Cilostazol ameliorates high free fatty acid (FFA)-induced activation of NLRP3 inflammasome in human vascular endothelial cells

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\textbf{ABSTRACT}

Cardiovascular disease is recognized as a leading cause of death worldwide, but the risk of death is 2–3 times higher for individuals with diabetes. NLRP3 inflammasome activation is a leading pathway of vascular damage, and new treatment methods are needed to reduce NLRP3 inflammasome expression, along with a detailed understanding of how those treatments work. In a series of assays on human vascular endothelial cells that were exposed to high concentrations of free fatty acids (FFA) to induce a diabetes-like environment, we found a significant impact of cilostazol, a vasodilator widely used to treat blood flow problems and well-tolerated medication. To our knowledge, this study is the first to demonstrate the effects of cilostazol in primary human aortic endothelial cells. We found that cilostazol significantly reduced NLRP3 inflammasome activation, as well as the activity of other related and harmful factors, including oxidative stress, expression of NADPH oxidase 4 (NOX-4), thioredoxin-interacting protein (TxNIP), high mobility group box 1 (HMGB-1), interleukin 1β (IL-1β) and IL-18. Cilostazol also protected the functionality of sirtuin 1 (SIRT1), which serves to restrict NLRP3 inflammasome activity, when exposure to FFAs would have otherwise impaired its function. Thus, it appears that cilostazol's mechanism of action in reducing NLRP3 inflammasome activation is an indirect one; it protects SIRT1, which then allows SIRT1 to perform its regulatory job. Cilostazol has potential as an already-available, well-tolerated preventive medication that may alleviate some of the adverse vascular effects of living with diabetes. The findings of the present study lay the groundwork for further research on the potential of cilostazol as a safe and effective treatment against diabetic endothelial dysfunction and vascular disease.

\section*{Introduction}

Cardiovascular disease is recognized as a leading cause of death worldwide, but the risk of death by cardiovascular disease is 2–3 times higher for individuals with diabetes [1]. Thus, it is urgent to identify ways by which the cardiovascular sequelae of diabetes can be prevented. Interventions targeting glucose levels, blood pressure, and oxidative stress do not reduce the incidence of cardiovascular deaths among diabetics [2]. However, activation of the nucleotide oligomerization domain (NOD)-like receptor pyrin domain-containing 3 (NLRP3) inflammasome is known to be one of the leading pathways through which the diabetic cardiovascular system is compromised, and there exists direct evidence that inhibiting NLRP3 inflammasome activation reduces inflammation, making it a ripe target for intervention [2–4]. NLRP3 inflammasome inhibitors exist but have been shown to be largely ineffective in clinical settings [5]. Type I interferons (IFNs) have good efficacy but come with substantial side effects in nearly every organ system [6]. In contrast, the phosphodiesterase-3 inhibitor cilostazol, a vasodilator widely used to treat blood flow problems, has anti-inflammatory effects in vascular smooth muscle cells and its side effect profile is mild [7,8]. Cilostazol protects against vascular dysfunction in diabetes, but its mechanisms are not well understood [9]. The purpose of this study to explore the impact of cilostazol on NLRP3 inflammasome activation, and the mechanisms by which that impact occurs.

Plasma levels of free fatty acids (FFA) are considerably increased in type II diabetes patients and are considered to be a highly accurate diagnostic indicator of disease progression [10]. High FFA levels are a key indicator of cardiovascular risk and have been shown to cause NLRP3 inflammasome activation [11,12]. In the present study, we administered high FFAs to induce NLRP3 inflammasome activation and examined the impact of cilostazol on several variables including oxidative stress, production of proinflammatory cytokines,
and the involvement of the class III histone deacetylase sir-tuin 1 (SIRT1). Oxidative stress is well recognized as an inducer of chronic inflammation and diabetic endothelial dys-
function [2]. NADPH oxidase 4 (NOX-4) is an enzyme that promotes the production of reactive oxygen species (ROS), thereby bringing on substantial oxidative stress and enhan-
cing endothelial dysfunction [13]. Previous research has shown that suppression of NOX-4 reduces oxidative stress-induced vascular degeneration [14]. Thioredoxin-interacting protein (TxNIP) is a key part of the mechanism by which NLRP3 is activated [15]. It is a known mediator of NLRP3 inflamma-
some-induced damage to endothelial cells and promotes oxi-
dative stress by inhibiting the antioxidant thioredoxin [16,17].

