Oxidative Medicine and Cellular Longevity

Oxidative Stress and Cardiometabolic Diseases: Role of the Nuclear Receptor Superfamily

Lead Guest Editor: Jun Pu
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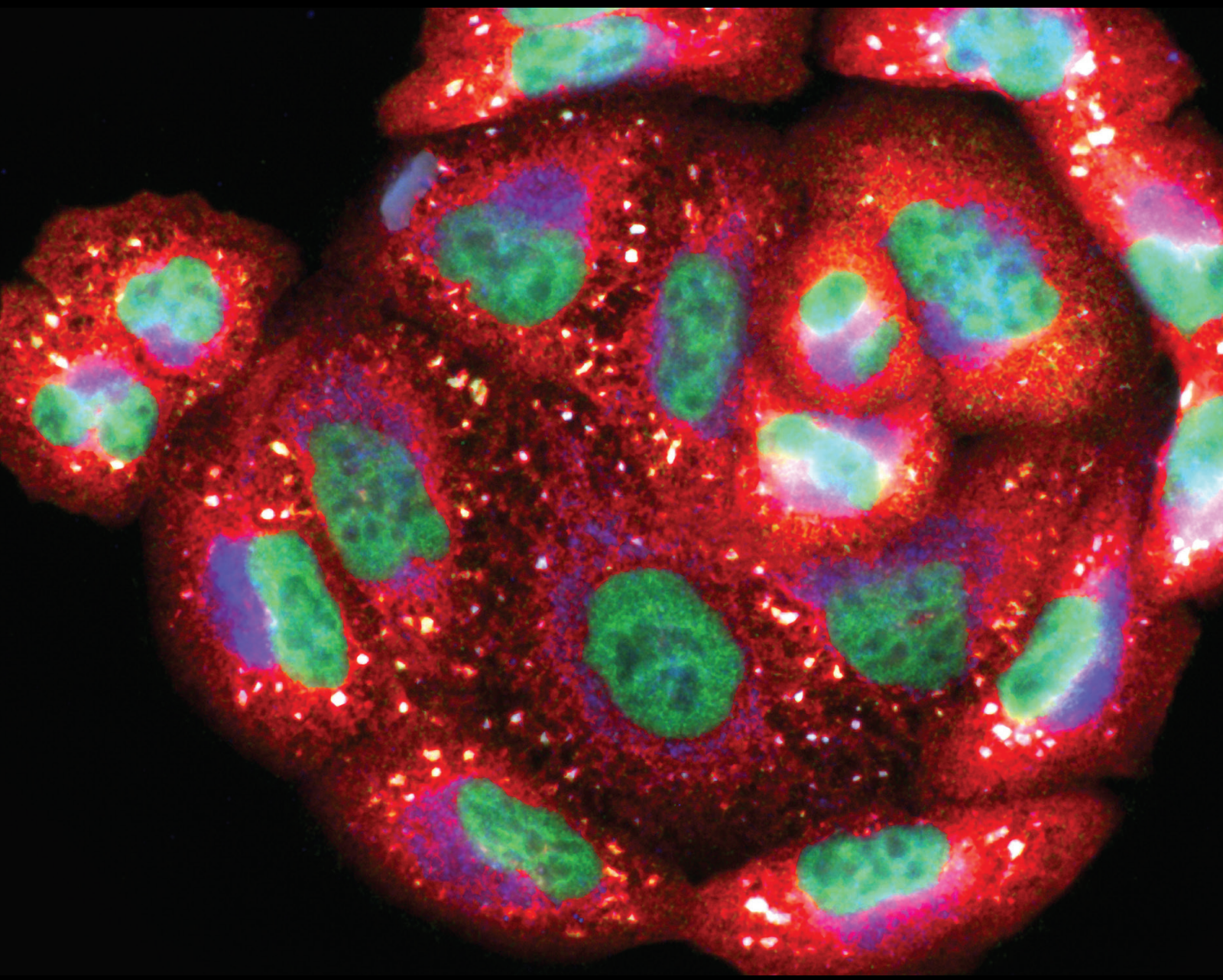

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Research Article

Apolipoprotein (a)/Lipoprotein(a)-Induced Oxidative-Inflammatory α7-nAChR/p38 MAPK/IL-6/RhoA-GTP Signaling Axis and M1 Macrophage Polarization Modulate Inflammation-Associated Development of Coronary Artery Spasm

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Objective. Apolipoprotein (a)/lipoprotein(a) (Lp(a)), a major carrier of oxidized phospholipids, and α7-nicotinic acetylcholine receptor (α7-nAChR) may play an important role in the development of coronary artery spasm (CAS). In CAS, the association between Lp(a) and the α7-nAChR-modulated inflammatory macrophage polarization and activation and smooth muscle cell dysfunction remains unknown. *Methods*. We investigated the relevance of Lp(a)/α7-nAChR signaling in patient monocyte-derived macrophages and human coronary artery smooth muscle cells (HCASMCs) using expression profile correlation analyses, fluorescence-assisted cell sorting flow cytometry, immunoblotting, quantitative real-time polymerase chain reaction, and clinicopathological analyses. *Results*. There are increased serum Lp(a) levels (3.98-fold, $p = 0.011$) and macrophage population (3.30-fold, $p = 0.013$) in patients with CAS compared with patients without CAS. Serum Lp(a) level was positively correlated with high-sensitivity C-reactive protein ($r^2 = 0.48$, $p < 0.01$), IL-6 ($r^2 = 0.38$, $p = 0.03$), and α7-nAChR ($r^2 = 0.45$, $p < 0.01$) in patients with CAS, but not in patients without CAS. Compared with untreated or low-density lipoprotein- (LDL-) treated macrophages, Lp(a)-treated macrophages exhibited markedly enhanced α7-nAChR mRNA expression ($p < 0.01$) and activity ($p < 0.01$), *in vitro* and *ex vivo*. Lp(a) but not LDL preferentially induced CD80+ macrophage (M1) polarization and reduced the inducible nitric oxide synthase expression and the subsequent NO production. While shRNA-mediated loss of α7-nAChR function reduced the Lp(a)-induced CD80+ macrophage pool, both shRNA and anti-IL-6 receptor tocilizumab suppressed

Lp(a)-upregulated α7-nAChR, p-p38 MAPK, IL-6, and RhoA-GTP protein expression levels in cultures of patient monocyte-derived macrophages and HCASMCs. *Conclusions*. Elevated Lp(a) levels upregulate α7-nAChR/IL-6/p38 MAPK signaling in macrophages of CAS patients and HCASMC, suggesting that Lp(a)-triggered inflammation mediates CAS through α7-nAChR/p38 MAPK/IL-6/RhoA-GTP signaling induction, macrophage M1 polarization, and HCASMC activation.

1. Introduction

Studies in patients with coronary artery spasm (CAS), an intense coronary vasoconstriction, have substantially contributed to the understanding of myocardial ischemia [1–3]. CAS is an inflammatory disease characterized by elevated peripheral monocyte count [4], plasma levels of high-sensitivity C-reactive protein (hs-CRP) [5, 6], and interleukin- (IL-) 6 [7], while enhanced serum IL-6 and hs-CRP levels attenuate endothelial nitric oxide (NO) synthase activity and suppress NO production [8–10], leading to CAS development. IL-6, a primary determinant of hepatic production of CRP, not only contributes to the inflammatory response but also has been shown to be associated with endothelial dysfunction and consequently plays an important role in CAS [2].

Cholinergic signaling and nicotinic acetylcholine receptors (nAChRs) have recently gained focus in cardiovascular morbidity and mortality [11, 12]. The α7-nAChRs, encoded by the CHRNA7 gene, are of relevance in inflammation [9] and expressed by mononuclear inflammatory cells, including monocytes and monocyte-derived macrophages [13, 14]. We previously demonstrated that the activation of monocytic α7-nAChR exacerbated oxidative stress and promoted CAS through a p38 mitogen-activated protein kinase- (MAPK-) dependent mechanism [9], which is in line with the studies of the nicotinic atherogenic effects of nAChR [11, 12]. Furthermore, the excessive vascular smooth muscle cell (VSMC) contraction in CAS has been related to Ras-homologous (Rho) A GTPase/Rho-kinase (ROCK1, ROCK2) pathway, which can induce inflammation and oxidative stress [15, 16]. Notably, α7-nAChR is involved in the activation of the Rho GTPase pathway and the downstream signaling pathway in VSMCs [17], which may lead to CAS. Despite the important role of α7-nAChR in CAS, the development of molecular modulators of α7-nAChR and the associated therapeutic translational research in CAS has remained largely unknown [18]. Therefore, we further explored probable druggable molecular modulators of α7-nAChR underlying CAS development.

