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The anti-inflammatory activity of palmitoleic acid from macadamia nuts

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Macadamia nuts are rich in a variety of nutrients and are prone to oxidative deterioration during storage and transportation. They contain a high level of monounsaturated fatty acids, whose composition may change during oxidation. In this study, we determined the oxidation indicators of macadamia nuts and the changes in the proportion of monounsaturated fatty acids. In addition, we demonstrated that POA intervention reduced excessive mitochondrial superoxide production, thereby alleviating oxidative stress and mitigating inflammation. The alleviation of inflammation was associated with reduced lysosomal damage, as evidenced by increased expression of *LAMP2* and *ATP6V1A*, decreased *CTSB* levels, and enhanced lysosomal biogenesis. *In vitro* assays further revealed that POA treatment increased *NRF2* expression while decreasing *TBK1* expression. Molecular docking analysis suggested that the interaction between POA and NRF2 may involve key residues including valine (VAL-418, VAL-512 and VAL-465). Overall, POA alleviated cell inflammation through the NRF2/TBK1 signaling pathway. This study provides insights into the anti-inflammatory mechanisms of POA, highlighting its potential as a therapeutic agent.

1. Introduction

Macadamia nuts have high nutritional value but are susceptible to oxidation during storage and transportation, which impairs their quality.[1] They are high in oil, especially dominated by monounsaturated fatty acids (MUFAs). Oxidative deterioration may affect the quality and composition of monounsaturated fatty acids in macadamia nut oil. Monounsaturated fatty acids, as a category of functional fatty acids and essential fatty acids for human beings, contribute to maintaining a favorable blood lipid profile, enhancing insulin sensitivity, and promoting better glycemic control.[2–5] Palmitoleic acid (16:1n-7,POA) has received considerable attention due to its recently proven benefits in immune system function, despite its metabolism having been first described in the 1960s.[5] Endogenous POA in the human body primarily originates from *de novo* fatty acid synthesis mediated by stearoyl-CoA desaturase, which catalyzes the biosynthesis of MUFAs from saturated fatty acids (SFAs). POA is renowned for its exceptional stability and is also found in animals and plants, particularly at high concentrations in macadamia nut oil, cod liver oil, and sea buckthorn oil.[6]

In recent years, multiple pieces of evidence have demonstrated that POA can effectively improve certain chronic subhealth conditions, *e.g.*, disorders of glycolipid metabolism and inflammation. POA alleviated liver damage by adjusting the composition and distribution of the gut microbiota.[7] Exogenous POA supplementation reduced systolic pressure and improved aortic remodeling by restraining NF-κB-mediated inflammation.[5] POA exerted a salutary influence against hepatic lipotoxicity by suppressing lipid metabolism-regulated ferroptosis.[8] *In vitro*, macrophages treated with POA showed reduced inflammatory gene expression and inflammatory cytokine production. POA has been demonstrated to drive macrophages toward an anti-inflammatory phenotype.[9] Supplementing with POA could help reduce inflammation in mouse models of inflammatory bowel disease (IBD).[10] Mechanistically, POA markedly elevated the transcriptomic profiles associated with cell division and biosynthetic processes in *Akkermansia muciniphila*, selectively promoted its proliferation and relative abundance within the gut microbiota, and ultimately reshaped the overall composition and structure of the intestinal microbial community.

In recent years, chronic inflammation has been reported to disrupt cellular homeostasis and lead to the development of metabolic and inflammatory diseases, including obesity, dia-

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betes, cardiovascular disease and metabolic dysfunction-associated steatotic liver disease (MASLD).[11] Beyond its well-known role in digestion and nutrient absorption, the gut is equally vital as a central regulator of immune function and microbial ecology. Intestinal inflammation disrupts the intestinal microenvironment, leading to the above-mentioned metabolic diseases, and even affecting the development of the nervous system.[12] Intestinal epithelial cells are the "initiators" of intestinal inflammation and subsequently become the "targets" of immune attack. Such ongoing damage is a key driver that initiates and perpetuates chronic and relapsing inflammation of the gastrointestinal tract, *e.g.*, inflammatory bowel disease (IBD).[13] POA can improve inflammation, but the mechanism of POA alleviating intestinal epithelial cell inflammation is not fully understood.

In this study, we aimed to investigate the changes in monounsaturated fatty acids (oleic acid and palmitoleic acid) during oxidation of macadamia nuts and to explore the molecular mechanism underlying the anti-inflammatory effect of palmitoleic acid (POA). Molecular biology assays and molecular docking analysis revealed that POA alleviated cell inflammation through the NRF2/TBK1 signaling pathway. Our research provides a new perspective on the anti-inflammatory effects of POA.

2. Materials and methods

2.1 Materials

Macadamia nuts were provided by Yunnan Puer Kangtai Fruit Co., Ltd, and were mechanically harvested in 2024. A total of 5 kg of macadamia nut samples were shelled manually, and the moldy, insect damaged and deformed kernels were removed. The macadamia nuts were sealed and stored in a −20 °C refrigerator until the experiment began. Palmitoleic acid (POA) was acquired from Sigma-Aldrich. LPS (*Escherichia coli* 055:B5) was provided by Shanghai Yuanye Bio-Technology Co., Ltd. Antibodies were provided by Proteintech Group, Inc. (China). Reagent kits were provided by Beyotime and Nanjing Jiancheng Bioengineering Institute. Caco-2 cells were provided by Wuhan Punosai Life Technology Co., Ltd (China).

2.2 Storage conditions of Macadamia nuts

Macadamia nuts were separated into six groups, placed in heat- and moisture-resistant sterilization bags (45 cm × 50 cm), and incubated at 60 °C for 0, 7, 14, 21, 28, and 35 days, respectively. After sampling, all specimens were sealed, protected from light, and stored at −20 °C until further analysis.

