 1 μg/mL LPS for 24 h, based on previous studies demonstrating the activation of the NLRP3 inflammasome by LPS without transfection in PMA-differentiated THP-1 cells.[22–24] LPS (from *Escherichia coli* O111:B4) was purchased from InvivoGen (SanDiego, CA, USA). Imeglimin was obtained from AdooQ BIOSCIENCE (Irvine, CA, USA).

2.2. Cell viability assays

The effect of imeglimin on cell viability was assessed via the cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan). THP-1 macrophages were plated in 96-well microtiter plates at a density of 1×10^4 cells per well and treated with varying concentrations of imeglimin for 24 h. Subsequently, 10 μL of CCK-8 solution was added to each well, followed by a further 4-h incubation period. Absorbance at 450 nm was recorded using a microplate reader (Bio-Tek Instruments, VT, USA).

2.3. Real-time quantitative PCR (RT-qPCR)

Total RNA extraction was performed using RNAiso Plus (Takara Bio Inc., Shiga, Japan) and RNeasy columns (QIAGEN, Hidden, Germany). The concentration of RNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). cDNA was synthesized utilizing a PrimeScript II 1st strand cDNA kit (Takara Bio) and 1 μg of RNA. RT-qPCR was conducted using the TB Green premix Ex Taq (Tli RNaseH Plus) mix (Takara Bio) via a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific). The expression levels of all genes were normalized to the expression of glyceraldehyde 3-dehydrogenase (GAPDH). Primer sequences are provided in Table 1.

2.4. Western blot analysis

After the previously described pretreatment, THP-1 macrophages were rinsed and lysed in ice-cold CelLytic M cell lysis buffer (Sigma-Aldrich), supplemented with protease and phosphatase inhibitors (Cell Signaling Technology Inc., Boston, MA, USA). Protein concentration was assessed using a BCA assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Total cell lysates (10 μg) were loaded onto a NuPAGE™ 4–12% bis-tris gradient gel (NOVEX by Life Technologies, Carlsbad, USA), separated under reducing conditions, and transferred to nitrocellulose (NC) membranes (GE Amersham, MA, USA). After blocking with 5% BSA at room temperature for 1 h, membranes were incubated overnight at 4 °C with specific primary antibodies. The antibodies used were as follows: anti-NLRP3 (AG-20B-0014-C100, 1:5000; AdipoGen, San Diego, CA, USA); anti-caspase-1 (sc56036, 1:100; Santa Cruz Biotechnology, Dallas, TX, USA); anti-IL-1β (12242, 1:1000), anti-TNF-α (3707, 1:1000), JNK (9252, 1:1000), anti-p-JNK (4668, 1:1000), anti-p38 (8690, 1:1000), anti-p-p38 (4511, 1:1000), anti-ERK (4695, 1:1000), anti-p-ERK (4370, 1:2000) (Cell Signaling Technology); anti-β-actin (ab8227, 1:10,000), and anti-IL-6 (ab259341, 1:1000) (Abcam, Cambridge, UK). After washing, membranes were incubated with the corresponding horseradish peroxidase linked secondary antibodies for 1 h at room temperature. Signals were developed using a standard chemiluminescence (ECL) detection reagent (Thermo Fisher Scientific, Rockford, IL, USA). Band intensities were quantified using ImageJ software (NIH, Bethesda, MD, USA).

2.5. Quantification of IL-1β by ELISA

THP-1 macrophages were seeded into 6-well plates at a density of 2×10^6 cells per well and treated as described above. Cell culture supernatants were collected and centrifuged at 10,000 ×*g* for 5 min at 4 °C. IL-1β concentration in cell supernatants was determined using a human IL-1β Quantikine ELISA kit (R&D Systems, Inc. Minneapolis, MN, USA), according to the manufacturer's instructions.

2.6. Determination of intracellular and mitochondrial ROS levels

Intracellular levels of ROS were assessed using the fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate,

Table 1
Primer sequences used for real-time quantitative PCR.

Gene	GenBank Accession No.	Primer sequences
NLRP3	NM 001127462	F: ACAGCCACCTCACTTCCAG R: CCAACCACAATCTCCGAATG
Caspase-1	NM 001257119	F: GCACAAGACCTCTGACAGCA R: TTGGGCAGTTCTTGGTATTC
IL-1β	NM 000567	F: GCCCTAAACAGATGAAGTGCTC R: GAACCAGCATCTTCCCAG
GAPDH	NM 002046	F: CATGAGAAGTATGACAACAGC R: AGTCCTTCCACGATACCAAAGT

acetyl ester (CM-H_2DCFDA; Invitrogen by Thermo Fisher Scientific, Eugene, OR, USA), following the manufacturer's instructions. THP-1 macrophages were seeded into 6-well plates at a density of 1×10^6 cells per well and treated as described above. After washing twice with HBSS buffer, 10 μM CM-H_2DCFDA was added, and plates were kept for 30 min at 37 °C in the dark. Cells were then washed three times with HBSS buffer and analyzed by flow cytometry using a FACSAria III cell sorter (BD Biosciences, San Jose, CA, USA).