On the other hand, lipoprotein(a) (Lp(a)), a major carrier of oxidized phospholipids, has been observed to play an important role in CAS development and related myocardial infarction [19, 20]. Moreover, elevated Lp(a) level is a causal risk factor for coronary artery disease (CAD) and may similarly play an important role in other atherothrombotic disorders [21]. While atherogenic lipoproteins significantly modulate vascular tone, oxidized Lp(a) is more potent than oxidized LDL [22]. The disproportionately large impact of Lp(a) on cardiovascular disease risk compared with low-density lipoprotein implies that additional pathogenic pathways need to be considered. Moreover, despite the implication of α7-nAChR and Lp(a) in CAS and shared molecular mediators [7–9, 20] between them, it remains undetermined whether CAS is due to the 2 conditions sharing common inflammatory factors or whether shared inflammatory factors provide the link. There is increasing evidence that blood monocyte function may be changed by dyslipidemia [23]. Hence, the present study examined probable interaction and modulatory loop between Lp(a) and monocytic α7-nAChR in patients with CAS.

The agonists of the nAChR include nicotine, epibatidine, choline [24], and the endogenous agonist acetylcholine, which has been used to provoke and diagnose CAS during coronary angiography [3]. Because nicotine is the major reinforcing component and psychoactive drug of tobacco smoke [25], both nicotine in the tobacco smoke [26] and endogenous acetylcholine may contribute to the CAS development. We previously demonstrated that activation of the monocytic α7-nAChRs modulates oxidative stress and inflammation-associated development of CAS via a p38 MAP-kinase signaling-dependent pathway [9]. In addition, positive interactions exist among CRP, hemoglobin, and platelet in women with CAS, but not in men [27]. While hemoglobin is a modifier in CAS development in women, platelet count is an independent risk factor for men [27]. Because hemoglobin levels and platelet counts have been found to vary substantially according to age, gender, and race/ethnicity [28, 29], population-based studies are needed for hemoglobin and platelet to differentiate the causality from predisposing factors through biomarkers to the occurrence of CAS. On the other hand, the potential role for platelet-released factors in CAS would not necessarily imply an abnormality in platelet function [30]. The primary abnormality might be increased sensitivity of VSMCs to normal levels of vasoconstrictive agents such as thromboxane A2 [30]. Furthermore, CAS could be the primary event and the CAS-induced "stasis" in the coronary artery might lead to an increase in the numbers of circulating platelet aggregates, suggesting a potential causal role of platelets in CAS [30]. Loscalzo et al. have demonstrated that intravenous administration of nitroglycerin inhibits cyclic blood flow responses caused by periodic platelet thrombus formation in stenosed canine coronary arteries [31], and N-acetylcysteine markedly potentiates the inhibition of platelet aggregation by nitroglycerin [32]. While platelet resistance to NO is aggravated during acute symptomatic CAS episodes, mast cell activation and damage to both vasculature and platelets also occur [33]. N-Acetylcysteine, via release of H2S, reverses platelet resistance to NO and terminates glycocalyx shedding during symptomatic CAS crises, suggesting that H2S donors may correct the pathophysiological anomalies [33]. Forman et al. [34] revealed that more mast cells were found in the adventitia of the involved artery in

patients with CAS than in patients with CAD and sudden death but without CAS or in normal controls who died in accidents, which raised the possibility that products derived from mast cells (histamine, prostaglandin D2, and leukotrienes C4 and D4) may partly mediate CAS in some patients. These important studies suggest that the loss of NO effect predisposes coronary vessels towards microthrombosis, endothelial damage, and ongoing inflammation. Notably, Kounis syndrome is defined as the coexistence of acute coronary syndromes including CAS, acute myocardial infarction, and stent thrombosis, with allergic or hypersensitivity conditions associated with mast cell and platelet activation [35]. Collectively, although endothelial cell dysfunction might favor the induction of CAS, other factors may also be involved in the pathogenesis of CAS. On the other hand, the involvement of the α7-nAChR in the development of atherosclerosis is yet an expanding field, as both atheroprotective and proatherogenic roles are attributed to the stimulation of α7nAChRs, and their role in the genesis and progression of atheromatous plaque is still under debate. In vivo studies revealed both anti- and proatherogenic effects [36]. In vitro studies indicated that the activation of α7-nAChRs regulates the function of different cells involved in a variety of pathways linked to plaque progression [36]. Stimulation of vascular α7-nAChRs contributes to angiogenesis and proliferation of VSMCs and may promote atherogenesis [36]. High Lp(a) concentrations (>50 mg/dL) are associated with significantly increased risk of myocardial infarction in all populations except Arabs and Africans [37]; however, the relationship between Lp(a) and α7-nAChR remains largely unknown. We recently demonstrated that garcinol attenuates Lp(a)-induced oxidative stress and inflammatory cytokine production in ventricular cardiomyocyte through α7-nAChR-mediated inhibition of the p38 MAPK and NF-κB signaling pathways in a mouse model of myocardial infarction [38], suggesting an important role of α7-nAChR and its downstream signaling mechanisms in regulating Lp(a)-induced cardiomyocyte apoptosis and inflammation. In addition, CAS is considered as one of the causes of acute coronary syndrome with plaque rupture [39] or myocardial infarction with nonobstructive coronary artery [40]. Therefore, while scarce data are available on Lp(a) in relation to α7-nAChR and CAS, more studies are warranted before Lp(a)/α7-nAChR-mediated responses could be considered as a therapeutic target for CAS.

Clinically, CAS is characterized by transient myocardial ischemia followed by reperfusion [41]. Repeat ischemia-reperfusion can stimulate proinflammatory responses from coronary VSMCs [42], which may increase the risk of developing CAS [43]. As part of the pathogenesis of atherosclerosis, inflammatory signals stimulate proinflammatory responses from macrophages and VSMCs [44], which in turn may exacerbate CAS. Taken together, these observations suggest a role for coronary VSMC-related inflammation in CAS development. On the other hand, Lp(a) acts in a species-specific manner on cultured rat and human VSMCs [45]. To date, no information is available concerning the effects of Lp(a) on monocyte-derived macrophages in patients with CAS and human coronary artery smooth muscle cells (HCASMCs). We, therefore, analyzed the protein expression levels of Lp(a) and α7-nAChR in the monocytes of patients with CAS. Furthermore, we investigated the effects of Lp(a) on monocyte-to-macrophage differentiation and polarization based on CD80 or CD206 positivity and α7-nAChR-dependent activation of the p38 MAPK signaling in monocyte-derived macrophages and primary HCASMCs.

2. Material and Methods

2.1. Cells, Compounds, and Reagents. The primary HCASMCs (ATCC® PCS-100-021™, American Type Culture Collection, Manassas, VA, USA) were cultured in smooth muscle cell growth medium 2 (#C-22062, PromoCell GmbH, Heidelberg, Germany), and all patient monocyte-derived macrophage cells were cultured in the RPMI-1640 culture media (Sigma-Aldrich Corporation, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 μg/mL streptomycin, and 50 U/mL penicillin in 5% CO_2 humidified atmosphere incubator at 37°C to 98%-100% confluence. Cells were subcultured and culture media changed every 48 h. Human CRP (#C1617, Sigma-Aldrich Corporation, St. Louis, MO, USA), human IL-6 (#407652, purity ≥ 95% by SDA-PAGE, Sigma-Aldrich Corporation, St. Louis, MO, USA), human low-density lipoprotein (LDL) (#LP2, purity ≥ 95% by SDA-PAGE, Sigma-Aldrich Corporation, St. Louis, MO, USA), methyllycaconitine (MLA), a selective and potent antagonist of the α7-nAChR, (351344-10-0, caymanchem, USA), and anti-IL-6 receptor antibody (tocilizumab) were also obtained from Sigma-Aldrich. Stock solutions of tocilizumab were prepared at a concentration of 10 mM in double-distilled water (ddH_2O) and stored at -20 °C until use. Methylergonovine (Methergine®) was obtained from Novartis (Novartis Pharmaceuticals Corp., Basel, Switzerland) and nitroglycerin from G. Pohl-Boskamp (Millisrol®; G. Pohl-Boskamp, Hohenlockstedt, Germany).