2.3 Oil extraction and evaluation of their quality

30 g of macadamia nut samples were ground using an ultrafine pulverizer (24 000 rpm; FW100; Tianjin Taisite Instrument Co., Ltd, Tianjin, China). Subsequently, 180 mL of petroleum ether was added to the powder, and the mixture was stirred continuously for 3 h. After phase separation, the supernatant was collected, and the above extraction procedure was repeated. The petroleum ether in the filtrate was removed *via* vacuum rotary evaporation at 40 °C to yield the oil sample, which was subsequently stored at 4 °C.

2.3.1 Peroxide value (PV) and acid value (AV). The PV and AV of macadamia nut oil were measured following the method described by Food Safety Standards (GB5009.227—2023 and GB5009.229—2016).

2.4 Observation of the microstructure of macadamia nuts

Initially, half of the macadamia nut tissue blocks were embedded in an OCT compound, mounted on a small metal stage, and rapidly frozen at −80 °C. The frozen samples were fixed in a specimen chuck and sectioned into 20 μm slices using a CM1950 cryostat (Leica Microsystems, Vienna, Austria). The sections were then mounted onto microscope slides. For morphological observation, cell walls were stained with 5 mg mL^{-1} calcofluor white, and lipid droplets were labeled with 5 mg mL^{-1} Nile red (w/v). Fluorescence imaging was performed using a Zeiss LSM 710 confocal microscope (Carl Zeiss Jena, Germany).

2.5 Analysis of the fatty acid composition

Fatty acid analysis of macadamia nut oil was performed by gas chromatography. Total lipids were subjected to methylation according to an official method. The resulting methyl-esterified fatty acids were filtered into sample vials and subjected to GC analysis for identification and quantification. Both the injection and detection ports were maintained at 260 °C. The oven temperature program was initially held at 110 °C for 3 min, followed by ramping to 220 °C at a rate of 4 °C min^{-1} and holding for 15 min. A flame ionization detector (FID) was used with hydrogen (H_2) flow rates of 30 mL min^{-1} and 400 mL min^{-1}, respectively. High-purity helium was used as the carrier gas with a constant flow rate of 1 mL min^{-1}. Peak retention times were identified using fatty acid methyl ester (FAME) standards, and the results were expressed as relative percentages of total fatty acids.

2.6 Cell culture conditions

Caco-2 cells were cultivated in Minimal Essential Medium (MEM) containing 20% fetal bovine serum (Gibco) and 1% penicillin–streptomycin. Cell culture was performed at 37 °C under a humidified 5% CO_2 atmosphere.

2.7 Reagent configuration

First, 1 mg mL^{-1} LPS stock solution was prepared, and then the stock solution was divided into small portions and stored at −20 °C. In subsequent studies, the stock solution was diluted with MEM to the desired concentration for use. In brief, pure POA was blended with 0.1 mol L^{-1} NaOH, followed by addition to 10% BSA. The control solution consisted of a mixture of 10% BSA and 0.1 mol L^{-1} NaOH.

2.8 Cell viability

The cell growth rate was assessed using a CCK-8 assay (Beyotime, China). Following seeding in 96-well plates, the

cells were treated with POA at various concentrations (0, 6.25, 12.5, 25, 50, 100, and 200 μM) for two time points (12 and 24 h). Then 10 μL of CCK-8 solution was added and the cells were incubated at 37 °C for 30 min. The absorbance was then measured at 450 nm. Cell viability was calculated based on the absorbance values.

2.9 Real-time quantitative PCR (RT-qPCR)

RT-qPCR was applied to determine the transcript abundance of target genes in Caco-2 cells. Total RNA was extracted using a MolPure® 5 min Flash Cell RNA Kit (Yeasen, China). After determining the purity and concentration of RNA, total RNA was reverse transcribed into cDNA (4 × Hifair® III SuperMix Plus: Buffer, dNTP, Hifair® III Reverse Transcriptase, RNase inhibitor, Random primers/Oligo (dT)18 primer mix). Quantitative RT-qPCR amplification was performed using a QuantStudio 3 real-time PCR system and Hieff® qPCR SYBR Green Master Mix (Yeasen, China). The primers for IL-6, IL-8, and other genes used are shown in Table 1. GAPDH was employed as the endogenous control for normalization of gene transcription data. Target gene expression levels were quantified using the $2^{-\Delta\Delta CT}$ method.

2.10 LysoTracker staining

Cells were seeded in confocal microplates. Following 24 hours of treatment, they were washed twice with MEM. Subsequently, 500 μL of assay buffer was added to each well, followed by 500 μL of PKH67 staining solution (2×). The cells were incubated at 37 °C for 5 minutes and then at 4 °C for 15 minutes. After one wash with complete medium, MEM containing 50 nM LysoTracker Red DND-99 was added. Cells were incubated at 37 °C for 30 minutes and washed three times with PBS. Lysosomal images were then acquired using a fluorescence microscope. Finally, ImageJ software was employed to quantify and compare the relative fluorescence intensities among the treatment groups.

2.11 Assessment of lysosomal enzyme activity

According to the manufacturer's instructions, the supernatant was collected to detect the activities of acid phosphatase (ACP, A060-1-1) and β-*N*-acetylglucosaminidase (NAG, A031-1-1).

2.12 Mitochondrial ROS and total intracellular ROS assay

Cells were stained with 5 μM MitoSOX™ Red dye solution (S0061S, Beyotime) for 30 min, followed by three washes with PBS. Subsequently, Hoechst 33342 staining solution (C1025, Beyotime) was added to Caco-2 cells in each group and incubated for 15 min, followed by three PBS washes. Fluorescence images were acquired using a fluorescence microscope and analyzed using ImageJ software. For total intracellular ROS measurement, cells were incubated with DCFH-DA (S0033S, Beyotime) at 37 °C for 30 min. After incubation, the cells were collected, and intracellular reactive oxygen species (ROS) levels were measured using a fluorescence microplate reader.

2.13 SOD assay

Intracellular superoxide dismutase (SOD) activity was measured using a commercial assay kit according to the manufacturer's instructions. The OD value was measured using a microplate reader to determine the change in enzyme activity.