High mobility group box 1 (HMGB1) protein can be charac-
terized as an alarmin that signals the need for initiation of
the inflammatory response, thereby contributing to chronic
inflammation and endothelial dysfunction [18]. Interleukins 1-
beta (IL-1β) and 18 (IL-18) are proinflammatory cytokines [19]
produced by NLRP3 that play a critical role in the pathogen-
esis of numerous inflammatory diseases. The NLRP3 inflam-
masome manufactures pro-IL-1β and pro-IL-18, which
become mature IL-1β and IL-18 upon cleavage induced by
caspase 1 [3,20]. SIRT1, a known protector against vascular
harm [21], is typically inhibited by NLRP3 and rescue of SIRT1
expression is regarded as a promising strategy to mitigate
NLRP3 inflammasome-induced endothelial dysfunction. Our
findings demonstrate that cilostazol significantly ameliorates
oxidative stress and inflammation by ameliorating activation
of the NLRP3 inflammasome via rescue of SIRT1 function.
Thus, cilostazol may serve as a safe and effective therapy
against endothelial dysfunction and vascular disease induced
by exposure to high FFA.

**Materials and methods**

**Cell culture and treatment**

Human aortic endothelial cells (HAECs) were obtained from
Lonza (Basel, Switzerland). HAECs were cultured in endothe-
lial growth media (EGM2) in low passage numbers (<10) in a
5% CO2 humidified incubator at 37°C and stimulated with
high FFA (1 mM) in the presence or absence of 5 or
10 μM cilostazol. HAECs were then fixed with 4% paraformal-
dehyde for 10 min at RT followed by permeabilization with
0.1% Triton X-100 in Tris-buffered saline and Tween 20 (TBST)
for 15 min. Cells were then blocked with 5% bovine serum
albumin (BSA) and 2.5% FBS in TBST, followed by incubation
with the primary anti-4HNE monoclonal antibody overnight
at 4°C. After being washed 3 times, cells were incubated
with Alexa-488 conjugated secondary antibodies for 1 h at
room temperature (RT).

**Real-time polymerase chain reaction (PCR)**

Total RNA was extracted from HAECs using a micro RNA
Micro Kit in accordance with the manufacturer’s instructions
(Qiagen (Cat.74004), USA). RNA concentrations were quanti-
fied using Nanodrop. A total of 1 μg RNA was used to synthe-
size complementary DNA (cDNA) using iScript™ Reverse
Transcription Supermix (BioRad, #1708840) for quantitative
real-time PCR analysis (Invitrogen). SYBR-based real-time PCR
experiments were performed to detect the total transcripts of
mRNA using the ABI 7500 platform. The experimental results
were analyzed by normalizing to GAPDH using the
2^{ΔΔCt} method.

**Western blot analysis**

HAECs under the different treatment conditions were lysed
using radioimmunoprecipitation assay (RIPA) buffer with
protease and phosphatase inhibitors. Nuclear extracts were
obtained by removing the cytoplasm via cell lysis with hypo-
tonic buffer. A total of 20 μg cell lysates or nuclear extracts
were immobilized via 10% sodium dodecyl sulfate-polyacyr-
amide gel electrophoresis (SDS-PAGE). The separated protein
mixtures were then transferred to polyvinylidene fluoride
(PVDF) membranes and blotted against their specific antibod-
ies as well as their corresponding secondary antibodies.
Immunoblots were visualized using Pierce™ ECL Plus western
blotting substrate (Catalog # 32132). The primary antibodies
used in this study are:

**Enzyme-linked immunosorbent assay (ELISA)**

The secreted levels of IL-1β, IL-18, and HMGB-1 were mea-
sured by collecting the culture media of the HAECs for an-
alysis. ELISA kits were purchased from R&D Systems and the
procedure was performed in accordance with the manufac-
turer’s instructions. 96-plate reader spectrometry was used to
collect the data.