2.2. Study Population. This prospective cohort study was carried out with the approval of the Taipei Medical University Joint Institutional Review Board (approval number: TMU-JIRB N201903036). All patients provided signed informed consent regarding use of their blood in scientific research, and the study was compliant with the guidance of the Declaration of Helsinki for biomedical research involving human subjects. A total of 64 patients (45 men and 19 women), who had chest pain and suspected ischemic heart disease on noninvasive tests, undergoing diagnostic coronary angiography with or without established CAS, but without obstructive stenosis, from July 2017 to March 2019 were enrolled in this study. Study subjects were stratified into control ($n = 32$) and CAS ($n = 32$) groups. Among them, 9 from the control and 10 CAS subjects were active smokers. Inclusion criteria for patients with CAS included spontaneous chest pain at rest associated with ST-segment elevation or depression on electrocardiogram that was relieved by sublingual administration of nitroglycerin, no angiographic evidence of obstructive CAD after intracoronary nitroglycerin administration, and a positive result on

intracoronary methylergonovine provocation testing. CAS was not induced in the remaining 32 patients (non-CAS, control), which consisted of patients who presented with atypical chest pain, no angiographic evidence of obstructive CAD, and negative results on intracoronary methylergonovine provocation testing (no CAS). Atypical chest pain was defined as spontaneous chest pain at rest and/or provoked by exertion that was eased by sublingual administration of nitroglycerin [46] but not linked with ST-segment change on resting electrocardiogram. Exclusion criteria included the presence of obstructive CAD, coronary microvascular spasm [47], inflammatory manifestations probably associated with noncardiac diseases (e.g., infections and autoimmune disorders), liver disease/renal failure (serum creatinine level > 2.5 mg/dL), collagen disease, malignancy, and loss of blood samples. None of our patients had allergic or hypersensitivity conditions.

2.3. Data Collection. For this study, patients' demographic, anthropometric, and laboratory data as well as details of their comorbidities, medicine use, habits, and number of functional units were collected. Current smoking was defined as having smoked a cigarette within 3 weeks of cardiac catheterization. Diabetes mellitus was diagnosed when the fasting glucose level was ≥126 mg/dL on >2 occasions or was defined from dietary treatment and/or medical therapy. Baseline seated blood pressure was derived from the mean of 6 readings obtained during the first 2 office visits at 2 weeks apart. Hypertension was defined as a blood pressure > 140/90 mmHg on >2 occasions or receiving antihypertensive treatment.

2.4. Spasm Provocation Test Protocol. The standard Judkins technique was employed for coronary angiography [8]. Nitrates and calcium antagonists were withdrawn for ≥24 h before the procedure. Left ventricular ejection fraction was calculated using Simpson's method. Obstructive CAD was defined as a ≥50% decrease in luminal diameter after administration of intracoronary nitroglycerin [9]. If no obstructive CAD was discovered, intracoronary methylergonovine (Methergine®; Novartis, Basel, Switzerland) was given stepwise (1, 5, 10, and 30 μg) first into the right coronary artery and subsequently into the left coronary artery. CAS was defined as a >70% reduction in luminal diameter compared with postintracoronary nitroglycerin, with associated angina and/or ST depression or elevation [9]. Provocation testing was stopped with an intracoronary injection of 50–200 μg of nitroglycerin (Millisrol®; G. Pohl-Boskamp, Hohenlockstedt, Germany).

2.5. Isolation of Monocytes from Human Peripheral Blood Mononuclear Cells. Following overnight fasting just before coronary angiography, blood was collected in BD Vacutainer® CPT™ mononuclear cell preparation tubes (#362753, BD Diagnostics, Sparks Glencoe, MD, USA) and centrifuged at $1800 \times g$ at room temperature for 20 min. After removing the upper layer containing plasma and Ficoll™Hypaque™ and without disturbance of the red lowest layer, the opaque interface containing the mononuclear cells was carefully transferred to a new 50 mL conical tube. The mononuclear cells were washed twice with phosphate-buffered saline (PBS). Subsequently, monocytes were isolated using Invitrogen™ Dynabeads® CD14 superparamagnetic beads (#11149D, Thermo Fisher Scientific Inc., Waltham, MA, USA) and magnetic activated cell sorting (MACS). Isolated monocyte purity was assessed by flow cytometry of fluorescein-labeled CD14-positive cells. Finally, isolated monocytes were resuspended in Invitrogen™ TRIzol™ reagent (#15596026, Thermo Fisher Scientific Inc., Waltham, MA, USA), and the total RNA extract was stored at −80°C until use.

2.6. Differentiation of Monocytes to Macrophages. For differentiation of monocytes to macrophages, monocytes were enriched by allowing adherence in 5% CO_2 atmosphere incubator at 37°C for 2 h. While nonadherent cells with the supernatant were carefully discarded, adherent monocytes were carefully washed with prewarmed 15 mL PBS and washing solution aspirated. Thereafter, the ImmunoCult™-SF macrophage medium (#10961, STEMCELL Technologies Inc., Kent, WA, USA) was used for monocyte differentiation to macrophages following manufacturer's instruction. M1 macrophages were obtained by treatment with 10 ng/mL lipopolysaccharides (LPS) (#L2630, Sigma-Aldrich Corporation, St. Louis, MO, USA) and 5 U/mL human recombinant interferon- (IFN-) γ (#I17001, Sigma-Aldrich Corporation, St. Louis, MO, USA), while M2 macrophages were obtained by treatment with 20 ng/mL human recombinant IL-4 (#I4269, Sigma-Aldrich Corporation, St. Louis, MO, USA). Then, the M1 or M2 cells were incubated in 5% CO_2 atmosphere incubator at 37°C for 24 h, supernatant discarded, and fresh RPMI-1640 (Sigma-Aldrich Corporation, St. Louis, MO, USA) supplemented with 5% FBS, 2 mM glutamine, 50 μg/mL streptomycin, 50 U/mL penicillin, and 0.05 mM β-mercaptoethanol (β-ME) for expansion.

2.7. Lp(a) Isolation and Detection. Lp(a) was isolated from pooled plasma sample from healthy subjects ($n = 7$) with Lp(a) > 50 mg/dL. The isolation of Lp(a) from the pooled plasma was carried out strictly as previously described [48]. The concentration of Lp(a) was measured using the Human Lipoprotein an ELISA Kit (ab212165, Abcam plc., Cambridge, UK); the lower limit of detection was 17.2 ng/mL.

2.8. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). RNA extraction and qRT-PCR were performed as previously described [8]. The specific primer sequences used are as follows: human α7-nAChR (forward: 5′-GGC AGA TAT CAG TGG CTA TAT C-3′, reverse: 5′-CTT CAT TCG CAG GAA CC-3′); human IL-6 (forward: 5′-CCA GCT ATG AAC TCC TTC TC-3′, reverse: 5′-GCT TGT TCC TCA CAT CTC TC-3′); and human GAPDH (forward: 5′-ACC CAC TCC TCC ACC TTT GA-3′, reverse: 5′-CTG TTG CTG TAG CCA AAT TCG T-3′). The PCR product amplification procedure is as follows:1 cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 57°C for 1 min, and 72°C for 1 min. Postamplification melting curve analysis was performed to verify amplicon accuracy. GAPDH served as the internal control.