2.14 Western blot analysis

Proteins were obtained from cells, and the concentrations of the proteins were quantified. Subsequently, the proteins were denatured by mixing each sample with five-fold loading buffer and boiling for five minutes. Following separation by 10% SDS-PAGE, the proteins were electrotransferred onto PVDF membranes at 250 mA for 1 hour. PVDF membranes were immersed in TBST for 1–2 min and then incubated with a specified volume of protein-free rapid blocking solution on a shaker for 30 min. Next, the membranes were incubated with the appropriate primary antibodies overnight at 4 °C.

Table 1 Primer sequences

Gene	Forward primer (5′ to 3′)	Reverse primer (3′ to 5′)
IL-6	CGCAGTTCTACAGCCGCTCAC	CTGAGTTTCCTCTGACTCCATCGC
IL-8	ACCACCGGAAGGAACCATCTCAC	GAAATCAGGAAGGCTGCCAAGAGAG
IL-1β	GGACAGGATATGGAGCAACAAGTGG	TCATCTTTCAACACGCAGGACAGG
COX-2	CCCCTTCTGCCTGACACCTTTC	CAGCAACCCTGCCAGCAATTTG
iNOS	TGCCCTGCTTTGTGCGGAATG	TTGCTGCCAGAAACTGCGGAAG
TLR4	TGGTGTGTCGGTCCTCAGTGTG	AGCCAGCAAGAAGCATCAGGTG
LAMP-1	GGCTCTGTGGAGGAGTGTCTG	CCGACGAGGTAGGCGATGAG
LAMP-2	TGGCACAGTGAGCACAAATGAG	TGGAGTAGGTGTTGTAGTAGGAGATG
ATP6V1A	TGTCACTGAGAAGCTGCCAG	CTCCCTGGACACACGGAAAA
ATP6V1C2	CCTGACTTCAAGGTGGGGAC	ATGACTTCCACCACGCTCTG
ATP6V1H	GGAATGGAGTCCTGTGCACA	TCGTGAGCAGCAACAGCTAA
TBK1	ATGCAGAGCACTTCTAATCATCTG	CTAAAGACAGTCAACGTTGCGA
NRF2	GGTTCCAAGTCCAGAAGCCA	GGTTGGGGTCTTCTGTGGAG
CTSB	CGTCACCGGAGAGATGATGG	AGAAGCCATTGTCACCCCAG
CTSL	CTGGTGGTTGGCTACGGATT	CTCCGGTCTTTGGCCATCTT
CTSD	CCAGTGCTTCACAGTCGTCT	CACGTAGGTGCTGGACTTGT
SLAMF7	CCAACATGCCTCACCCTCAT	GGAACCGACCAGCTCTTTCA
ALG2	ATGGCGGAGGAGCAGGGC	TTATACCAGCAGTTTGGTAACATATC

Following primary antibody incubation, the membranes were washed three times with TBST. Subsequently, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (H + L) at room temperature for 2 hours. The membranes were washed three times with TBST for 10 minutes, and the protein bands were visualized using an ultrasensitive ECL assay kit (Beyotime, China). Quantification of the protein bands was performed using ImageJ software. Protein expression levels were calculated after normalization relative to the internal reference protein. The raw data obtained from western blotting are provided in the SI.

2.15 Molecular docking

The canonical SMILES structure of POA was obtained from the PubChem database and converted into an SDF file using NovoPro to prepare the putative ligand. The crystal structure of NRF2 was obtained from the PDB database, and the receptor structure was processed using the visualization software PyMOL to remove water molecules and unwanted heteroatoms. AutoDock Vina was used to perform molecular docking after adding hydrogen atoms and charges. Finally, the binding affinity of POA for NRF2 was calculated using a grid box covering the entire protein structure.

2.16 Statistical analysis

Each set of experiments included a minimum of three biological replicates. GraphPad Prism 10.1.2 was used to depict the experimental results, and the data are presented as mean ± SEM. Statistical analysis was performed using IBM SPSS Statistics 27 software. One-way ANOVA followed by Duncan's multiple range test was employed for intergroup comparisons. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1 Oxidation and monounsaturated fatty acid changes in macadamia nut oil

Throughout the entire storage period, the AV of MO continued to rise (Fig. 1A). From 0 d to 14 d, the AV of MO increased from 0.277 mg g^{-1} to 0.510 mg g^{-1}. Compared with fresh macadamia nuts, the AV of macadamia nuts stored for 35 days underwent a significant change (Fig. 1A). During the entire storage period, the PV of MO increased from 0.183 mmol kg^{-1} to 0.956 mmol kg^{-1}, indicating a significant increase in the peroxide value of the oil (Fig. 1B).

Based on the above results, we observed the microstructure of macadamia nuts at three key time points: 0 d, 14 d, and 35 d. CLSM results showed that a series of changes occurred in the cell structure during the oxidation of stored macadamia nuts. As shown in Fig. 1C, the blue regions indicate cell walls, and the red regions represent oil bodies. With prolonged storage, the oil bodies in macadamia nuts gradually decomposed and lipids aggregated, thereby accelerating the oxidation process. Next, we analyzed the changes in palmitoleic acid and oleic acid (OA) in macadamia nuts. The results showed that OA remained relatively stable after 35 days of accelerated oxidation, whereas POA underwent a certain degree of degradation (Fig. 1D and E). Although POA is less stable than OA, its physiological activity remains of considerable interest.