Mitochondrial ROS was determined using the MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen). Pretreated cells were washed three times with HBSS buffer, incubated with 2.5 μM MitoSOX™ for 20 min at 37 °C in the dark, washed again with HBSS buffer (three times), and analyzed by flow cytometry using a FACSAria III cell sorter. Data were processed utilizing FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Mitochondrial H_2O_2 production was determined using an Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen). Briefly, THP-1 macrophages seeded in black 96-well Costar plates were washed with HBSS buffer, and H_2O_2 levels were measured in the presence of 50 μM Amplex Red reagent and 0.1 U/mL horseradish peroxidase in a reaction buffer containing 145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 1.22 mM MgSO4, 0.54 mM $CaCl_2$, and 5.5 mM glucose (pH 7.35). Glutamate (5 mM) and malate (5 mM) were used as complex I substrates, while succinate (5 mM) was used as complex II substrates. Complex I and complex II-derived H_2O_2 production with and without rotenone (1 μM) was detected at an excitation wavelength range of 530–560 nm and an emission wavelength of 590 nm using a GloMax Discover GM3000 microplate reader (Promega, Madison, WI, USA). H_2O_2 concentrations were calculated by comparison with a standard curve generated from known concentrations of H_2O_2.

2.7. Detection of mitochondrial membrane potential (MMP)

MMP was measured using a MitoProbe JC-1 assay kit (Molecular Probes by Life Technologies) according to the manufacturer's instructions. Briefly, pretreated THP-1 macrophages were washed (three times) and resuspended in PBS, and 1×10^6 cells were stained with 2 μM JC-1 for 25 min at 37 °C in the dark. For the positive control, cells were treated with a potent mitochondrial membrane disruptor, CCCP, and incubated at 37 °C for 5 min. After washing, cells were resuspended in 0.5 mL PBS and analyzed by flow cytometry using a FACSAria III cell sorter. Mitochondrial depolarization can be determined by the JC-1 red/green fluorescence ratio because JC-1 aggregates exhibit excitation and emission in the red spectrum, therefore, the higher the red to green ratio, the higher the membrane potential. Accordingly, as JC-1 molecules exhibit green fluorescence, JC-1 monomer accumulation reflects a loss of membrane potential. The data were analyzed using FlowJo software.

2.8. mPTP opening changes

mPTP opening was assessed with a MitoProbe™ Transition Pore assay kit (Molecular Probes), according to the manufacturer's instructions. Briefly, pretreated THP-1-derived macrophages were washed three times with HBSS with Ca^{++} and Mg^{++}. Well-dispersed cells in HBSS were incubated with 2 μM calcein-AM in the presence of cobalt chloride to quench cytosolic fluorescence while maintaining mitochondrial fluorescence. As a control, cells were treated with the ionophore ionomycin to induce a massive loss of calcein fluorescence (reflecting mPTP opening). Cells were incubated for 15 min at 37 °C in the dark, washed with 3.5 mL of HBSS with Ca^{++} and Mg^{++}, and resuspended in 0.4 mL of HBSS. The fluorescence intensity of calcein-AM was measured by flow cytometry using a FACSAria III cell sorter. Data were analyzed using FlowJo software.

2.9. Statistical analysis

The data were presented as mean ± SEM from a minimum of three independent experiments. Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software, Inc., San Diego, USA). One-way ANOVA followed by Tukey's multiple comparison test was employed for data analysis. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Imeglimin protected LPS-induced cell death in THP-1 macrophages

Cytotoxicity of imeglimin was determined by the CCK-8 assay. THP-1 macrophages were treated with 50, 100, 500, and 1000 μM of imeglimin for 24 h. Cells treated with up to 100 μM imeglimin for 24 h did not show any cell viability differences compared with those of control cells (Fig. 1A). In contrast, THP-1 macrophages pretreated with 100 μM imeglimin prior to LPS exposure showed increased viability compared to that of THP-1 macrophages treated with LPS alone (Fig. 1B).

3.2. Imeglimin attenuated proinflammatory cytokine production in LPS-treated THP-1 macrophages

LPS treatment (1 μg/mL) markedly increased IL-1β, IL-6, and TNF-α protein expression in THP-1 macrophages. However, treating THP-1 macrophages with imeglimin (50 and 100 μM) reduced the expression of LPS-induced pro-inflammatory cytokines (Fig. 2). As we observed that the 100 μM imeglimin treatment did not affect the viability of THP-1 macrophages but effectively suppressed the LPS-induced proinflammatory response, we used 100 μM imeglimin in the following experiments.

3.3. Imeglimin inhibited LPS-induced NLRP3 inflammasome activation in THP-1 macrophages

We further investigated the effect of imeglimin on the mRNA expression of NLRP3 inflammasome components. As illustrated in Fig. 3A, imeglimin significantly downregulated the mRNA levels of NLRP3, caspase-1, and IL-1β induced by LPS. Subsequently, we used western blotting to analyze the corresponding protein levels in cell lysates. We found that imeglimin treatment resulted in decreased expression of LPS-induced NLRP3 inflammasome component proteins (Fig. 3B and C). ELISA analysis showed that imeglimin effectively suppressed the secretion of IL-1β due to LPS stimulation in culture supernatants (Fig. 3D).