2.9. Proinflammatory IL-6 Cytokine Assay and Fluorescence-Activated Cell Sorting (FACS) Flow Cytometry. After 15 min fixation of 2×10^6 cells in 4% formaldehyde (pH 7.5) at room temperature, the cells were incubated in blocking solution containing 1% bovine serum albumin (#A7030, Sigma-Aldrich Corporation, St. Louis, MO, USA) and 1% goat serum in PBS for 30 min, followed by 2 h incubation in primary antibodies against α7-nAChR (1 : 100, Abcam) or Lp(a) (1 : 100, #ab125014, Abcam plc.). After washing twice with PBS, cells were incubated in PBS/fluorescein isothiocyanate-conjugated IgG solution for 1 h and then cell surface marker expression levels analyzed using the BD FACSCalibur™ modular analyzer (BD Biosciences, San Jose, CA, USA). The concentration of intracellular IL-6 was measured using the sandwich Human Interleukin-6 DuoSet ELISA Development Immunoassay Kit (#DY206, R&D Systems Inc., Minneapolis, Minnesota); the lower limit of detection was 0.70 pg/mL.

2.10. NO Analytical Measurements. The NO level was detected using ready kits (Abcam, Co., Cambridge, MA, USA; ab65328) following the manufacturer's protocols. Briefly, the nitrate is catalyzed with nitrate reductase into nitrite. Later, total nitrite is converted into a deep purple azo compound (azo chromophore) with Griess Reagents. The absorbance of the purple azo compound is measured at 540 nm, where the absorbance of the azo compound is directly proportional to NO production. The detection limit of the assay is approximately 1 nmol nitrite/well or 10 μM.

2.11. α7-nAChR Luciferase Activity Reporter Assay. Both monocyte-derived macrophages were stably transfected with α7-nAChR luciferase reporter plasmids (GeneCopoeia Inc., Rockville, MD, USA) containing an Invitrogen™ pcDNA™3.1$^{(+)}$-derived neomycin-resistant thymidine kinase (TK) cassette (pCHRNA7neo-luc) (#V79020, Thermo Fisher Scientific Inc., Waltham, MA, USA). The viable transfected (resistant) cells were expanded and subcultured severally (12 passages) in neomycin containing RPMI-1640. For the α7-nAChR luciferase activity reporter assay, after pretreating cells with 500 nM LDL or Lp(a) for 30 min, treatment media were decanted, cells were washed with ice-cold 1x PBS thrice, lysed with passive lysis 5x buffer from the luciferase assay system (#E1941, Promega Corporation, Fitchburg, WI, USA), and then, the cell lysates were used to determine the α7-nAChR luciferase activity following the manufacturer's protocol.

2.12. Western Blot Assays. Western blot analyses were performed according to standard protocol [49] using the following antibodies against: α7-nAChR (ab216485; 1 : 1000), p38 (ab31828; 1 : 1000), p-p38 (phospho T180+Y182) (ab4822; 1 : 1000), IL-6 (ab9324; 1 : 1000), inducible NO synthase (ab3523; 1 : 1000), and GAPDH (ab9484; 1 : 10,000), purchased from Abcam (Abcam plc., Cambridge, UK), and RhoA (#2117; 1 : 1000), RhoA-GTP (#8820; 1 : 1000), ROCK1 (#4035; 1 : 1000), ROCK2 (#9029; 1 : 1000), t-MBS (#2634; 1 : 1000), and p-MBS (#3040; 1 : 1000) from Cell Signaling Technology (Cell Signaling, Danvers, MA, USA) in Supplementary Table S1. The protein bands were pictured using enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, NJ, USA) and quantified using ImageJ software (https://imagej.nih.gov/ij/).

2.13. Immunofluorescence. Briefly, 5×10^3 cells were plated into 6-well plates containing 1–2 mL medium. After 24–36 h, Lp(a) was added and cells were incubated for another 48 h. For nicotinic acetylcholine receptor staining, cells were incubated with either Alexa Fluor 488 α-bungarotoxin, a competitive antagonist to nAChR (α-BTX, green fluorescence; B-13422, Thermo Fisher Scientific Inc., Waltham, MA, USA), or α7-nAChR antibody (red fluorescence; 21379-1-AP, Thermo Fisher Scientific Inc., Waltham, MA, USA). For the staining of nuclei, sections and/or cells were incubated with 50 μg/mL DAPI in PBS and then mounted with an antifade mounting medium (0.1 M Tris, pH 9.0).

2.14. Short Hairpin RNA (shRNA) Transfection. The shRNA specifically targeting CHRNA7 was using the Nicotinic Acetylcholine Receptor Alpha 7 Human shRNA Plasmid Kit (Locus ID 1139) (ORIGENE, Rockville, MD, USA). For CHRNA7 silencing, HCASMCs grown to ~80% confluence were transfected with CHRNA7 shRNA. Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) was used for the transfection of the shRNAs according to the manufacturer's protocol. The total RNA or protein was extracted 48 h after transfection and used for the western blot analyses.

2.15. Statistical Analysis. All assays were performed at least 3 times in triplicates. Continuous variables are expressed as the mean ± standard deviation (SD) or median (2-quartile), and positively skewed variables were log-transformed for subsequent intergroup Student's t-test. Discrete variables were expressed as numbers and percentages (%) of the total sample and comparisons made using the two-tailed Fisher's exact test, while categorical variables were analyzed using the χ^2 test. Correlation between the levels of serum Lp(a) and hs-CRP, IL-6, or α7-nAChR expression in patients with CAS were determined by Spearman correlation. The coefficient of determination (r^2) and associated p value were calculated using linear regression analysis. All statistical analyses were performed with SPSS (IBM Corp., released 2017, IBM SPSS Statistics for Windows, Version 25.0, Armonk, NY: IBM Corp.). A two-tailed p value < 0.05 was considered statistically significant.

3. Results

3.1. Study Cohort Baseline Characteristics. A total of 64 patients were enrolled in the study (median age = 58.0 years ; interquartile range, 49.2–65.0 years). Patients in the CAS group compared with patients in the control group had significantly higher Lp(a) levels ($p = 0.011$) (Table 1). The leukocyte, monocyte, and macrophage count and hs-CRP values were also significantly higher in the CAS group than in the control group. Single-vessel spasm was the most common finding in the CAS patients, and spasm was provoked mostly in the right coronary artery. No difference in medication use before coronary angiography was observed between

Table 1: Baseline characteristics of the study cohort.

(a)

	Controls ($n = 32$)	CAS ($n = 32$)	p value
Age (years)	62.5 (54.0–66.5)	53.5 (46.3–63.8)	0.07
Male sex, n (%)	24 (75)	21 (66)	0.41
Body mass index (kg/m^2)	27.3 ± 4.2	27.2 ± 3.4	0.86
Current smoker, n (%)	9 (28)	10 (31)	0.78
Diabetes mellitus, n (%)	9 (28)	3 (9)	0.06
Hypertension, n (%)	17 (53)	11 (34)	0.13
Left ventricular ejection fraction, %	65 ± 6	66 ± 6	0.74
Total cholesterol (mg/dL)	173 ± 33	191 ± 40	0.05
Triglyceride (mg/dL)	131 ± 100	170 ± 219	0.37
LDL cholesterol (mg/dL)	107 ± 26	119 ± 42	0.15
HDL cholesterol (mg/dL)	44 ± 16	43 ± 9	0.83
Lipoprotein(a) (mg/dL)	102 ± 49	406 ± 164	0.011
Peripheral leukocytes (/mm^3)	6066 ± 1671	6994 ± 1943	0.045
Monocytes (/mm^3)	465 ± 183	568 ± 220	0.046
Macrophage (/mm^3)	120 ± 16	397 ± 147	0.013
Lymphocytes (/mm^3)	1673 ± 615	2036 ± 886	0.06
Hemoglobin (g/dL)	13.9 ± 1.4	14.0 ± 1.8	0.85
Hematocrit (%)	41.3 ± 6.4	40.4 ± 5.2	0.54
Platelet (×10^3/mm^3)	217 ± 57	242 ± 62	0.1
hs-CRP (mg/L*)	0.80 (0.50–2.28)	1.04 (1.00–2.03)	0.044
Coronary artery with lesion			
Left anterior descending artery, n (%)		4 (11)	
Left circumflex artery, n (%)		3 (9)	
Right coronary artery, n (%)		25 (78)	
Number of spastic arteries			
One-vessel spasm, n (%)		27 (84)	
Two-vessel spasm, n (%)		4 (14)	
Three-vessel spasm, n (%)		1 (4)	