3.2 POA ameliorated intestinal cell inflammation

To determine the protective effect of palmitoleic acid (POA) against inflammation, Caco-2 cells were stimulated with lipopolysaccharide (LPS) at a series of concentrations (1, 5, 10, 25, 50, 100, and 200 μg mL^{-1}) for 24 h to induce inflammation. Among

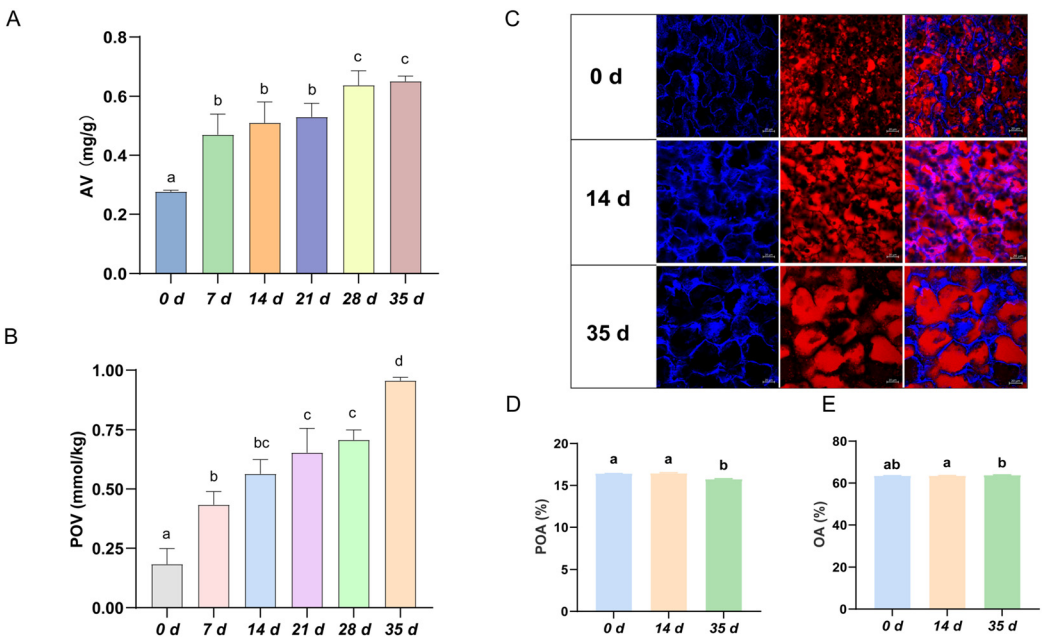

Fig. 1 Oxidation and monounsaturated fatty acid changes in macadamia nut oil. (A) AV. (B) PV. (C) The microstructure of macadamia nuts. (D) POA (%). (E) OA (%). Different letters (a–d) above the bars indicate statistically significant differences between groups.

the tested concentrations, 25 μg mL^{-1} exhibited the most significant effect without inducing cytotoxicity (Fig. S1 in the SI). Analysis of the CCK-8 assay indicated that low concentrations of POA exhibited no cytotoxic effects on the cells, whereas the cell viability decreased significantly at concentrations of 50 μM and above (Fig. S2). Based on previous studies[14] and our experimental results, two intermediate concentrations (12.5 μM and 25 μM) were selected for subsequent experiments.

To investigate whether POA can inhibit inflammation in intestinal epithelial cells *in vitro*, POA was added simultaneously with LPS and co-cultured for 24 h. As shown by RT-qPCR analysis, the expression levels of proinflammatory cytokines, including *IL-8*, *IL-6*, and *IL-1β*, were markedly upregulated in the model group at the gene expression level. The addition of POA significantly reduced the LPS-induced overexpression of these proinflammatory cytokines (Fig. 2A). Moreover, in LPS-induced cells, the suppression of proinflammatory cytokine expression was more pronounced with 12.5 μM POA intervention compared with 25 μM POA intervention (Fig. S2). In addition, the expression levels of two inflammation-related enzymes, *COX-2* and *iNOS*, were evaluated. The results indicated that the POA intervention effectively retarded the LPS-induced upregulation of COX-2 and iNOS at both gene and protein levels (Fig. 2B and C). LPS can activate the key receptor *TLR4*, thereby directly driving the gene expression of core inflammatory mediators through downstream signaling pathways. POA inhibited *TLR4* activation, thereby attenuating the progression of inflammation (Fig. 2D).

3.3 POA affected mitochondrial function and oxidative stress

To investigate whether POA modulates intracellular oxidative stress levels, we measured intracellular ROS levels and SOD activity. A significantly elevated ROS level was observed in the LPS group, indicating increased oxidative stress. POA treatment significantly reduced this increase (Fig. 3A). Conversely, SOD activity was markedly lower in the LPS group than in the CON and POA groups (Fig. 3B). Additionally, confocal microscopy was used to assess mitochondrial superoxide levels. Compared with the LPS group, the POA group exhibited significantly weaker red fluorescence (Fig. 3C), suggesting that POA intervention effectively mitigated the excessive generation of mitochondrial ROS induced by LPS.

Measurements of cathepsins—key effector and regulatory molecules linking immunity, cellular stress, and tissue destruction—revealed that LPS induced the upregulation of cathepsin B (*CTSB*) and significant downregulation of cathepsin L (*CTSL*), without exerting a significant effect on cathepsin D. More importantly, POA attenuated the aberrant upregulation of *CTSB* but did not significantly affect *CTSL* or *CTSD* levels (Fig. 3D).

3.4 POA repaired lysosomal damage

Lysosomal content was assessed using a fluorescent semi-quantitative method. As shown in Fig. 4A, the lysosomal red fluorescence intensity was markedly diminished in the LPS group relative to the control group. In contrast, the fluorescence intensity in the POA group remained comparable to that in the control group. LPS induction led to lysosomal damage, resulting in a reduction in lysosomal content. POA treatment attenuated the extent of lysosomal damage. Under conditions of lysosomal damage, the activities of lysosomal enzymes were altered accordingly. ACP and NAG activities were significantly reduced in the model group. Moreover, POA maintained the stability of ACP activity to a certain extent but did not significantly improve NAG activity (Fig. 4B and C).

