Feprazone Prevents Free Fatty Acid (FFA)-Induced Endothelial Inflammation by Mitigating the Activation of the TLR4/MyD88/NF-κB Pathway

Min Song, Liukun Meng, Xiaoxi Liu, and Yan Yang*

ABSTRACT: Increased levels of free fatty acid (FFA)-induced endothelial dysfunction play an important role in the initiation and development of atherosclerosis. Feprazone is a nonsteroidal anti-inflammatory compound. However, the beneficial effects of feprazone on FFA-induced endothelial dysfunction have not been reported before. In the current study, we found that treatment with feprazone ameliorated FFA-induced cell death of human aortic endothelial cells (HAECs) by restoring cell viability and reducing the release of lactate dehydrogenase (LDH). Importantly, we found that treatment with feprazone ameliorated FFA-induced oxidative stress by reducing the production of mitochondrial reactive oxygen species (ROS). In addition, feprazone prevented FFA-induced expression and secretion of proinflammatory cytokines and chemokines, such as chemokine ligand 5 (CCL5), interleukin-6 (IL-6), and interleukin-8 (IL-8). We also found that feprazone decreased the expression of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9). Interestingly, we found that feprazone reduced the expression of cell adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1). Our results also demonstrate that feprazone prevented FFA-induced activation of the toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/nuclear factor kappa-B (NF-κB) signaling pathway. These findings suggest that feprazone might serve as a potential agent for the treatment of atherosclerosis by improving the endothelial function.

1. INTRODUCTION

Atherosclerosis (AS) is characterized by the accumulation of fatty plaque and immune cells in the intimal endothelial space of large- and medium-sized arteries. Progressive disease can lead to arterial narrowing or occlusion, which results in the occurrence of stroke and myocardial infarction in the advanced stages.1 Recent reports show that approximately 31% of all deaths globally can be attributed to AS, making it one of the most deadly diseases worldwide. However, the development of effective and reliable treatments remains challenging.2 Recent research has focused on mitigating the effects of free fatty acids (FFAs) or nonesterified fatty acids. FFA is a byproduct of lipid metabolism and a major metabolic energy source. Elevated plasma levels of FFA are associated with an increased risk of CVDs and metabolic disorders, including obesity, type II diabetes mellitus (T2DM), and coronary artery disease (CAD), and play a critical role in the initiation and progression of AS.

In the early stages of AS, exposure to FFA induces endothelial cell (EC) apoptosis/necroptosis, adversely affects EC progenitor cells, and induces EC dysfunction, which is associated with dysregulated nitric oxide (NO) production and irregular vasodilation/constriction.3 Additionally, FFAs induce the production of reactive oxygen species (ROS) by mitochondria. Increased levels of ROS can shift the oxidant/antioxidant balance toward a state of oxidative stress and trigger an inflammatory response.4 Proinflammatory cytokines and chemokines, including C–C chemokine ligand 5 (CCL5), interleukin-6 (IL-6), and interleukin-8 (IL-8), play a major role in the pathogenesis of AS. Activated platelets that adhere to the arterial wall release chemokine CCL5 to recruit leukocytes and other immune cells to invade the intimal space.5 Platelets also release IL-6 and IL-8, which play a major role in AS. An increased plasma IL-6 level is an independent risk factor for AS, as IL-6 has been shown to activate ECs and promote thrombosis, smooth muscle proliferation, and macrophage foam cell formation.5,6 IL-8 is highly expressed in human atherosclerotic lesions and has been used as a marker for subclinical AS.8
Rupture of atherosclerotic plaques due to lesion formation results in myocardial infarction, stroke, and often death. Matrix metalloproteinases (MMPs) play a complex role in determining plaque vulnerability. While some MMPs have been shown to promote plaque stability, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) contribute to plaque rupture and lesion formation by degrading extracellular matrix, respectively. These enzymes have been shown to be significantly upregulated in patients with unstable plaques. Cellular adhesion molecules including vascular cellular adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 play an active role in leukocyte invasion of the vascular wall, thereby contributing to atherosclerotic plaque rupture. VCAM-1 has been suggested as a serum marker to determine the severity of lesion formation. The expression of proinflammatory cytokines, chemokines, and adhesion molecules is largely mediated through nuclear factor (NF)-κB signaling. Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that mediate the innate immune response to stimuli including pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). TLRs have been shown to play a pathological role in AS. For example, TLR3 decreases plaque stability by upregulating MMP-2 and MMP-9 expression, while TLR4 triggers nuclear translocation of p65 protein and subsequent activation of the proinflammatory NF-κB signaling pathway through myeloid differentiation factor 88 (MyD88). Modifying the activity of TLR-mediated pathways is considered as a potential strategy for the treatment or prevention of AS.