(b)

Medications	A	D	A	D	A	D
β-Blockers, n (%)	21 (66)	13 (41)	21 (66)	6 (19)	1.0	0.06
Calcium-channel blockers, n (%)	16 (50)	18 (56)	11 (34)	31 (97)	0.21	<0.001
Angiotensin receptor blocker, n (%)	17 (53)	19 (59)	11 (34)	12 (38)	0.13	0.08
Nitrates, n (%)	16 (50)	5 (16)	10 (31)	13 (41)	0.13	0.03
Statins, n (%)	6 (19)	13 (41)	11 (34)	20 (63)	0.16	0.08
Aspirin, n (%)	30 (94)	26 (81)	30 (94)	31 (97)	1.0	0.05
Diuretics, n (%)	2 (6)	2 (6)	0 (0)	0 (0)	0.15	0.15

Values are expressed as *mean* ± *SD* or median (interquartile range). A: before angiography; *CAS: coronary artery spasm; D: at discharge; hs-CRP: high-sensitivity C-reactive protein; LDL: low-density lipoprotein; HDL: high-density lipoprotein; Lp(a): lipoprotein(a). *Log-transformed values were used for the analyses.

the 2 groups. However, after coronary angiography, the number of patients being treated with calcium channel blockers and nitrates was significantly higher in the CAS group than in the control group (Table 1).

3.2. Lp(a) Levels Positively Correlate with Monocytic α7-nAChR Levels and Are Implicated in Inflammation-Associated CAS. Lp(a) levels were significantly higher in patients with CAS than in the control group ($p = 0.011$)

(Table 1, Figure 1(a)). There was a significantly positive correlation between Lp(a) and hs-CRP ($r^2 = 0.48$, $p < 0.01$) (Figure 1(b)) or IL-6 ($r^2 = 0.38$, $p = 0.03$) (Figure 1(c)). Furthermore, Lp(a) levels were positively correlated with α7-nAChR expression ($r^2 = 0.45$, $p < 0.01$) in patients with CAS (Figure 1(d)). However, the correlation between Lp(a) and IL-6 ($r^2 = 0.20$, $p = 0.27$) or α7-nAChR ($r^2 = 0.11$, $p = 0.54$) (Figures 1(e) and 1(f)) was markedly reduced in the control group.

3.3. The Apolipoprotein (a) Component of Lp(a) Interacts with and Induces α7-nAChR Expression in the Monocyte-Derived Macrophages of Patients with CAS. Compared with the untreated control or the treatment of 500 nM LDL, exposure to 500 nM Lp(a) induced ~10-fold increase ($p < 0.01$) in the expression levels of α7-nAChR mRNA in CAS patient monocyte-derived macrophages (Figure 2(a)). Lp(a) significantly induced higher α7-nAChR activity in the CAS monocyte-derived macrophages than in the LDL-treated or untreated cells (~126-fold, $p < 0.01$) (Figure 2(b)). In parallel assays, exposure to 100 nM–1 μM Lp(a) increased dose-dependently ($p < 0.001$) α7-nAChR mRNA expression level (Figure 2(c)) and luciferase activity (Figure 2(d)) in CAS monocyte-derived macrophages. Methyllycaconitine (MLA) dose dependently inhibited the Lp(a)-induced activation of α7-nAChR (Figure 2(e)). By using the Edu PyMoL molecular graphics system version 1.7.4, based on a clustering root mean squared deviation of 4.0 A, we demonstrated that the ligand-binding domain of pentameric α7-nAChR directly interacts with the kringle KIV_7, KIV_{10}, and KV domains of the apolipoprotein (a) component of Lp(a) with a geometric shape complementarity score of 21,956, a complex interface area of ~4525.80 $Å^2$, and an atomic contact energy of 372.29 kcal/mol (Figure 2(f)). Immunofluorescence image demonstrated Lp(a)-induced expression of α7-nAChR, which had a high correlation with α7-nAChR protein localization with α-BTX using fluorescent-protein tagging, original magnification ×200. DAPI (blue) served as a nuclear marker (Figure 2(g)).

3.4. Lp(a) Preferentially Induces Patient Monocyte-Derived Macrophage M1 Polarization. To evaluate if and how Lp(a) modulates macrophage activities in CAS, which has not been studied previously, we used CAD-specific functional genomics data from the National Center for Biotechnology Information Gene Expression Omnibus website (https://www.ncbi.nlm.nih.gov/geo/) to perform comparison of knowledge between CAD and CAS. Our reanalysis of the GSE9820/GPL6255/GDS3690 dataset ($n = 153$), which originally analyzed various circulating mononuclear cells from patients with severe CAD, revealed that the expression of α7-nAChR, CD163, CD206, and CD80 was the highest in macrophages, compared to the CD14+ resting monocytes, CD34+ stem cells, LPS-stimulated monocytes, or CD4+ T helper cells (Figures 3(a) and 3(b)). In addition, the median CD80+ macrophage population was 1.49-fold more than the CD206+ macrophages (Figures 3(c) and 3(d)). In our CAS monocyte-derived macrophages, 500 nM Lp(a) compared with the 500 nM LDL elicited a 3.16-fold stronger shift in fluorescence intensity of the CD80+ macrophage population ($p < 0.01$) (Figure 3(e)). However, exposure to neither LDL nor Lp(a) had any apparent effect on the median fluorescence intensity (MFI) of the CD206+ macrophage population (Figure 3(f)), suggesting that Lp(a) preferentially induces M1 macrophage polarization in patients with CAS.

3.5. Lp(a) Promotes Inflammation in Patient Monocyte-Derived Macrophages and HCASMCs by Inducing α7-nAChR-Dependent Activation of p38 MAPK Signaling. Exposure to Lp(a) significantly increased the macrophage expression of α7-nAChR, phosphorylated p38 (p-p38) MAPK, and IL-6 proteins in a dose-dependent and time-dependent manner (Figure 4(a)). A similar dose- and time-dependent induction of the expression of α7-nAChR/p38 MAPK/IL-6/RhoA-GTP was observed in HCASMCs (Figure 4(b)). To further understand the influence of Lp(a) on the Rho GTPase, we examined the activation of an important Rho GTPase, RhoA-GTP, and its downstream effector, ROCK. Lp(a) dose dependently activated ROCK (Figure 4(c)). While treatment with 1 μM Lp(a) enhanced the expression of α7-nAChR, p-p38 MAPK, and RhoA-GTP protein in unsilenced negative control HCASMCs by ~4-fold, ~3.2-fold, and ~2.5-fold, respectively, compared to the untreated cells, its enhancing effect on α7-nAChR, p-p38 MAPK, and RhoA-GTP protein level was significantly reduced by shRNA of CHRNA7 function (shCHRNA7) (Figure 4(d)), suggesting that the activation of p38 MAPK signaling in macrophages and HCASMCs by Lp(a) is α7-nAChR-dependent. On the other hand, while shCHRNA7 exhibited no suppressive effect on the CD80+ M1 macrophages in the absence of Lp(a), exposure to Lp(a) significantly enhanced the ability of shCHRNA7 to suppress the fluorescence intensity of CD80+ M1 cells (1.73-fold, $p < 0.01$) (Figure 4(d)). When patient monocyte-derived macrophages were treated with increasing concentrations of Lp(a) (0 to 2 μM), the NO production and the expression of inducible NO synthase were dose-dependently inhibited (Figures 4(e) and 4(f)).