Additionally, we analyzed changes in the expression of genes (*ATP6V1A*, *ATP6V1C2*, and *ATP6V1H*) associated with the

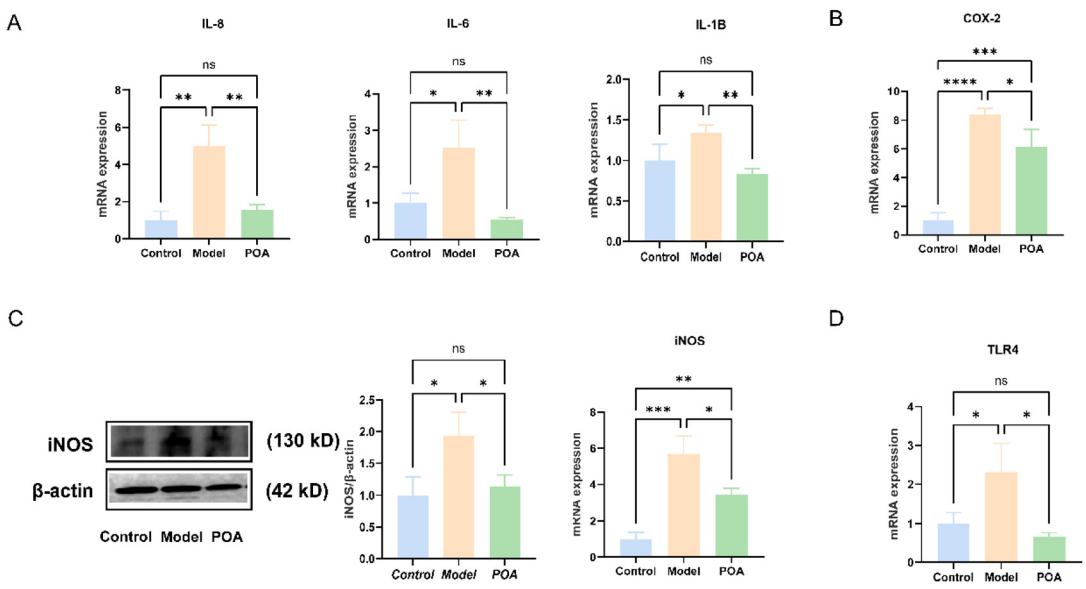

Fig. 2 Effects of POA on cellular inflammation. (A) Expression of inflammatory genes. (B) Expression of the *COX-2 gene*. (C) Expression of the *iNOS* gene and protein. (D) Expression of the *TLR4* gene. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

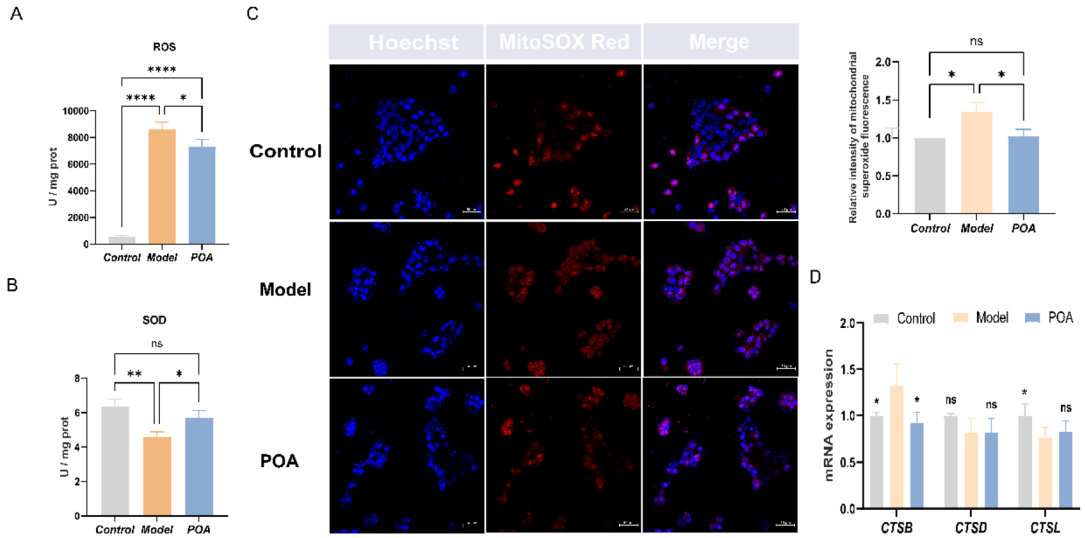

Fig. 3 Effects of POA on cellular oxidative stress and mitochondrial dysfunction. (A) Intracellular ROS levels. (B) SOD activity. (C) Nuclear and mitochondrial superoxide staining. (D) Expression of the cathepsin genes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

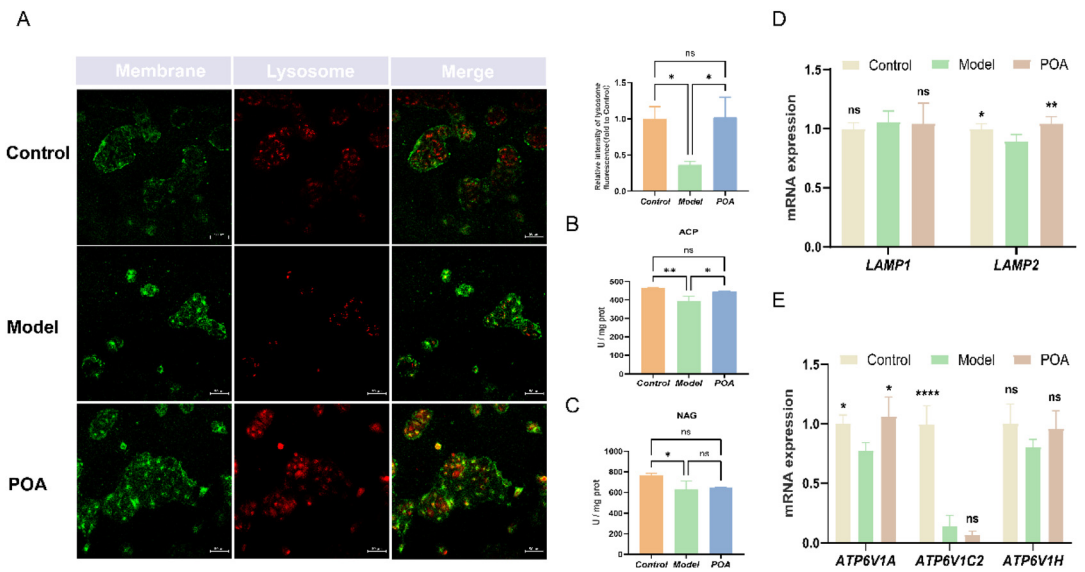

Fig. 4 Effects of POA on cellular lysosomal damage. (A) Staining of the cell membrane and lysosome. (B) ACP activity. (C) NAG activity. (D) Expression of the lysosome-associated membrane protein genes. (E) Expression of the lysosomal V-ATPase pump-related genes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