3.4. Imeglimin reduced LPS-induced mitochondrial ROS generation

LPS is known to induce ROS generation, which is associated with activation of the NLRP3 inflammasome.[18] To investigate the effect of imeglimin on LPS-induced ROS generation, intracellular ROS levels were examined by flow cytometry with CM-H_2DCFDA. The results showed that imeglimin treatment significantly reduced the generation of intracellular ROS induced by LPS in THP-1 macrophages (Fig. 4A).

Given that mitochondria are the main contributors to ROS production, generating approximately 90% of cellular ROS,[25] we further examined the effects of imeglimin on mitochondrial ROS using MitoSOX-based flow cytometry and the Amplex Red horseradish peroxidase method. The results showed that treating THP-1 macrophages with imeglimin significantly inhibited mitochondrial ROS generation by LPS (Fig. 4B). As shown in Fig. 4C, imeglimin did not inhibit H_2O_2 production when LPS-stimulated THP-1 macrophages were incubated in the presence of glutamate/malate (complex I substrates), either before or after the addition of rotenone (a complex I inhibitor). However, H_2O_2 production was increased in LPS-stimulated THP-1 macrophages in the presence of succinate (a complex II substrate) compared

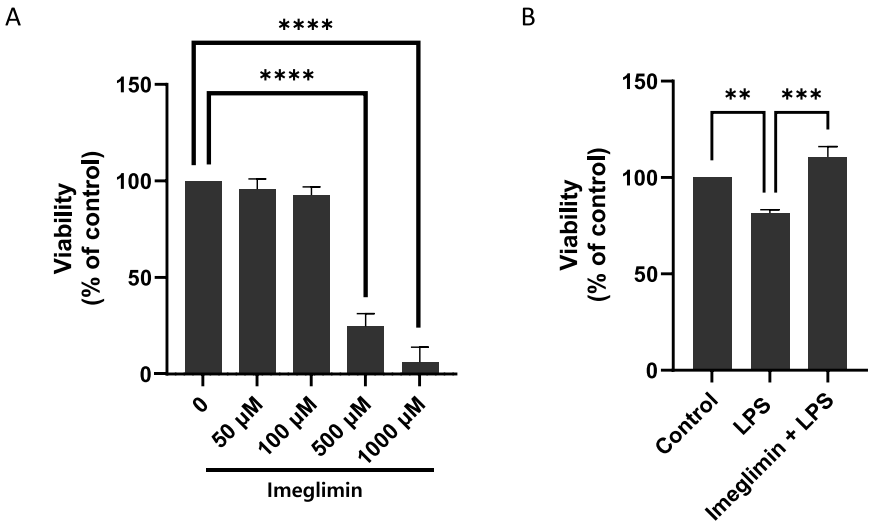

Fig. 1. Effect of imeglimin on the on the viability of THP-1 macrophages. (A) Cytotoxic effect of imeglimin on THP-1 macrophages. THP-1 cells were cultured at different concentrations of imeglimin for 24 h. Cell viability was assessed using the CCK-8 method. (B) Protective effect of imeglimin against LPS-induced cell death in THP-1 macrophages. THP-1 cells were cultured with 100 μM imeglimin in the absence or presence of 1 μg/mL LPS for 24 h. Cell viability was assessed using the CCK-8 method. The percentage of viability was determined by comparison with that of control cells. Data represent the mean ± SEM of at least three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

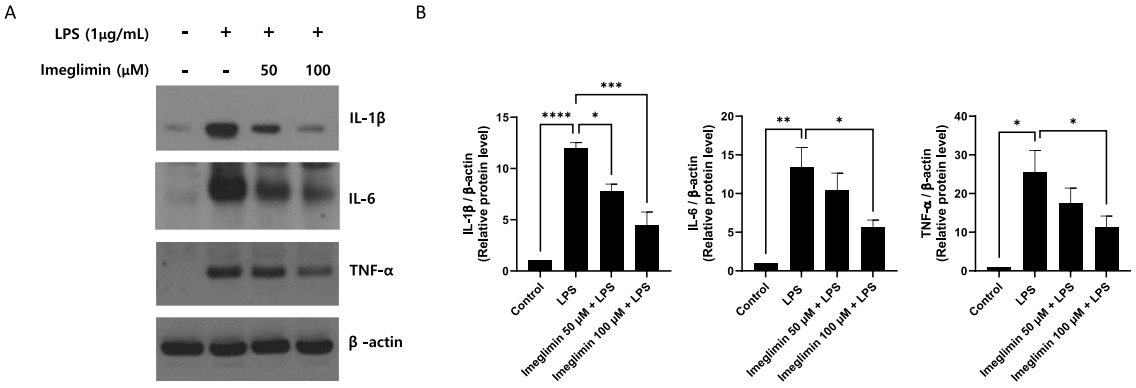

Fig. 2. Imeglimin inhibits the expression of LPS-induced inflammation markers in THP-1 macrophages. THP-1 macrophages were pretreated with or without imeglimin (50 and 100 μM) for 24 h and then exposed to 1 μg/mL LPS for 24 h. (A) Western blot analysis was performed using antibodies against mature IL-1β, IL-6, and TNF-α. (B) Protein expression levels of mature IL-1β, IL-6, and TNF-α were quantified using ImageJ. Data represent the mean ± SEM of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

with that in the control group (Fig. 4D). Moreover, imeglimin significantly decreased H_2O_2 production in LPS-stimulated THP-1 macrophages incubated in the presence of succinate. The addition of rotenone, which inhibited reverse electron flux from complex II to complex I,[26] also decreased H_2O_2 production. These findings suggest that imeglimin inhibits H_2O_2 production by interfering with reverse electron flux through complex I.