Feprazone, also known as prenazole, is a prenylated analogue of phenylbutazone and a nonsteroidal anti-inflammatory drug (NSAID) used for the treatment of joint and muscular pain. Feprazone acts by inhibiting the activity of cyclooxygenase (COX)-2, which is a precursor to prostaglandin production, and has been shown to have tenfold selectivity for COX-2 over COX-1. Many NSAIDs act by inhibiting the activity of prostaglandins. Prostaglandins are a type of fatty acid that trigger pain and inflammation and have been shown to contribute to atherosclerotic plaque rupture by mediating the expression of MMPs. Previous research has demonstrated the involvement of COX-2-mediated prostaglandin production in the pathological mechanism of AS. As a phenylbutazone derivative, feprazone has a similar structure to phenylbutazone, with the main difference lying in the replacement of the butyl located at the C4 position on the pyrazoline-2,5-dione skeleton with a 3-methylbutenyl substituent. Feprazone and other members of the pyrazolone family have been used for decades owing to their wide range of pharmacological activities, including antipyretic, analgesic, anti-inflammatory, antioxidant, anticancer, and many others. In the present study, we investigated whether feprazone might mitigate the effects of FFAs in human aortic endothelial cells (HAECs) and explored the underlying mechanism.

2. RESULTS

2.1. Feprazone Improves Cell Viability. Feprazone has a molecular structure of C_{20}H_{20}N_{2}O_{2} (Figure 1) and a molecular weight of 320.4 g/mol (PubChem). We began by exploring the potential protective effects of feprazone against FFA-induced reduced cell viability and increased release of lactate dehydrogenase (LDH). In this experiment, cells were treated with 2.5, 5, and 10 μM feprazone. As shown in Figure 2A, exposure to FFAs reduced the cell viability to 63% of baseline. However, although the protective effect of the low dose of feprazone was negligible, treatment with 5 and 10 μM feprazone exerted a much greater protective effect, rescuing cell viability to 81 and 93% of baseline. As shown in Figure 2B, feprazone dose-dependently reduced the release of LDH from HAECs exposed to insult from FFA, thereby demonstrating a notable protective effect of feprazone against FFA-induced cell death and apoptosis.

2.2. Feprazone Reduces FFA-Induced Oxidative Stress and Inflammation Factor Expression. Levels of ROS production were determined using MitoScene Red CMXRos staining. As shown in Figure 3, stimulation with 300 μM FFA increased ROS production by 3.4-fold, while 5 and 10 μM feprazone reduced ROS production to only 2.4- and 1.6-fold, respectively. Next, we measured the messenger RNA (mRNA) expression and secretion of CCL5, IL-6, and IL-8. The results of polymerase chain reaction (PCR) analysis in Figure 4A showed that while FFA exposure induced a significant increase in the expression of all three cytokines, this effect was reduced by feprazone treatment, with the higher dose mitigating the increase by approximately half. A similar inhibitory effect was observed at the protein level (Figure 4B).