V-ATPase pump, the most critical protein complex for maintaining lysosomal function. The results showed that LPS induced a downregulation of the *ATP6V1A* and *ATP6V1C2* levels. Following the POA intervention, the *ATP6V1A* expression was partially restored compared with the LPS-treated group (Fig. 4E). Simultaneously, RT-qPCR analysis revealed that LPS-treated Caco-2 cells exhibited reduced expression of the lysosomal membrane protein gene *LAMP-2* compared with untreated Caco-2 cells (Fig. 4D). These data indicate that lipopolysaccharides induce damage to cellular lysosomes, compromising lysosomal membrane permeability, while POA mitigated this damage to a certain extent.

3.5 POA improved inflammation by modulating the NRF2/TBK1 pathway

To elucidate the mechanism by which POA ameliorates LPS-induced inflammation in Caco-2 cells, we reviewed the literature to identify target genes associated with lysosomal damage.[15–19] Next, we quantified the transcription levels of these target genes. The results revealed that LPS induction modulated the expression of multiple genes, including *HSPA1B*, *SLAMF7*, *NRF2*, and *TBK1*, without affecting other genes. POA improved the expression of *NRF2* and *TBK1* but did not exert ameliorative effects on the expression of *HSPA1B* and

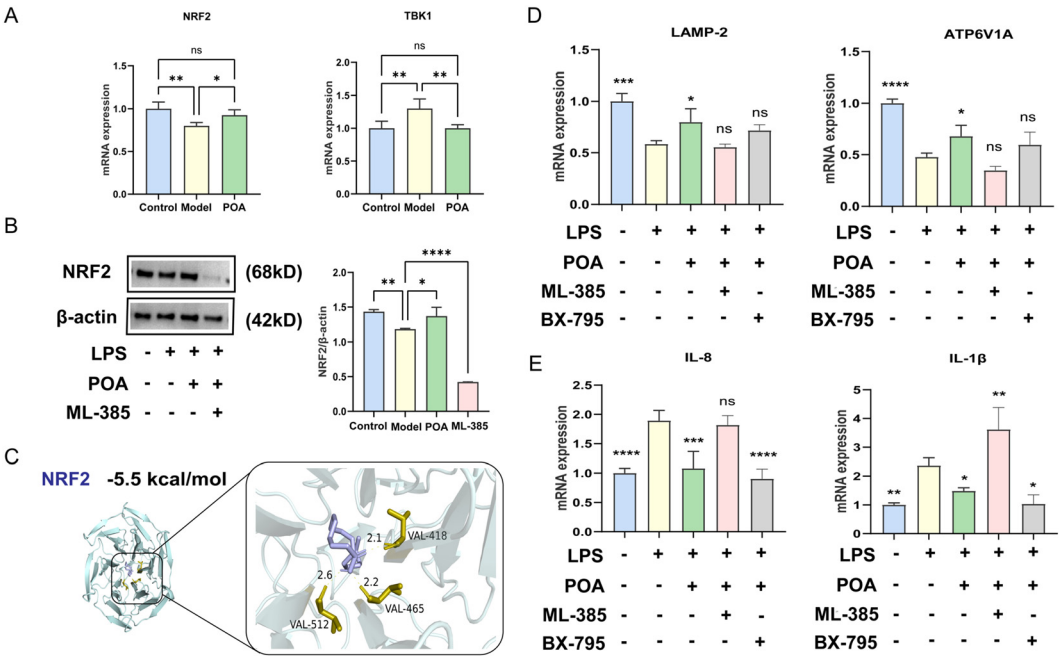

Fig. 5 POA improved inflammation and lysosomal damage by modulating the NRF2/TBK1 pathway. (A) Expression of *TBK1* and *NRF2* genes (without inhibitors). (B) Expression of NRF2 protein. (C) Molecular docking. (D) Expression of *ATP6V1A* and *LAMP-2* genes (with inhibitors). (E) Expression of inflammatory genes (with inhibitors). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

SLAMF7 genes (Fig. 5A). These findings suggest that POA may mitigate lysosomal damage through the modulation of *NRF2* and *TBK1*.

Then, the NRF2 inhibitor ML-385 was added to POA-treated cells and changes in NRF2 protein expression were determined. The results showed that ML-385 attenuated the effect of POA (Fig. 5B). Subsequently, molecular docking was used to predict the interaction modes and binding sites of POA with its protein targets. POA exhibited binding interactions with NRF2, forming hydrogen bonds with VAL-418, VAL-512, and VAL-465 (Fig. 5C). POA also formed a hydrogen bond with GLN-250 of TBK1 (Fig. S3).To further validate the relationship between these targets and lysosomal damage, ML-385 and BX-795 were added to the POA treatment group. The results showed that the NRF2 inhibitor downregulated the expression of the lysosomal membrane protein *LAMP-2* and V-type ATPase genes (Fig. 5D). To strengthen the association between lysosomal damage and inflammation, ML-385 and BX-795 were added again to POA treatment group, and the expression levels of inflammatory genes were measured. The NRF2 inhibitor upregulated the expression of inflammatory genes (Fig. 5E). These results indicate that POA intervention may retard LPS-triggered inflammatory responses through the NRF2/TBK1/lysosomal axis.