3.5. Imeglimin inhibited LPS-induced mitochondrial membrane depolarization

Oxidative stress in the mitochondria leads to subsequent mitochondrial damage,[27] and MMP is an indicator of the functional metabolic status of these organelles.[28] To investigate whether imeglimin could alleviate LPS-induced mitochondrial damage, we examined MMP by flow cytometry using JC-1, a fluorescence probe. JC-1 staining demonstrated that LPS induced a loss of MMP compared to that in control mitochondria (Fig. 5). In contrast, THP-1 macrophages treated with imeglimin showed a significant reversion of the loss of MMP compared to that of LPS-stimulated cells (Fig. 5). These results suggest that imeglimin prevents LPS-induced mitochondrial membrane depolarization.

3.6. Imeglimin inhibited LPS-induced mPTP opening in THP-1 macrophages

The accumulation of ROS in mitochondria poses a significant risk to cell viability because it may stimulate the opening of the mPTP, leading to the collapse of the MMP.[27,29,30] Therefore, we investigated the effect of imeglimin on mPTP opening in LPS-stimulated THP-1 macrophages by flow cytometry analysis using calcein-AM. As shown in Fig. 6, LPS-stimulated cells displayed a reduced calcein-AM fluorescence intensity compared to that of control cells, indicating the opening of mPTP. In contrast, imeglimin treatment significantly reduced the mPTP opening induced by LPS treatment in THP-1 macrophages.