**Statistical analysis**

Data are expressed as means ± standard derivation (SD). One-
way analysis of variance (ANOVA) followed by Bonferroni
post-test comparisons was used to determine statistical sig-
nificance. p ≤ .05 was considered statistically significant.
Cilostazol reduces FFA-induced activation of the NLRP3 inflammasome

Next, we investigated the effects of cilostazol on high FFA-induced activation of the NLRP3 inflammasome by measuring the levels of TxNIP and HMGB1. As demonstrated by the results of real-time PCR and western blot analysis in Figure 3, exposure to high FFA increased the expression of TxNIP to 3.8- and 3.5-fold at the mRNA and protein levels, respectively. Meanwhile, the introduction of 5 and 10 μM cilostazol potently ameliorated this effect in a dose-dependent manner, reducing the mRNA expression of TxNIP to only 2.7- and 1.9-fold, and the protein expression of TxNIP to 2.5- and 1.3-fold, respectively. The results in Figure 4 demonstrate a similar effect of cilostazol on high FFA-induced expression of HMGB1. Exposure to high FFA for 36h increased the secretion of HMGB1 to 4.2-fold baseline, which was ameliorated by treatment with the two doses of cilostazol to only 2.5-and 1.4-fold, thereby demonstrating strong inhibition of HMGB1 expression. Finally, we employed western blot analysis to determine the effects of cilostazol on high FFA-induced activation of the NLRP3 inflammasome by measuring the protein expressions of NLRP3, ASC, and cleaved caspase-1 (P10). As shown in Figure 5, exposure to high FFA increased NLRP3 expression to 3.8-fold, expression of ASC to 3.5-fold, and P10 expression to 4.1-fold. Meanwhile, the two doses of cilostazol significantly reduced the expression of these three factors in a dose-dependent manner. NLRP3 expression was reduced to 2.6- and 1.6-fold, ASC expression to 2.3- and 1.4-fold, and P10 expression to 2.6- and 1.5-fold.

Cilostazol reduces the production of IL-1β and IL-18

Next, we confirmed that inhibition of NLRP3 inflammasome activation by cilostazol suppressed the production of IL-1β and IL-18. As shown by the results of ELISA in Figure 6, the baseline concentration of IL-1β was 34.3 pg/ml, which was increased to 256.6 pg/ml upon exposure to high FFA for 36h. However, treatment with the two doses of cilostazol exerted a strong inhibitory effect, reducing the concentration of IL-1β to only 115.4 and 73.9 pg/ml in a dose-dependent manner. Similarly, the concentration of IL-18 was 56.2 pg/ml at baseline concentration of IL-18 which was increased to 186.7 pg/ml by exposure to high FFA for 36h, while the two doses of cilostazol significantly reduced the concentration of IL-18 to 105.4 and 79.6 pg/ml, respectively. Thus, our findings confirm that inhibition of NLRP3 inflammasome activation by cilostazol exerts a potent anti-inflammatory effect.