4. Discussion

Macadamia nut oil is rich in POA, which accounts for approximately 20% of its fatty acid composition. The oxidation of macadamia nuts is accompanied by changes in POA content. POA is a 16-carbon MUFA belonging to the omega-7 series. Previous studies have reported that POA exerts beneficial effects on the production of inflammatory cytokines and chemokines in BMDM cells.[20] Additionally, POA reduced the levels of VEGF, a growth factor associated with endothelial dysfunction and atherosclerosis formation.[21] In this research, to investigate the protective role of POA, we evaluated its effects on LPS-induced oxidative stress and cytokine expression. The results demonstrated that POA attenuated LPS-induced oxidative stress levels, promoted the recovery of mitochondrial function, mitigated lysosomal damage, reduced aberrant cathepsin expression, and inhibited the production and release of inflammatory mediators. Additionally, POA may alleviate lysosomal damage through the NRF2/TBK1 signaling pathway, thereby suppressing inflammatory response (Fig. 6). These findings enhance the understanding of the functions of POA and confirm its potential in reducing oxidative stress and improving lysosomal function, thereby alleviating cellular inflammation.

Mitochondria, as the main source of ROS, are highly sensitive to oxidative damage.[22] Continuously elevated ROS levels impair mitochondrial function, induce hypoxic conditions, and lead to cell death. Moreover, abnormalities in mitochondrial morphology and function impair the electron transport chain and oxidative phosphorylation. This impairment triggers excessive ROS generation, thereby exacerbating cellular damage and creating a vicious cycle.[23] Thus, mitochondrial dysfunction is frequently associated with excessive ROS production and oxidative stress.[24] It has been demonstrated that the interaction of ROSs with key intracellular macromolecules

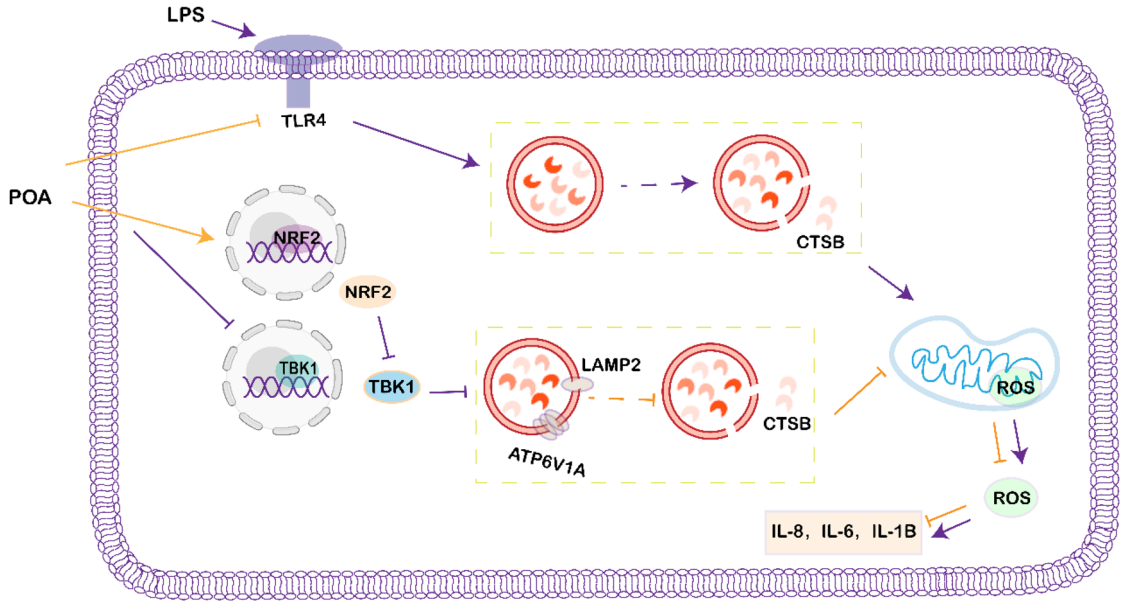

Fig. 6 The mechanistic diagram of POA regulating inflammation.

—proteins, nucleic acids, and lipids—can lead to the disruption of vital cellular processes.[25] Additionally, numerous studies support the notion that oxidative stress and inflammation are interdependent and interconnected processes.[26,27] ROSs activate the AP-1 signaling pathway, subsequently inducing the expression of pro-inflammatory genes.[24] At low levels, ROSs directly modify disulfide bonds within the AKT molecule. This oxidative modification liberates AKT from phosphatase 2A (PP2A) inhibition, ultimately resulting in its transient activation. Increased ROS levels activate NF-κB *via* promoting its phosphorylation and nuclear translocation. This subsequently promotes pro-inflammatory outcomes by modulating critical inflammatory mediators and leukocyte activity.[28] Consistent with these findings, a noticeable increase in mitochondrial superoxide and ROS levels was detected in the inflammation model group compared with the control group. After POA intervention, both levels were significantly reduced.