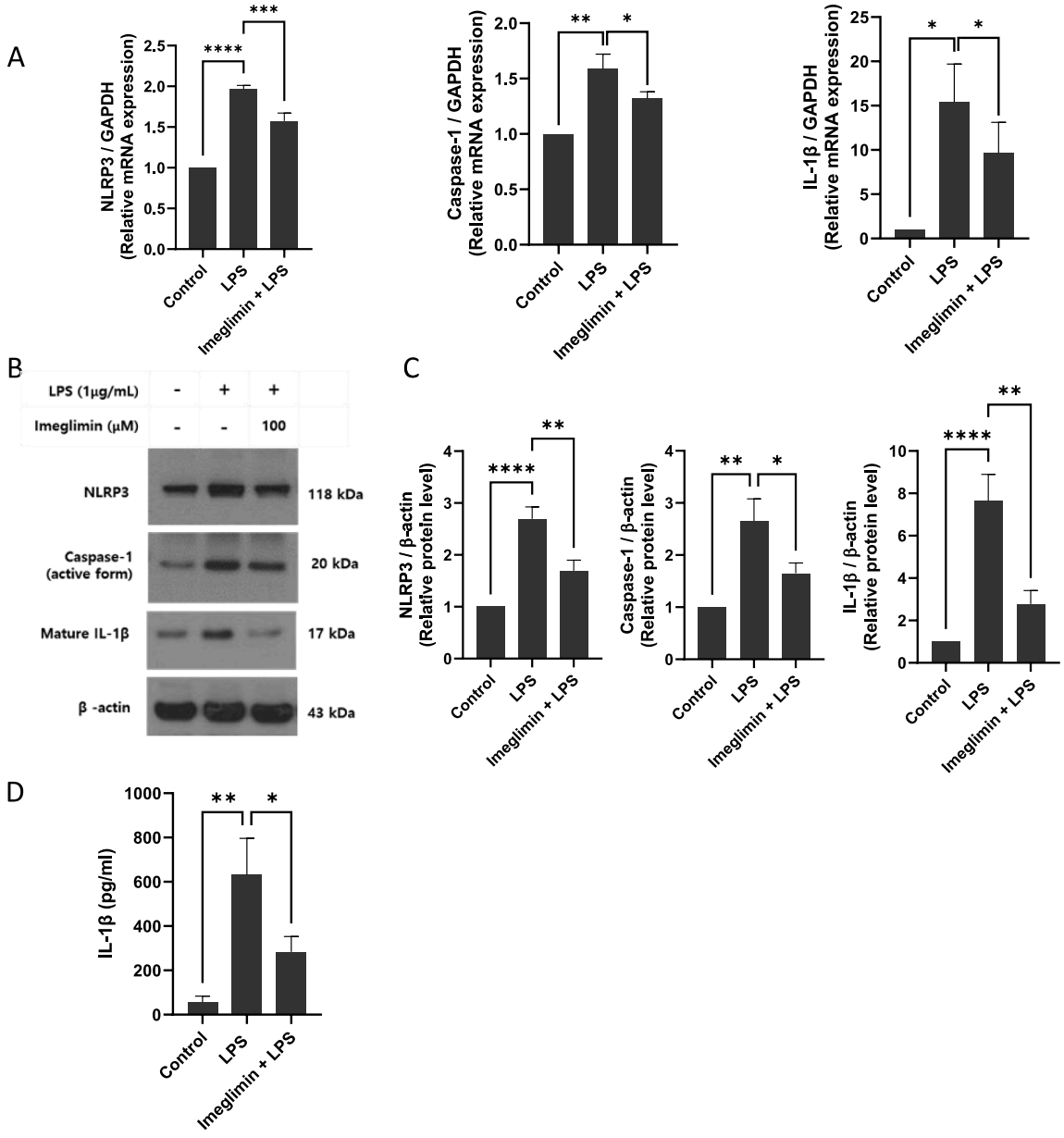

Fig. 3. Inhibitory effect of imeglimin on LPS-induced inflammasome activation in THP-1 macrophages. THP-1 macrophages were pretreated with or without 100 μM imeglimin for 24 h and then exposed to 1 μg/mL LPS for 24 h. (A) The mRNA expression levels of NLRP3, caspase-1, and IL-1β were examined by real-time quantitative PCR. (B) Western blot analysis was performed using antibodies against NLRP3, activated caspase-1, and mature IL-1β. (C) Protein expression levels of NLRP3 activated caspase-1 and mature IL-1β were quantified using ImageJ. (D) IL-1β levels in cell culture supernatants were measured by ELISA. Data represent the mean ± SEM of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

3.7. Imeglimin attenuated LPS-induced NLRP3 inflammasome activation via ROS/mitogen-activated protein kinase (MAPK) pathway

Excessive production of ROS can activate the MAPK signaling pathway, ultimately leading to NLRP3 activation.[31–33] We conducted experiments to explore the relationship between ROS, MAPK, and the NLRP3 inflammasome in LPS-induced THP-1 macrophage models. Inhibition of MAPK did not significantly suppress LPS-induced ROS production (Supplementary Fig. 1A). Consistent with previous findings, this suggests that ROS acts upstream of MAPK in LPS-induced THP-1 macrophage models.[34,35] Furthermore, the ERK inhibitor and JNK inhibitor significantly suppressed the protein expression of NLRP3 inflammasome components and IL-1β secretion (Supplementary Figs. 1B–D). Collectively, these results indicate that LPS induces IL-1β secretion by activating the ROS-MAPK-NLRP3 inflammasome signaling pathway in THP-1 macrophages. For this reason, we explored the effect of imeglimin on MAPKs in LPS-stimulated THP-1 macrophages. Western blot analysis was carried out to detect phosphorylated and total forms of p38 MAPK, ERK, and JNK. Our results showed that imeglimin significantly decreased the levels of phosphorylated p38 MAPK, ERK, and JNK but did not affect total form levels of p38 MAPK, ERK, and JNK in LPS-stimulated THP-1 macrophages (Fig. 7).

4. Discussion

Imeglimin, a recently launched therapeutic agent for the treatment of type 2 diabetes, has garnered significant attention in the field of diabetes research and clinical practice. However, there is a lack of