2.3. Feprazone Inhibits FFA-Induced Expression of Degradative Enzymes and Adhesion Molecules. Arterial remodeling mediated by MMPs and immune cell infiltration are significant factors in the pathogenesis of AS. To determine whether feprazone might protect against arterial remodeling, we measured its effects on the mRNA and protein expression of MMP-2 and MMP-9 induced by stimulation with FFAs. As shown in Figure 5A, B, PCR and enzyme-linked immunosorbent assay (ELISA) analyses revealed that FFAs increased mRNA expression levels of the two molecules were increased to 2.8- and 3.4-fold, respectively, while the addition of feprazone dose-dependently reduced the release of LDH from HAECs exposed to insult from FFA, thereby demonstrating a notable protective effect of feprazone against FFA-induced cell death and apoptosis.

Figure 1. Molecular structure of feprazone.
pathogenesis of AS. As shown in Figure 7, FFA stimulation increased the activity of TLR4 and MyD88 by roughly twofold, while the phosphorylation of NF-κB p65 protein increased by 2.5-fold. Indeed, the addition of feprazone exerted a notable inhibitory effect on the activity of TLR4 and MyD88 while reducing the phosphorylation of p65 and subsequent activation of NF-κB by minimizing the levels of all three to roughly 1.5-fold. Therefore, we hypothesize that feprazone may protect against AS by inhibiting the activation of the TLR4/MyD88/NF-κB pathway.

3. DISCUSSION

In the present study, we demonstrate that feprazone treatment could suppress the proatherosclerotic effects of exposure to FFAs in HAECS, such as increased cell death; oxidative stress; expression of proinflammatory cytokines, chemokines, and adhesion molecules; and activation of the NF-κB pathway through TLR4/MyD88 signaling. Additionally, we show that feprazone treatment could prevent FFA-induced cell death and oxidative stress in vitro. Oxidative stress due to overproduction of ROS acts as a key pathological mechanism in all stages of AS. While in normal physiology, ROS are important reactive molecules that regulate various cellular functions and processes, ROS-induced oxidative stress leads to vascular injury, inflammation, and foam cell formation. Exposure to FFAs is well recognized as a trigger for overproduction of ROS, inflammatory response, and endothelial cell dysfunction. Recent research has suggested the use of various types of NSAIDs to inhibit oxidative stress in patients with AS Here, we report that feprazone might protect against ROS-mediated vascular injury by inhibiting the generation of ROS induced by FFAs.

Chronic inflammation serves as the cornerstone of numerous diseases, including AS, so it follows that anti-inflammatory medications such as NSAIDs are an important part of disease management. Chemokines, such as CCL5 and IL-8, play a key role in inflammation and contribute to atherogenesis by recruiting immune cells to infiltrate the arterial wall. CCL5 is also known as regulated upon activation, normal T-cell expressed and secreted (RANTES) and has been...
suggested as a therapeutic target to slow the progression of AS. CCL5 expression is regulated by p65 Rel protein, the same involved in the activation of inflammatory NF-κB signaling, and is increased in atherosclerotic plaques. Antagonism of CCL5 and its receptor CCR5 has been shown to reduce atherosclerotic burden and hinder disease progression. In the present study, we found that exposure to FFAs significantly upregulated the mRNA and protein expression of CCL5. Additionally, we found that feprazone could suppress the expression of CCL5 induced by FFAs.

IL-6 is regarded as one of the main upstream cytokines involved in the chronic inflammatory response in AS. IL-6 is highly expressed in atherosclerotic lesions and is known to affect a variety of different cell types. Although IL-6 is most well recognized for its role in promoting atheroma formation by activating ECs and promoting thrombosis, smooth muscle cell migration, and lipid accumulation, recent research has raised some controversy regarding the potential protective role of IL-6, as it has also been shown to aid in macrophage cholesterol efflux via ATP-binding cassette transporter (ABC)A1. Inhibition of IL-6 has been suggested as a treatment approach for AS. Chemokine IL-8 binds to its receptors CXC chemokine receptor 1 (CXCR1) and CXC chemokine receptor 2 (CXCR2) to initiate various biological functions, including inflammation, angiogenesis, mitosis, etc. IL-8 has been shown to contribute to AS via neutrophil extracellular trap formation, which further upregulates IL-8 expression through TLR9/NF-κB signaling, thereby creating a pathological positive feedback loop. Inhibition of COX-2 is an established anti-inflammatory treatment, and COX-2 inhibitors