3.6. The Human Monoclonal Antibody, Tocilizumab, Disrupts Lp(a)-Induced α7-nAChR/p38 MAPK Signaling by Attenuating Inflammation in Patient Monocyte-Derived Macrophages and HCASMCs. For the rational selection of therapeutic monoclonal antibodies to examine whether Lp(a) could be a potential target, tocilizumab, a 148 g/mol anti-IL-6 receptor monoclonal antibody with the chemical structure $C_{6428}H_{9976}N_{1720}O_{2018}S_{42}$, was used (Figure 5(a)). Treatment with 1.25 μM–10 μM tocilizumab exhibited no observable toxic effects to the patient monocyte-derived macrophages and HCASMCs (Figure 5(b)). Furthermore, exposure to 2.5 μM–10 μM tocilizumab dose dependently and significantly reversed the Lp(a)-induced upregulation of α7-nAChR, p-p38 MAPK protein expression levels in both patient monocyte-derived macrophages and HCASMCs, and additionally RhoA-GTP in HCASMCs (Figures 5(c) and 5(d)).

4. Discussion

In this translational work, we demonstrated that elevated serum Lp(a) levels were positively correlated with the levels

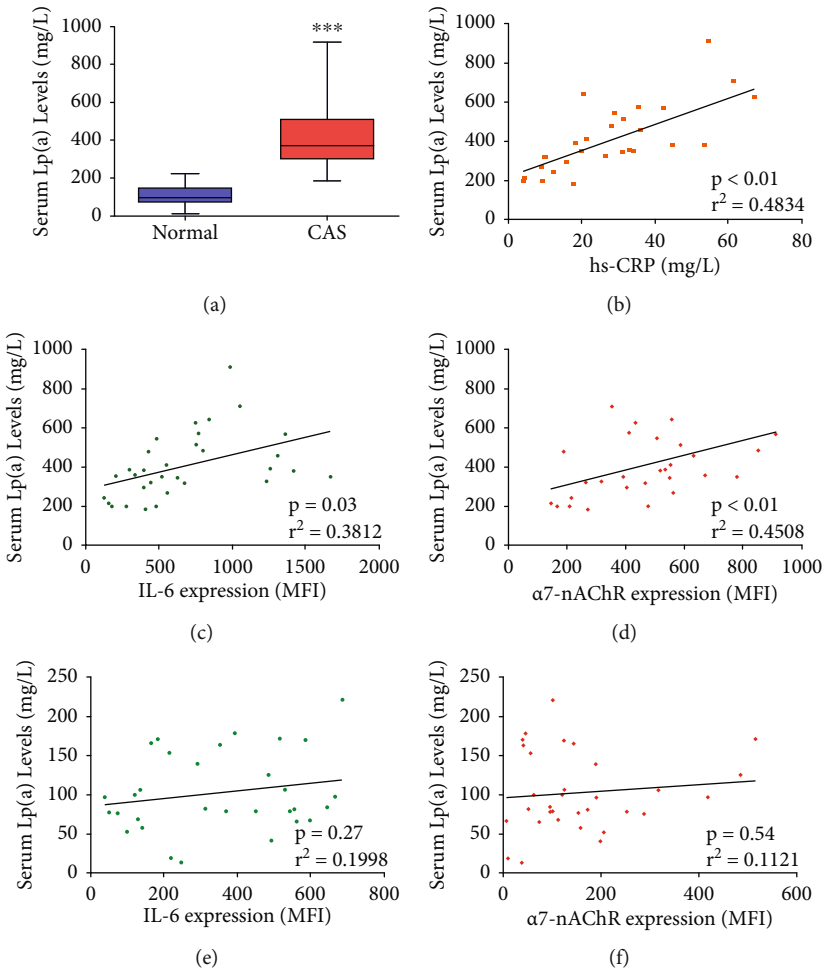
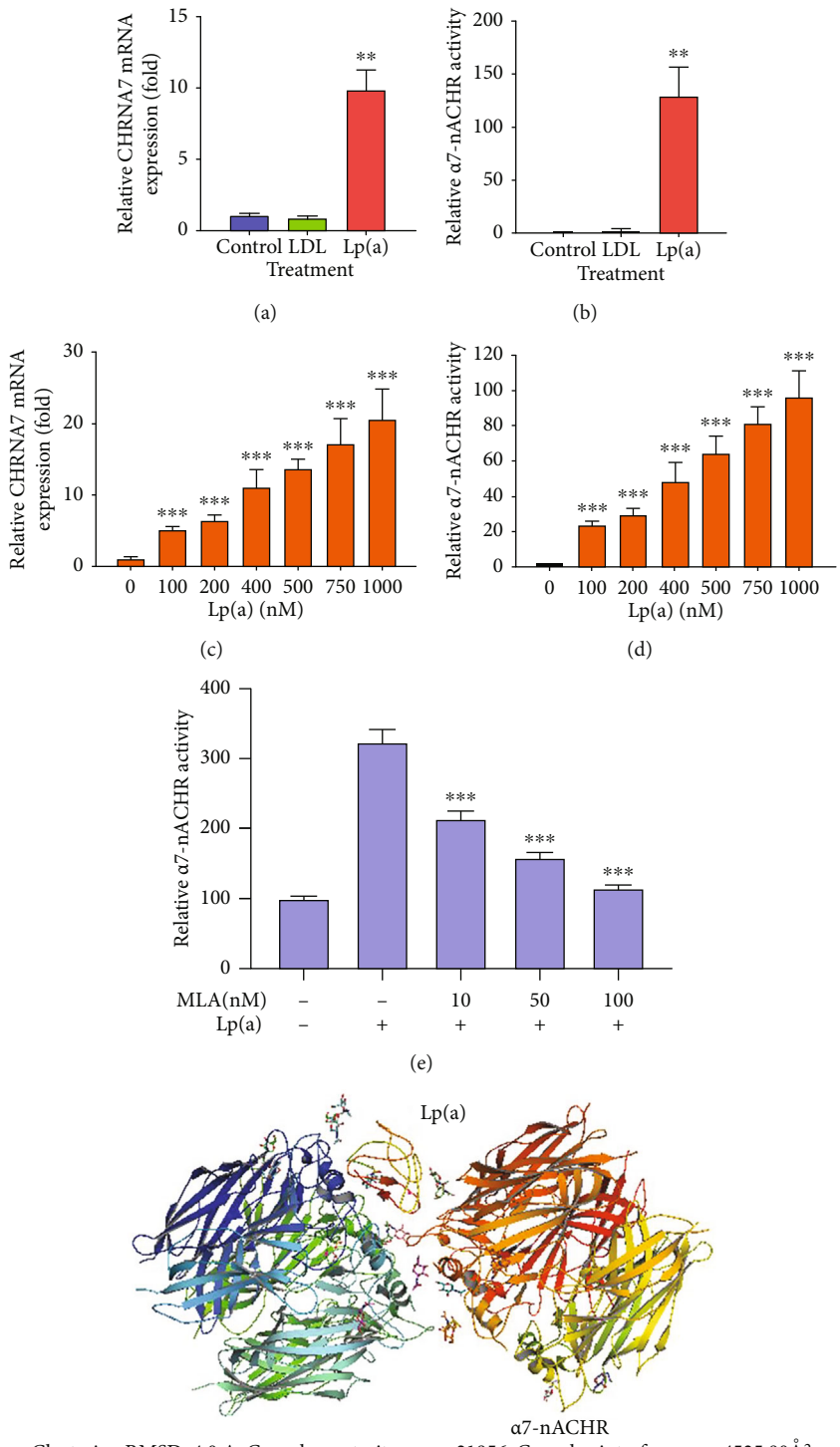


FIGURE 1: Lp(a) levels positively correlate with patient monocytic α7-nAChR levels and are implicated in inflammation-associated CAS. (a) Box and whisker plot of the differential serum Lp(a) levels in control subjects and patients with CAS. Spearman dots and regression line plots showing the correlation between serum Lp(a) levels and (b) hs-CRP levels, (c) IL-6 expression, or (d) monocytic α7-nAChR expression, in patients with CAS. Spearman dots and regression line plots showing the correlation between serum Lp(a) levels and (e) IL-6 expression or (f) monocytic α7-nAChR expression, in the controls. R^2: coefficient of determination; MFI: median fluorescence intensity; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

of CRP, IL-6, and monocytic α7-nAChR in CAS. Lp(a), through its apolipoprotein (a) chain, increased the expression of α7-nAChR in the monocyte-derived macrophages of patients with CAS and HCASMCs. Lp(a), in synergism with α7-nAChR, induced the proinflammatory activation of patient monocyte-derived M1 macrophages and HCASMCs through p38 MAPK/IL-6/RhoA-GTP. Lp(a) dose dependently reduced the inducible NO synthase expression level in monocyte-derived macrophages derived from CAS patients. Tocilizumab, a monoclonal antibody against IL-6 receptor, reduced Lp(a)-associated expression of α7nAChR-dependent activation of p38 MAPK, IL-6, and additionally RhoA-GTP in HCASMCs. To the best of our knowledge, this is the first study demonstrating the mechanism by which interactions of Lp(a)/monocyte/HCASMC and the subsequent expression of α7-nAChR/p38 MAPK/IL-6/RhoA-GTP contribute to VSMC dysfunction and the development of CAS.