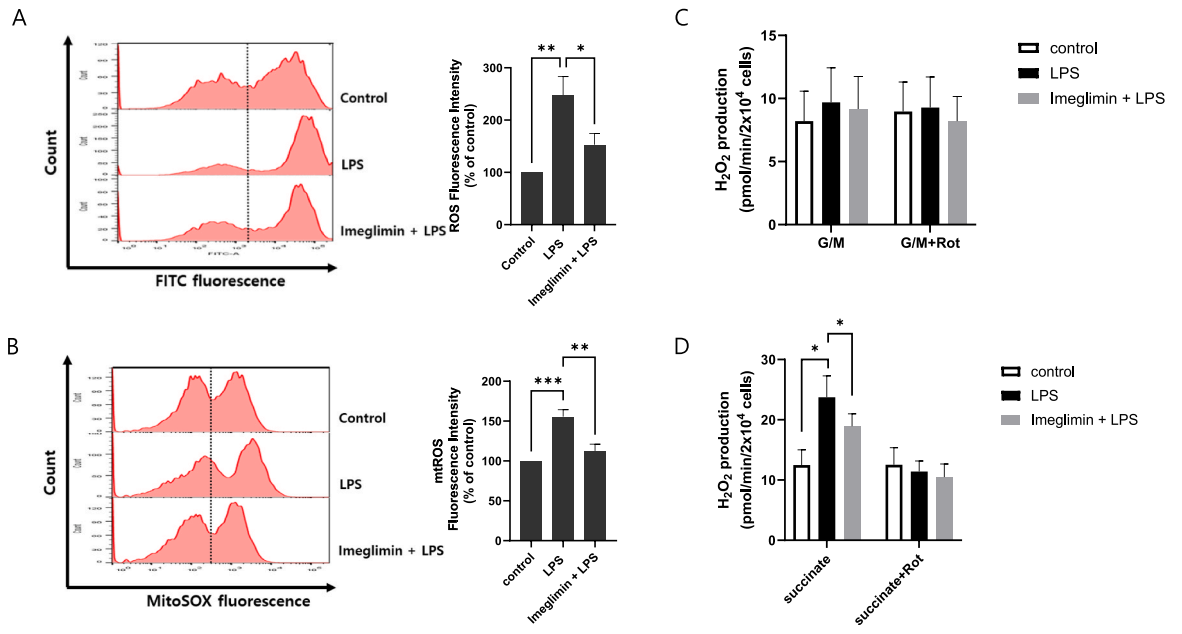

Fig. 4. Effect of imeglimin on LPS-induced ROS generation. (A) Intracellular ROS was measured using CM-H_2DCFDA and flow cytometry. (B) Mitochondrial (mt) ROS was analyzed using MitoSOX and flow cytometry. Both intracellular and mt ROS level mean fluorescence intensities are presented as percentages of those in control cells. (C, D) The H_2O_2 production rate was measured using Amplex Red and horseradish peroxidase with (C) glutamate/malate as complex I substrates and (D) succinate as complex II substrates before and after the addition of the complex I inhibitor rotenone (Rot). Data represent the mean ± SEM of at least three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

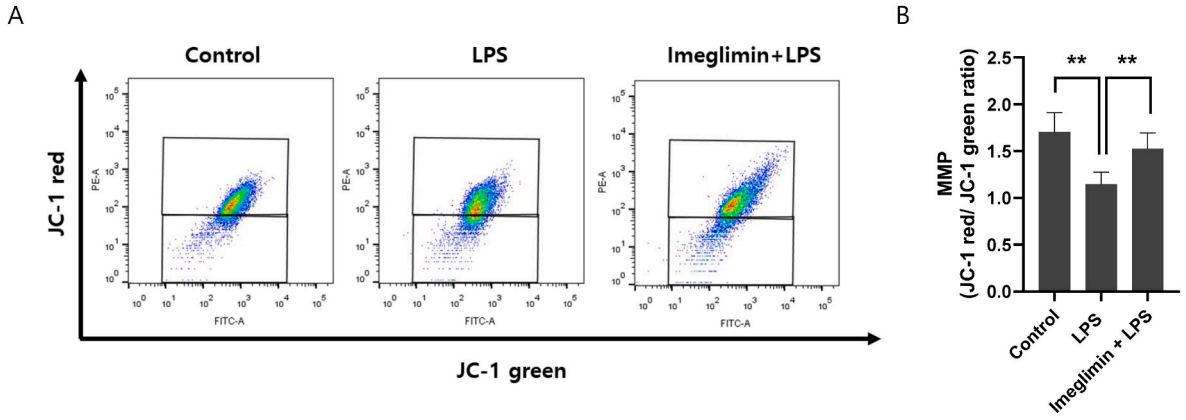

Fig. 5. Effect of imeglimin on mitochondrial membrane potential (MMP). (A) MMP was assessed by flow cytometry using JC-1 dye to measure mitochondrial depolarization. (B) Changes in MMP were determined by the JC-1 red to green fluorescence ratio. Data represent the mean ± SEM of at least three independent experiments. $^{**}p < 0.01$.

research on both the glucose-lowering effects of this medication and its potential additional effects. To the best of our knowledge, this is the first study to demonstrate that imeglimin has anti-inflammatory properties by inhibiting LPS-induced NLRP3 inflammasome activation in macrophages. Moreover, we found that imeglimin suppressed mitochondrial ROS generation and restored mitochondrial function by inhibiting mPTP opening. In addition, imeglimin suppressed MAPK phosphorylation in LPS-stimulated THP-1 macrophages (Fig. 8).

The activation of the NLRP3 inflammasome is a critical step in the development of inflammation; it leads to the maturation and secretion of IL-1β, which is involved in the progression of diabetes and atherosclerotic diseases.[36,37] A recent experimental study partially reported the effects of imeglimin on the NLRP3 inflammasome in beta cells[38]; it demonstrated that imeglimin treatment reduced the mRNA expression of NLRP3 and IL-1β in pancreatic β-cells from type 2 diabetic *db/db* mice, suggesting an inhibitory effect of imeglimin on the NLRP3 inflammasome. However, protein expression levels of NLRP3 inflammasome components and secretion of IL-1β after imeglimin treatment were not fully investigated. The present study demonstrated that imeglimin suppressed the expression of NLRP3, caspase-1, and IL-1β at the protein level while inhibiting the secretion of IL-1β in LPS-stimulated THP-1 macrophages. In addition, imeglimin administration attenuated NLRP3 inflammasome activation induced by LPS/ATP, but not by imiquimod in THP-1 macrophages (Supplementary Fig. 2). This result suggests that the effect of imeglimin on NLRP3 activation may vary depending on the NLRP3 activator. The NLRP3 inflammasome plays a crucial role in various metabolic, neurological, cardiovascular, and inflammatory diseases.[39] Imeglimin has the potential to be beneficial in diseases associated with NLRP3 inflammasome activation, but further research is needed to elucidate its mechanism of action for each specific disease. Imeglimin did not significantly suppress the activation of the LPS-induced TLR 4/MyD88/NF-kB pathway (Supplementary Fig. 3).