Figure 5. Feprazone inhibited FFA-induced expression of MMP-2 and MMP-9 in HAECs. Cells were stimulated with 300 μM FFAs in the presence or absence of feprazone (5, 10 μM) for 24 h. (A) mRNA levels of MMP-2 and MMP-9 and (B) protein levels of MMP-2 and MMP-9 (***, P < 0.001 vs vehicle group; #, ##, P < 0.05, 0.01 vs FFA treatment group).

Figure 6. Feprazone inhibited FFA-induced expression of VCAM-1 and E-selectin in HAECs. Cells were stimulated with 300 μM FFAs in the presence or absence of feprazone (5, 10 μM) for 24 h. (A) mRNA levels of VCAM-1 and ICAM-1 and (B) protein levels of VCAM-1 and ICAM-1 (***, P < 0.001 vs vehicle group; #, ##, P < 0.05, 0.01 vs FFA treatment group).

Figure 7. Feprazone prevented FFA-induced activation of the TLR4/MyD88/NF-κB pathway in HAECs. Cells were stimulated with 300 μM FFAs in the presence or absence of feprazone (5, 10 μM) for 6 h. Expression of TLR4, MyD88, and p-NF-κB p65 was measured (***, P < 0.001 vs vehicle group; #, ##, P < 0.05, 0.01 vs FFA treatment group).
have been shown to reduce early atherosclerosis in mouse models. In the present study, we found that the COX-2 inhibitor feprazone could inhibit the expression of IL-6 and IL-8 in HAECs challenged with FFAs. Thus, the anti-inflammation effects of feprazone may be harnessed to inhibit atherogenesis.

Proinflammatory NF-κB signaling is one of the most well-known and thoroughly studied inflammatory signaling mechanisms involved in AS. Activation of NF-κB can occur through several intercellular signaling pathways in AS, and inhibiting its activity has been well-documented as a potential therapeutic strategy to halt or prevent disease progression. Previous research has shown that exposure to FFAs increases NF-κB activation, thereby driving EC dysfunction and atherogenesis. Recently, a mouse model study demonstrated that inhibition of NF-κB could help protect against vascular dysfunction in diabetic mice via COX-2 inhibition. In AS, after immune cells are recruited to the vascular wall through chemokine signaling, adhesion molecules including ICAM-1 and VCAM-1 induce cells to roll along and cling to the endothelial cells of the arterial wall, followed by intimal infiltration and lesion formation. Modifying TLR4/NF-κB signaling has been shown to attenuate atherosclerosis by inhibiting the expression of VCAM-1 and ICAM-1. Previous research has revealed the association between COX-2 inhibition and reduced expression of cellular adhesion molecules. Here, we found that treatment with feprazone not only suppressed FFA-induced expression of adhesion molecules but also inhibited activation of NF-κB signaling through TLR4/MyD88.

Together, our findings provide evidence for a novel antiatherosclerotic mechanism of the COX-2 inhibitor and NSAID feprazone against FFA-induced development of AS. As the diet of the global population trends toward a high-fat western diet, the prevalence of AS and related metabolic disorders is likely to increase, making therapies against such disease highly valuable. Here, we found that feprazone could attenuate several pathological mechanisms associated with AS, including cell death and apoptosis, oxidative stress, inflammation, and monocyte adhesion to ECs. However, the present study was only performed using an in vitro model of FFA-induced AS. Future studies identifying the underlying molecular mechanism and exploring the effects of feprazone on AS in vivo are needed to better understand its therapeutic potential. In the meantime, this research lies the groundwork for such investigation.