The diagnosis of CAS by coronary angiography in the catheterization laboratory was not rare in the 1970s and 1980s. It soon became clear that CAS could occur in patients with atherosclerotic obstructive CAD [50, 51], nonobstructive CAD, or angiographically normal coronary arteries [3]. Hence, coronary lesions are dynamic [52]. Among the mechanisms of angina pectoris, CAS had long been considered the chief one [53], although it was yet unproved until 1940s when the observation of angina-associated fixed atherosclerotic stenosis at autopsy led to a revision of the theory that CAS may produce paroxysmal myocardial ischemia [54–56]. Lp(a) is now established as an independent risk factor for myocardial infarction and ischemic heart disease [57]. Genetic studies have provided strong evidence of causality; however, the disease-causing mechanism is to some extent still unknown [57]. Besides, considering CRP from a genetic perspective, investigators have found that specific polymorphisms in the CRP gene associate with plasma levels



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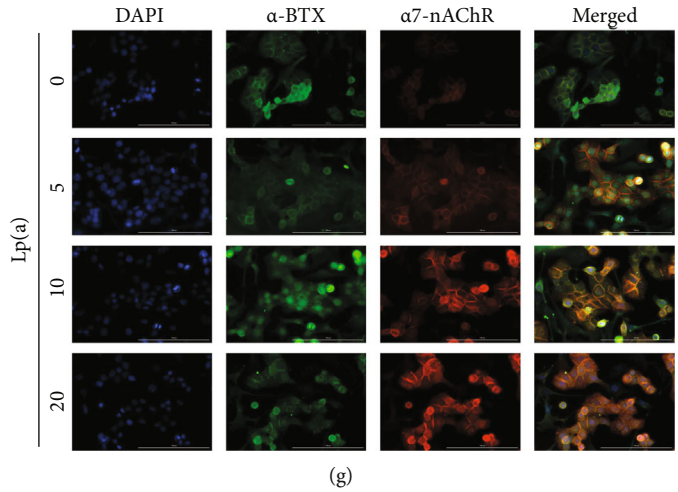


Figure 2: The apolipoprotein (a) component of Lp(a) interacts with and induces α7-nAChR expression in the monocyte-derived macrophages of patients with CAS. Graphical representation of the effect of 500 nM LDL or Lp(a) on the (a) relative expression of CHRNA7 mRNA or (b) relative luciferase reporter activity of α7-nAChR in the patient monocyte-derived macrophages. Histograms showing the effect of 100 nM–1000 nM Lp(a) on the (c) relative expression of CHRNA7 mRNA or (d) relative luciferase reporter activity of α7-nAChR in the patient monocyte-derived macrophages. (e) Methyllycaconitine (MLA) dose dependently inhibited the Lp(a)-induced activation of α7-nAChR. (f) Visualization of the direct molecular interaction between Lp(a) and α7-nAChR using the PyMoL molecular docking and visualization software. Complex formation criteria are indicated. (g) Immunofluorescence demonstrated induced expression of α7-nAChR after Lp(a) treatment and fluorescent-protein tagging showed a high correlation for protein localization with α-BTX. Original magnification ×200. DAPI (blue) served as a nuclear marker. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Lp(a): lipoprotein(a); CHRNA7: gene encoding α7-nAChR.

of CRP and predict future events, suggesting a potentially causal link between CRP and atherothrombosis [58]. While Lp(a) appears to be a largely inherited basis for premature atherogenesis, a very different process to that of CAS, the relationship between Lp(a) levels and inflammation, as reflected by elevated CRP levels, is somewhat unclear [57]. Previous studies have only investigated this association in highly selected groups such as patients with rheumatoid arthritis [59, 60] or patients undergoing hemodialysis [61]. Notably, Lp(a) levels from the Danish general population are minimally increased at increased levels of CRP [57]. Regarding the differential development of atherosclerosis and CAS, smoking and CRP have been demonstrated to be the 2 important risk factors for both diseases [3, 62, 63]. Therefore, it remains unknown whether they represent a risk continuum of atherosclerosis or completely different diseases. On the other hand, there is no clear boundary between stable and unstable angina and some overlap must be taken into account in the natural history of CAD [64]. As a result, dynamic stenosis can be caused by (1) "physiologic" increase of coronary tone, as in stable angina, (2) spasm, as in variant angina, and (3) thrombosis, usually combined with "physiologic" changes in tone or with spasm, or both, as in unstable angina [1]. Hence, this "atherosclerotic continuum" has been proposed as one of the most promising research target [65]. Furthermore, studies of genetic mutations or polymorphisms in the pathogenesis of CAS have been inconsistent [66]. Mutations or polymorphisms of the endothelial NO synthase gene [10, 67, 68] and polymorphisms of paraoxonase I gene [69] are significantly associated with CAS. However, NO gene polymorphisms are found in only one-third of patients [70]. Gene polymorphisms of other proteins that have been described in CAS contain adrenergic and serotoninergic receptors [71, 72], angiotensin-converting enzyme [73], and inflammatory cytokines [74, 75]. In a Japanese cohort analysis, the NADH/NADPH oxidase p22 phox gene is a predisposition locus in men, while stromelysin-1 and interleukin-6 genes are predisposition loci in women [76]. Although CAS itself is rarely familial and family history is not a risk factor for CAS, there is familial evidence of CAS and possible involvement of HLA-DR2 linkage disequilibrium with a susceptibility gene of CAS in a Japanese study [77]. Familial migraine and CAS in 2 siblings have also been reported [78]. As researchers at or associated with the National Human Genome Research Institute unlock the secrets of the human genome (the complete set of human genes), nearly all diseases have a genetic component [79]. In addition, the fluctuations of CAS activity appear with circadian variations in the short term and active and inactive phases in the long term [80], suggesting gene-environment interactions may exist in the development of CAS [76].

Although Lp(a) levels are largely determined by genetic factors, Lp(a) is also induced by mediators of the innate immunity in several chronic inflammatory diseases such as rheumatoid arthritis [81] and Crohn's disease [82] and in patients undergoing hemodialysis [61], which could be responsible for the increased cardiovascular risk found in such subjects [82]. Moreover, there is evidence that Lp(a) levels also increase with other conditions such as surgery or myocardial infarction [83], all possibly being associated with induction of the innate immunity. While in subjects with CAD there is a lack of correlation of Lp(a) with CRP [84], Lp(a) levels from the Danish general population are minimally increased at increased levels of CRP [57], which