Mitochondria and lysosomes are two important organelles, and the interaction between them is essential for maintaining normal cell metabolism.[29] Cathepsins regulate a wide array of complex physiological reactions. They serve as pivotal molecules linking innate and adaptive immunity, tissue homeostasis and destruction, and acute inflammation and chronic disease progression. Dysregulation of cathepsins leads to mitochondrial dysfunction, thereby promoting the development of human inflammatory and metabolic diseases.[30,31] Mitochondrial dysfunction can be initiated when lysosomal damage leads to the release of proteases such as cathepsins into the cytosol, propagating a cascade of cellular injury.[32] Mitochondrial dysfunction further contributes to inflammatory responses.[33] As a predominant lysosomal cysteine protease, cathepsin B plays a critical role in mediating mitochondrial damage. In this study, we observed that CTSB expression was upregulated following LPS induction, while POA intervention attenuated this effect. This finding suggests that the intervention effect of POA may be associated with the regulation of lysosomal function. Previous studies have reported that fatty acids can regulate lysosomal function. PUFA deficiency may lead to the degradation of glucosinolates and sulfated glycosaminoglycans (sGAGs), processes associated with autophagy activation and generalized lysosomal hyperfunction.[34] Oleic acid (OA) regulates lysosomal dysfunction through TFEB, thereby alleviating hepatic lipotoxic injury.[35] In contrast, palmitic acid (PA) induces a significant decrease in LAMP-1 and LAMP-2 expression, resulting in lysosomal damage.[36] LAMP-1 and LAMP-2 are essential for maintaining lysosomal integrity.[32,37] Based on these findings, we hypothesized that POA can improve lysosomal function. Subsequently, we validated this hypothesis through biological experiments. In this study, we discovered that LPS exposure caused lysosomal damage, whereas POA exposure alleviated this damage, as evidenced by improvements in key parameters including acidification (*ATP6V1A*, *ATP6V1C2*, and *ATP6V1H*), enzyme activities (NAG and ACP), membrane permeability (*CTSB*), and overall abundance (LysoTracker). Moreover, ACP binds tightly to the lysosomal membrane, with certain isoenzymes functioning as "integral membrane proteins". The integrity and fluidity of the lysosomal membrane are crucial for its normal function. The aforementioned results may be related to the POA's ability to maintain the normal expression of lysosomal membrane proteins, thereby preserving the lysosomal membrane structure. Furthermore, these findings build upon previous studies that identified cathepsins as key molecular links connecting lysosomes and mitochondria.[38] In our study, LPS exposure induced mitochondrial dysfunction and elevated ROS levels. Compared to LPS treatment alone, the addition of POA attenuated excessive ROS production. This effect may be related to the fact that POA altered the abnormal expression of CTSB.

Lysosomal damage mediated mitochondrial dysfunction, thereby triggering oxidative stress responses that subsequently promoted inflammatory responses.[39] POA alleviated lysosomal damage and reduced inflammatory responses. Next, we reviewed the literature to identify target proteins associated with lysosomal damage. In this study, the transcription levels of these candidate genes were assessed. *In vitro* cellular experiments validated that POA treatment increased NRF2 expression while decreasing TBK1 expression. Consistent with the literature reports, TBK1 activation was crucial for lysosomal damage.[15] When NRF2 signaling is impaired, lysosomal acidification and lysosomal enzyme function are disrupted.[18] Previous studies have reported that NRF2 knockdown leads to excessive activation of the STING/TBK1 pathway, thereby exacerbating inflammation and liver injury.[40] Therefore, POA may mitigate lysosomal damage by acting on NRF2 target proteins. The NRF2 inhibitor ML-385 was added to POA-treated cells and changes in NRF2 protein expression were determined. The results showed that ML-385 attenuated the effect of POA, further supporting the above conclusion. In order to explore possible binding modes and binding sites, we performed molecular docking of POA and NRF2. The results showed that there was a covalent interaction between POA and NRF2. This correlation is mainly caused by valine, and POA can connect the three valines (VAL-418, VAL-512 and VAL-465) of NRF2 through hydrogen bonds. To further investigate the association between lysosomal damage and inflammation, ML-385 was administered to the POA group, and the transcription levels of lysosomal membrane protein genes and inflammatory cytokine genes were measured. NRF2 inhibition downregulated the expression of *LAMP-2* and *ATP6V1A* and upregulated the expression of inflammatory genes. The above results indicate that POA intervention may attenuate LPS-induced oxidative stress through the NRF2/TBK1/lysosomal axis, thereby alleviating inflammation (Fig. 6). Overall, our findings indicate that POA supplementation ameliorates lysosomal damage and mitochondrial dysfunction while exerting significant anti-inflammatory effects in the gut cells. This study confirms the association between POA and lysosomal function, thereby enhancing the understanding of the biological activities of POA.

5. Conclusion

In summary, this study has systematically elucidated the changes in palmitoleic acid during macadamia nut oxidation and investigated the *in vitro* anti-inflammatory mechanism of palmitoleic acid. Through the integration of existing literature, molecular docking techniques, and experimental validation, the NRF2/TBK1 pathway has been identified as a pivotal mechanism mediating the anti-inflammatory effects of POA. Exogenous addition of POA alleviated lysosomal damage and reduced cellular oxidative stress, thereby significantly suppressing LPS-induced inflammatory responses. After the addition of NRF2 inhibitors, the ability of POA to modulate inflammatory responses in Caco-2 cells was almost completely lost. This emphasized the crucial role of this target in mediating the anti-inflammatory effects of POA. These findings provide a novel perspective on the anti-inflammatory effects mediated by monounsaturated fatty acids. Taking measures to inhibit the oxidation of macadamia nuts and thus protect palmitoleic acid in the oil will be the focus of our future research, and this also represents a limitation of the present study.

Author contributions

Wen-Hui Sun: writing – original draft, conceptualization, data curation, and visualization. Ya-Zhuan Li: writing – original draft and data curation. Wen Dai: data curation and visualization. Chin-Ping Tan: writing – review and editing. Xiao-Song Rong: supervision and writing – original draft. Yong-Jiang Xu: supervision, writing – original draft, writing – review and editing, and project administration.

Conflicts of interest

The authors declare no competing financial interest.

Abbreviations

POA	Palmitoleic acid
MUFAs	Monounsaturated fatty acids
SFAs	Saturated fatty acids
LPS	Lipopolysaccharide
CTSB	Cathepsin B
CTSL	Cathepsin L
CTSD	Cathepsin D
ACP	Acid phosphatase
NAG	β-*N*-Acetylglucosaminidase
ROS	Reactive oxygen species
IBD	Inflammatory bowel disease

Data availability

The data supporting this article have been included as part of the supplementary information (SI). All external links (including any DOIs or repository links) are functional. See DOI: https://doi.org/10.1039/d6fo00826g.

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