4. MATERIALS AND METHODS

4.1. Cell Culture and Treatment. Human subject experiments were designed in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. All of the experiments were approved by the ethics committee of Fu Wai Hospital. Human aortic endothelial cells (HAECs) were supplied by the American Type Culture Collection (ATCC, Massachusetts). Cells were maintained in endothelial basal medium-2 (EBM-2) (Lonza, Switzerland) containing endothelial growth medium-2 supplements (0.004 mL/mL endothelial cell growth supplement, 10 ng/mL epidermal growth factor, 90 μg/mL heparin, and 1 μg/mL hydrocortisone), 5% fetal bovine serum (FBS), and 1% antibiotics (penicillin/streptomycin) in a humid atmosphere at 37°C and 5% CO₂. The medium was changed every 3–4 days. The cells were then stimulated with 300 μM FFAs in the presence or absence of feprazone (purity ≥98%, no. GC40565, GLPBIO) at concentrations of 2.5, 5, and 10 μM for the cell viability and apoptosis experiments and 5 and 10 μM for all other experiments.

4.2. MTT Assay. To assess the cell viability of FFA-induced HAECs treated with feprazone, we employed the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described. Prior to experimentation, HAECs were seeded into 96-well plates at a density of 2 × 10⁴ cells/well and treated with 300 μM FFA with or without 5 and 10 μM feprazone. In total, 20 μl of MTT solution (5 mg/ml, Sigma-Aldrich) was added to each well. The plates were then incubated in a 5% CO₂ incubator overnight at 37°C. The culture medium was then removed, and dimethyl sulfoxide (DMSO, 150 μL) was added to the wells to dissolve the precipitate. The optical density at 490 nm was measured using a microplate reader.

4.3. LDH Release. After the indicated treatment, the release of LDH was measured. Briefly, 1.5 × 10⁶ cells were seeded into 96-well plates, and 50 μL of supernatant was transferred to a new well, followed by the addition of 50 μL of LDH assay solution to each well. The plates were covered and allowed to process for 1 h, followed by the addition of 50 μL of stop solution. The rate of absorbance recorded at 570 nm was used to determine the release of LDH.

4.4. MitoScene Red CMXRos Staining. To determine levels of oxidative stress in HAECs, mitochondrial ROS was detected using MitoScene Red CMXRos staining. Briefly, after necessary treatment, cells were rinsed with PBS three times, followed by incubation with 1 μM MitoScene Red CMXRos for 30 min at 37°C in the dark. Fluorescent signals were visualized using a confocal microscope with emission/excitation wavelength of 510/580 nm.

4.5. Real-Time PCR. To determine the RNA expression of the target genes, total RNA was extracted from treated HAECs using an RNeasy Mini Kit in accordance with the manufacturer's instructions (Qiagen). Isolated RNA was used to synthesize complementary DNA (cDNA) using a Universal One-Step RT-qPCR Kit (Bio-rad). Then, 20 μg of cDNA was subjected to SYBR Green PCR using an ABI 7900HT system. The protocol consisted of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The 2⁻ΔΔCt method was used to determine the levels of mRNA. The following primers were used in this study: human GAPDH: forward: 5'-ACCCACTCCTCCACCTTTGGA-3', reverse: 5'-CTTTGCTGTAGCCAAATTCGG-3'; CCL5: forward: 5'-CCTGAGTCTTGGCCTACCTTCCTC-3', reverse: 5'-ACA-CCTTGGGCTTTCTTTGGA-3'; IL-6: forward: 5'-AGGA-TACCACTCCCAACAGACCT-3', reverse: 5'-CAAGTG-TCACTGTATGTTTTGAC-3'; IL-8: forward: 5'-GTTGAGTTTTGGCAAGGAAT-3', reverse: 5'-TTAT-GAATTTTCAGGCTTCTTCTAACAATCTCTC-3'; MMP-2: forward: 5'-ACTTTGATGGGAACTCAGAG-3', reverse: 5'-CAAGTTCATGTCAAGAGAG-3'; MMP-9: forward: 5'-GCCACTACTGTGGCTTTTGAG-3', reverse: 5'- GCTCACAGTACCAGGAGG-3'; CCL5: forward: 5'-ACATGGCAACCCCTTGTAG-3', reverse: 5'-ACCTCAGACATCCCTTGACA-3'; ICAM-1: forward: 5'-AGAAATTGTTGCTCATGGATGTC-3', reverse: 5'-CATCGACACCTCCTGTGACC-3'; VCAM-1: forward: 5'-TGACAAGTCCACATCGTTGA-3', reverse: 5'-AC-TGCGGACGACCATAAT-3'.