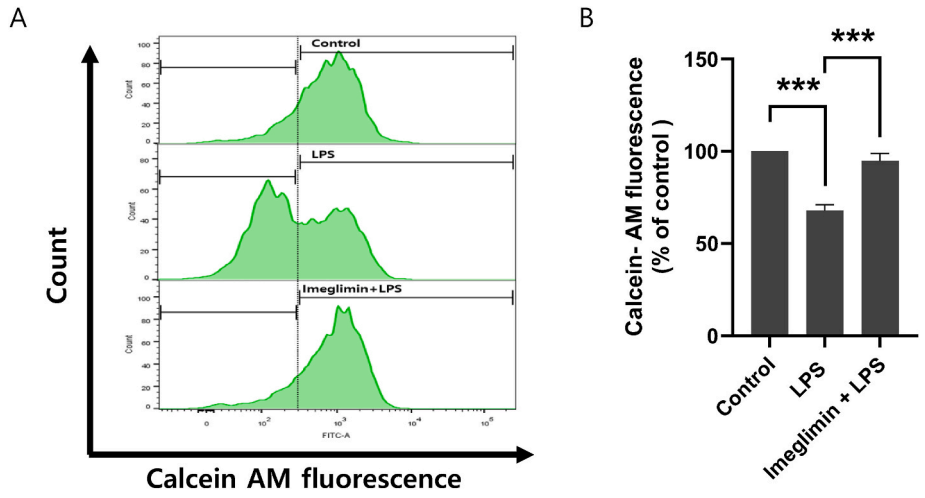

Fig. 6. Effect of imeglimin on mPTP opening. The mPTP opening status was evaluated in THP-1 macrophages by calcein-AM fluorescence in the presence of $CoCl_2$. (A) Representative flow cytometry graph and (B) statistical analysis of mPTP opening by flow cytometry. Data represent the mean ± SEM of at least three independent experiments. ***$p < 0.001$.

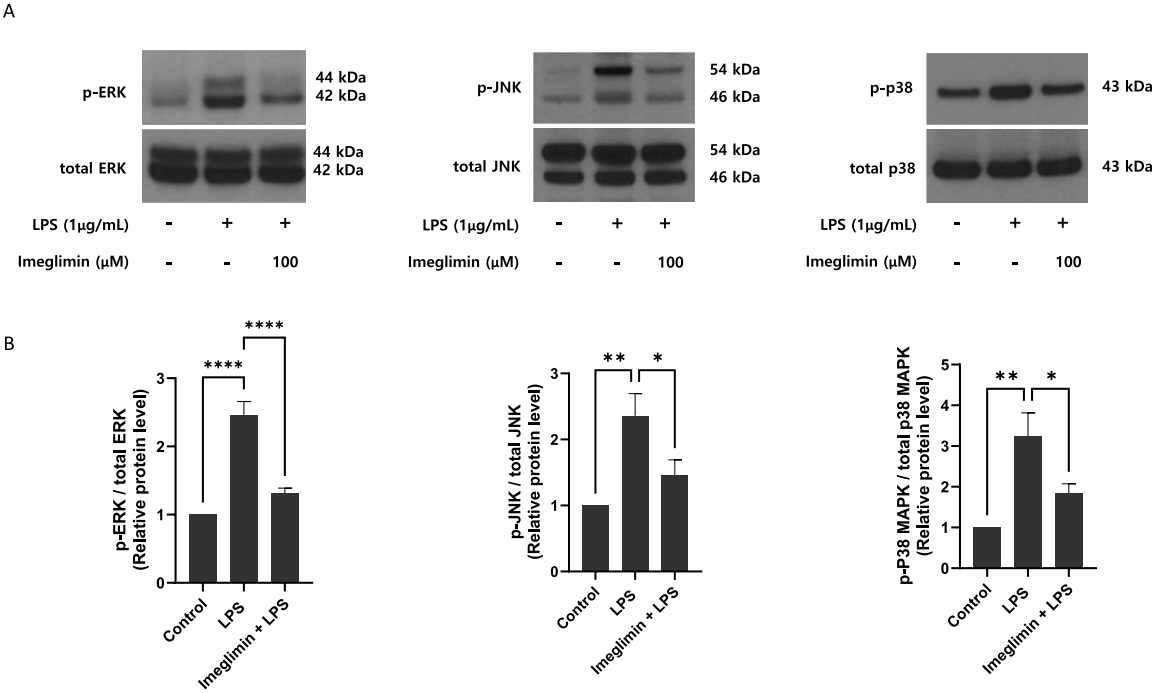

Fig. 7. Effect of imeglimin on LPS-induced MAPK activation. (A) Cell lysates were analyzed by Western blot using antibodies against ERK, JNK, p38 MAPK, phosphorylated-p38 MAPK (p-p38), phosphorylated-JNK (p-JNK), and phosphorylated-ERK (p-ERK). (B) Relative protein expression levels were determined using ImageJ. Data represent the mean ± SEM of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