The effects of cilostazol are mediated through SIRT1

Next, we investigated the effects of cilostazol on high FFA-induced reduced expression of the NLRP3 inhibitor SIRT1. As shown in Figure 7, the results of real-time PCR show that exposure to high FFA reduced the expression of SIRT1 to roughly 45%, which was recovered to 71% and 92% baseline at the mRNA level. Concordantly, the results of western blot analysis show that the protein expression of SIRT1 was reduced to 42% by high FFA exposure, while the two doses...
of cilostazol rescued SIRT1 protein expression to roughly 69% and 88% in a dose-dependent manner. Finally, we performed a SIRT1 inhibition experiment using nicotinamide to determine whether the effects of cilostazol are dependent on SIRT1. Briefly, HAECs were stimulated with 1 mM high FFA in the presence or absence of 10 μM cilostazol or 1 mM nicotinamide for 36 h. As demonstrated by the results of western blot analysis in Figure 8(A), the protein expression of NLRP3 was increased to 4-fold by high FFA exposure, which was reduced to 1.5-fold by the addition of cilostazol, while inhibition of SIRT1 by nicotinamide abolished the effects of cilostazol, resulting in 3.7-fold expression of NLRP3. Meanwhile, ASC and P10 expression were respectively increased to 3.8- and 4.3-fold by high FFA alone, decreased to 1.6-fold for both factors in the presence of high FFA plus cilostazol, and increased to 4.2- and 4.1-fold in the presence of high FFA with cilostazol plus nicotinamide. Concordantly, the results of ELISA in Figure 8(B) demonstrate that the inhibition of SIRT1 by nicotinamide exerted a similar effect on the protein secretion of IL-1β and IL-18. These findings demonstrate that expression of SIRT1 plays a vital role in facilitating the effects of cilostazol against high FFA-induced activation of the NLRP3 inflammasome.

Discussion

In this study, using primary human endothelial cells exposed to high FFA, we examined the impact of cilostazol on several variables related to cardiovascular risk, including oxidative stress, expression of proinflammatory cytokines, activation of the NLRP3 inflammasome, and the involvement of SIRT1. Our findings demonstrate a powerful effect of cilostazol at clinically relevant doses to inhibit all of these aspects of endothelial dysfunction. Oxidative stress is a major contributing factor in a wide range of diseases. In diabetes-related cardiovascular disease, increased levels of mitochondrial ROS and the reactive lipid 4-HNE have been shown to promote chronic inflammation and fibrosis in various tissues and drive the disease progression of diabetes [23,24]. Recent studies have demonstrated the ability of cilostazol to mitigate overproduction of ROS and 4-HNE in hepatocytes and oocytes [25,26]. Concomitant treatment with ginkgo balboa extract and cilostazol has been shown to potentially reduce vascular disease by suppressing the development of atherosclerotic lesions and oxidative stress in ApoE mice fed a high-fat diet [27]. Concordantly, our findings demonstrate that treatment with cilostazol alone significantly inhibits overproduction of ROS and 4-HNE induced by prolonged exposure to high FFA, thereby providing further evidence of a strong antioxidant effect of cilostazol. Additionally, we demonstrate that the antioxidant capacity of cilostazol may be a result of its
Figure 5. Cilostazol prevents FFA-induced activation of NLRP3 inflammasome in HAECs. Cells were stimulated with high FFAs (1 mM) with or without cilostazol (5, 10 \( \mu M \)) in HAECs for 36 h. Expression of NLRP3, ASC, and cleaved caspase 1 (P10) was measured by western blot analysis (*, #, $, p < .01 vs. previous group).

Figure 6. Cilostazol inhibits FFA-induced secretion of IL-1\( \beta \) and IL-18 in HAECs. Cells were stimulated high FFAs (1 mM) with or without cilostazol (5, 10 \( \mu M \)) in HAECs for 36 h. protein secretion of (A) IL-1\( \beta \) and (B) IL-18 was measured by ELISA (*, #, $, p < .01 vs. previous group).

Figure 7. Cilostazol protected against FFA-induced reduction of SIRT1 in HAECs. Cells were stimulated with high FFAs (1 mM) with or without cilostazol (5, 10 \( \mu M \)) in HAECs for 36 h. (A). mRNA of SIRT1 as determined by real-time PCR analysis; (B). Protein of SIRT1 as determined by western blot analysis (*, #, $, p < .01 vs. previous group).