4.6. ELISA. Enzyme-linked immunosorbent assay (ELISA) kits were used in accordance with the manufacturer's
instructions to determine the protein secretions of the target genes. Briefly, 50 μL of cell culture supernatant was collected and added to ELISA plates and incubated overnight at 4 °C. After that, the plates were incubated with primary antibody for 1 h followed by HRP-conjugated secondary antibodies for 30 min after a thorough washing. The reaction was stopped, and 100 μL of substrate buffer was added. The absorbance was recorded at 450 nm to index the concentrations of the target proteins.

4.7. Western Blot Analysis. After the indicated treatment, radioimmunoprecipitation assay (RIPA) buffer was used to obtain total protein from HAECs. Briefly, 20 μg of total protein was electrically separated onto a sodium dodecyl sulfate polyacrylamide gel and then transferred onto a poly(vinylidene difluoride) (PVDF) membrane, which was then blocked against nonspecific sites for 1 h using skimmed milk. The membranes were incubated overnight with primary antibodies and then washed three times before the addition of HRP-conjugated secondary antibodies for 30 min. Enhanced chemiluminescence was used to determine the fluorescent protein signals. The following antibodies were used in this study: TLR4 (1:2000, no. 14358, Cell Signaling Technology); Myd88 (1:2000, no. 4283, Cell Signaling Technology); p-NF-κB p65 (1:1000, no. 3033, Cell Signaling Technology); NF-κB p65 (1:2000, no. 8242, Cell Signaling Technology); β-actin (1:10 000, no. 4970, Cell Signaling Technology); anti-ribbit IgG, HRP-linked antibody (1:3000, no. 7074, Cell Signaling Technology); antirabbit IgG, HRP-linked antibody (1:3000, no. 7076, Cell Signaling Technology).

4.8. Statistical Analysis. The experimental data are presented as mean ± standard error of mean (SEM). Statistical analysis was carried out by analysis of variance (ANOVA) with Tukey’s posthoc test using SPSS software (Version 19.0). Results with a P value of <0.05 were regarded statistically significant.

■ AUTHOR INFORMATION

Corresponding Author
Yan Yang — Adult Cardiac Surgery Center, State Key Laboratory of Cardiovascular Diseases and FuWai Hospital, CAMS and PUMC, Beijing 100037, China; orcid.org/0000-0003-4862-064X; Phone: +86-010-88396565; Email: yangyan1445@163.com

Authors
Min Song — Adult Cardiac Surgery Center, State Key Laboratory of Cardiovascular Diseases and FuWai Hospital, CAMS and PUMC, Beijing 100037, China
Liukun Meng — Adult Cardiac Surgery Center, State Key Laboratory of Cardiovascular Diseases, National Center for Cardiovascular Diseases and FuWai Hospital, CAMS and PUMC, Beijing 100037, China
Xiaoxi Liu — Adult Cardiac Surgery Center, State Key Laboratory of Cardiovascular Diseases, National Center for Cardiovascular Diseases and FuWai Hospital, CAMS and PUMC, Beijing 100037, China

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c05826

Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work is funded by the National Center for Cardiovascular Diseases and FuWai Hospital, CAMS and PUMC.

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