ROS are formed as natural by-products of aerobic metabolism in cells and organelles, such as mitochondria, endoplasmic reticulum, and peroxisomes, and mitochondrial respiration is a major source of ROS.[40] Oxidative stress refers to an imbalance between ROS and the antioxidant defense system, resulting in increased oxidative damage within cells.[41] It is also known that ROS plays a crucial role in initiating the assembly and activation of the NLRP3 inflammasome in response to both exogenous stimuli and endogenously produced molecules from damaged cells.[42] Therefore, we hypothesized that the anti-inflammatory effect of imeglimin was related to the suppression of ROS generation. Our results demonstrated that imeglimin significantly inhibited mitochondrial ROS enhanced by LPS stimulation in THP-1 macrophages. In addition, imeglimin reduced mitochondrial ROS production in the presence of complex II substrates but not in the presence of complex I substrates. Upon activation with complex II substrates in the respiratory chain, most ROS generation results from reverse electron flow through complex I. Therefore, we suggest that imeglimin reduces mitochondrial ROS by interfering with reverse electron flux through complex I in THP-1 macrophages. To date, the effect of imeglimin on mitochondrial ROS in the presence of complex I or complex II substrates is inconclusive. Similar to our results, imeglimin reduced mitochondrial ROS production in the presence of complex II substrates but not in the presence of complex I substrates in permeabilized human endothelial cells and liver mitochondria of mice fed a high-fat and high-sucrose diet.[11,13] However, a recent study showed no difference in mitochondrial ROS levels between mice belonging to an ischemic brain injured group and control

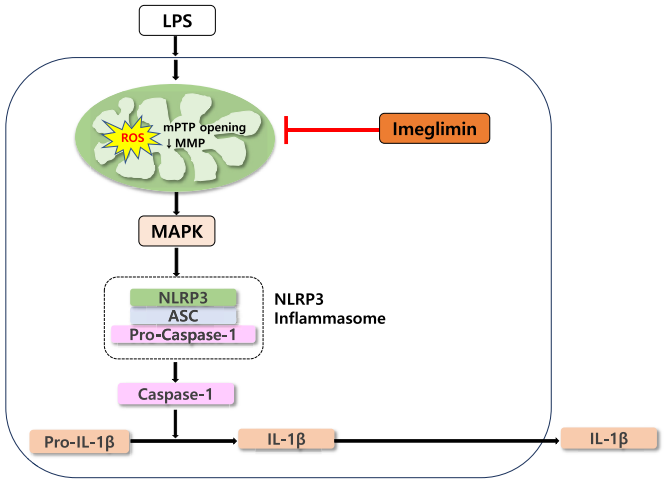

Fig. 8. The schematic diagram of the anti-inflammatory effect and mechanisms of imeglimin. Imeglimin exhibited inhibitory effects on LPS-induced NLRP3 inflammasome activation in THP-1 macrophages. The underlying mechanisms included the suppression of mitochondrial ROS generation, inhibition of MPTP opening, and mitigation of MMP loss. Consequently, imeglimin attenuated LPS-induced NLRP3 inflammasome activation mediated by MAPK activation via ROS generation.

group mice in the presence of complex I or complex II substrates. Moreover, ROS levels were similar even after imeglimin administration.[43]

MMP maintenance is critical for normal mitochondrial function.[27,29] It has been reported that increased ROS production leads to mPTP opening, which is responsible for mitochondrial membrane depolarization.[27,29] We found that imeglimin restored LPS-induced mitochondrial depolarization. We also found that imeglimin inhibited mPTP opening in LPS-stimulated THP-1 macrophages. These results are consistent with those of studies showing that mPTP opening is inhibited by imeglimin in cultured human endothelial cells,[13] neurons, and astrocytes.[43] Additionally, a previous study showed that blocking mPTP opening inhibited NLRP3-mediated activation of caspase-1.[44] Taken together, our result suggests that imeglimin preserves mitochondrial integrity by inhibiting mPTP opening and preventing MMP loss.

Research has demonstrated that the production of proinflammatory cytokines by ROS is associated with MAPK signaling pathways.[31,32] We, therefore, examined the effect of imeglimin on MAPK activation. We found that imeglimin treatment inhibited LPS-induced activation of p38 MAPK, JNK, and ERK. These results suggest that imeglimin mediates ROS-induced activation of the NLRP3 inflammasome by inhibiting the MAPK signaling pathway.

5. Conclusions

This study revealed that imeglimin can effectively modulate the immune response by reducing NLRP3 inflammasome activation in LPS-stimulated THP-1 macrophages. These results provide evidence for the potential use of imeglimin as a potent anti-inflammatory agent because activation of the NLRP3 inflammasome plays an important role in the initiation and progression of the inflammatory response in diabetes and its vascular-related complications. Although further studies are needed to confirm its therapeutic effect in inflammatory diseases *in vivo*, our results suggest that imeglimin may have potential therapeutic benefits in the treatment of vascular complications caused by diabetes, such as atherosclerosis and diabetic cardiovascular disease, besides its use as a glucose-lowering agent in the treatment of type 2 diabetes.

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Data availability

The data supporting the findings of this study have been completely included in the manuscript.

CRediT authorship contribution statement

Ji Yeon Lee: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Yup Kang:** Conceptualization, Writing – review & editing. **Ja Young Jeon:** Writing – review & editing. **Hae Jin Kim:** Writing – review & editing. **Dae Jung Kim:** Writing – review & editing. **Kwan Woo Lee:** Writing – review & editing. **Seung Jin Han:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphs.2024.03.004.

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