Figure 8. Inhibition of SIRT1 by nicotinamide abolishes the inhibitory effects of cilostazol in FFA-induced activation of NLRP3 inflammasome. HAECs were stimulated high FFAs (1 mM) in the presence or absence of cilostazol (10 \( \mu M \)) or nicotinamide (1 mM) for 36 h. (A). Protein expression of NLRP3, ASC, and cleaved caspase 1 (P10) as determined by western blot analysis (*, #, $, p < .01 vs. previous group); (B) Protein secretion of IL-1\( \beta \) and IL-18 as measured by ELISA (*, #, $, p < .01 vs. previous group).
suppression of high FFA-induced overexpression of NOX4, a key promoter of ROS production.

Increased expression of TxNIP and HMGB1 is recognized as a major event in high FFS-induced NLRP3 inflammasome activation. TxNIP also plays a key role in oxidative stress by inhibiting the expression of the antioxidants Trx-1 and Trx-2, thereby exacerbating mitochondrial ROS production. Expression of TxNIP has been shown to be involved in the regulation of numerous processes, including the inflammatory response, cellular redox balance, lipid metabolism, and cellular apoptosis [27]. However, to our knowledge, this is the first study to investigate the effects of cilostazol on TxNIP expression. Our findings demonstrate a strong ability of cilostazol to inhibit the expression of TxNIP, thereby further downregulating oxidative stress induced by high FFA exposure. HMGB1 is another key component of NLRP3 inflammasome activation. Recent research has demonstrated that HMGB1 has two separate redox states—a chemotactic reduced form and a proinflammatory disulfide form, the latter of which is involved in activating the NLRP3 inflammasome [28]. A recent study showed that cilostazol could suppress the secretion of HMGB1 induced by lipopolysaccharide exposure through AMPK activation and p38 MAPK expression [29]. Our results also demonstrate the ability of cilostazol to downregulate HMGB1 expression induced by high FFA.

Preventing activation of the NLRP3 inflammasome is considered a valuable treatment approach against numerous chronic inflammatory diseases, including while numerous studies have suggested inhibition of NLRP3 inflammasome activation as a means to mediate the inflammatory response, a safe and reliable treatment has yet to be pinpointed. Cilostazol was originally prescribed as an antiplatelet agent and to ameliorate intermittent claudication. Recently, it has also been shown to prevent the development of foot ulcers in type II diabetes patients with peripheral vascular disease [30]. Contemporary research using a rat model suggests that cilostazol can improve age-related endothelial dysfunction via suppression of oxidative stress, increased bioavailability of nitric oxide, and EDHF-like vasorelaxation. Cilostazol treatment has also been shown to be effective in preventing the development of atherosclerosis by downregulating the expression of cytokines and chemokines induced by glucose [31]. In the present study, we demonstrate for the first time to our knowledge, that cilostazol can inhibit activation of the proinflammatory NLRP3 inflammasome induced by high FFA exposure, thereby significantly suppressing endothelial dysfunction due to chronic inflammation and oxidative stress. Importantly, we also show that SIRT1 plays a vital role in this beneficial effect of cilostazol, as evidenced by the results of our SIRT1 knockdown experiment using nicotinamide. Rescue of SIRT1 is considered an attractive treatment option in numerous diseases, and cilostazol has been shown to increase SIRT1 activation in neuronal cells, osteoclasts, hepatocytes, and others [32–34]. However, our study is the first to our knowledge to demonstrate that cilostazol can increase SIRT1 expression in primary HAECs, thereby opening the way to a new potential use for cilostazol in SIRT1-mediated amelioration of vascular diseases. Further research is required to better understand the mechanisms through which cilostazol exerts these remarkable antioxidant, anti-NLRP3, and pro-SIRT1 effects in the context of high FFA-induced vascular disease.

Disclosure statement

No potential conflict of interest was reported by the authors.

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