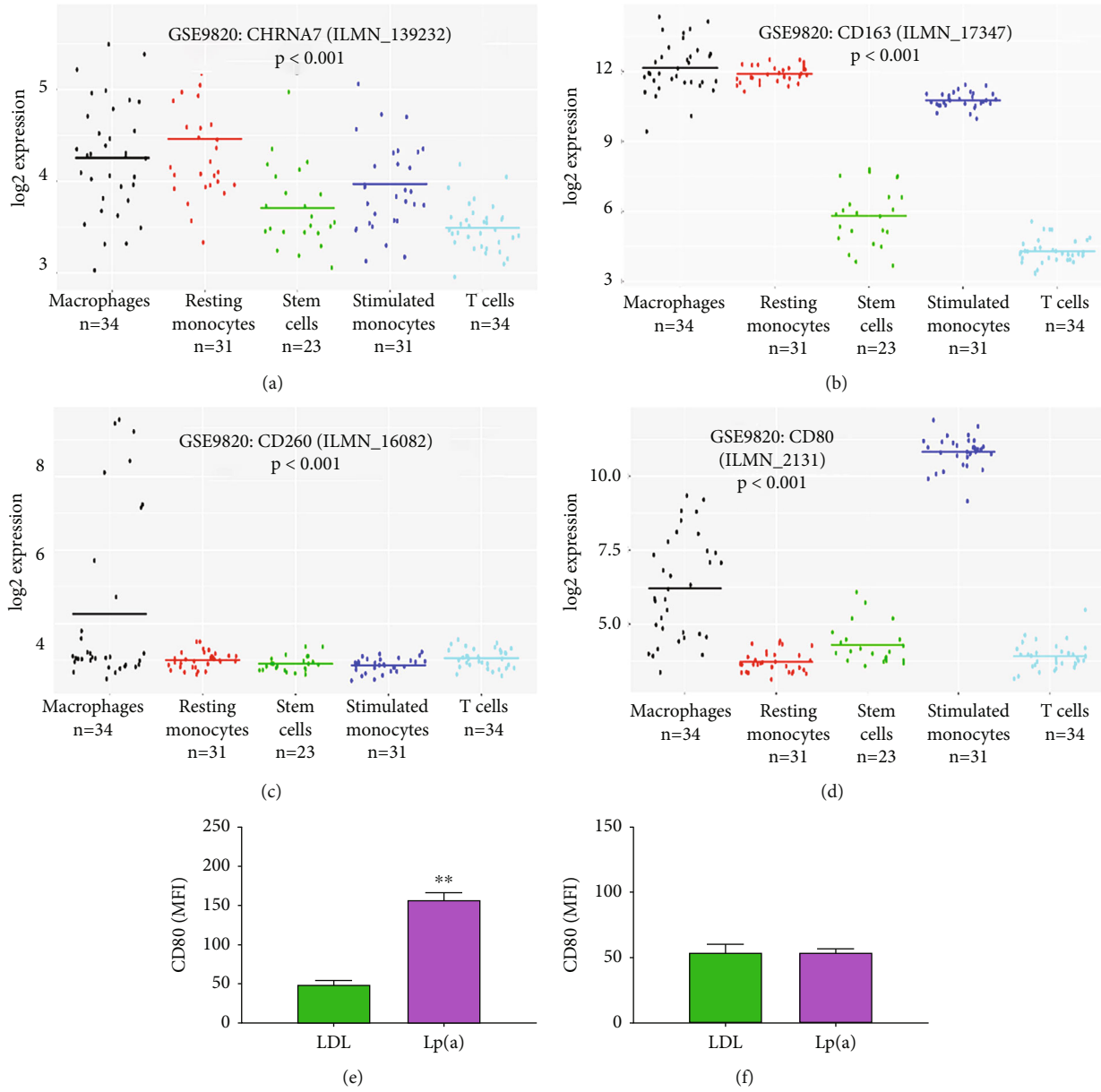
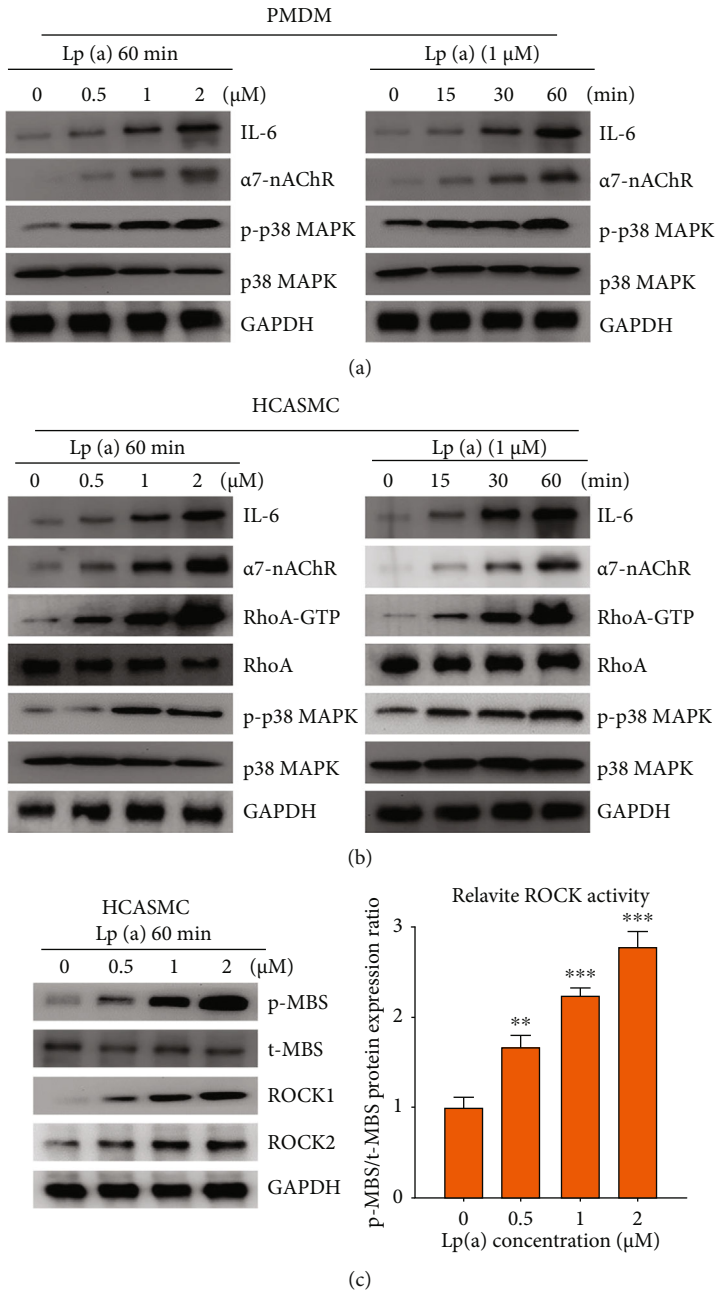


Figure 3: Lp(a) preferentially induces patient monocyte-derived macrophage M1 polarization. Dots and line plot showing the expression profile of (a) CHRNA7, (b) CD163, (c) CD206, or (d) CD80 in the macrophages, resting monocytes, stem cells, stimulated monocytes, and T cells of patients with CAD using the GSE9820/GPL6255/GDS3690 dataset, $n = 153$. Flow cytometry cell count polygons (*upper panel*) and histograms (*lower panel*) depicting the effect of treatment with 500 nM LDL or Lp(a) on the (e) CD80 median fluorescence intensity or (f) CD206 median fluorescence intensity of CAS monocyte-derived macrophages. Histogram colors are green for control antibody and purple for target antibody. The macrophages were exposed to either Lp(a) or LDL for 24 hours. LDL: low-density lipoprotein; PE: phycoerythrin. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

has been demonstrated to be an important risk factor for CAS. Because the correlation of Lp(a) with CRP is significant in our CAS patients, it suggests a different mechanism from CAD that leads to CAS. Lp(a) levels have been demonstrated to be more correlated to IL-6 compared with metabolic parameters, such as body mass index, insulin resistance, and triglyceride, indicating that Lp(a) serum concentrations are not only genetically determined but are also influenced by IL-6 [85]. Notwithstanding that the correlation of Lp(a) with α7-nAChR in humans has not been evaluated and requires further exploration, a positive feedback may exist between the 2 markers.

Lp(a) exerts both proatherogenic and prothrombotic effects, parts of which are primarily related to the LDL component whereas others are apolipoprotein (a)-dependent [86]. The competition with plasminogen for binding to endothelial cells and monocytes is mediated by apolipoprotein (a) [87], which supports a procoagulant/antifibrinolytic function for apolipoprotein (a), but there has been little progress in proving the pathophysiological relevance of the



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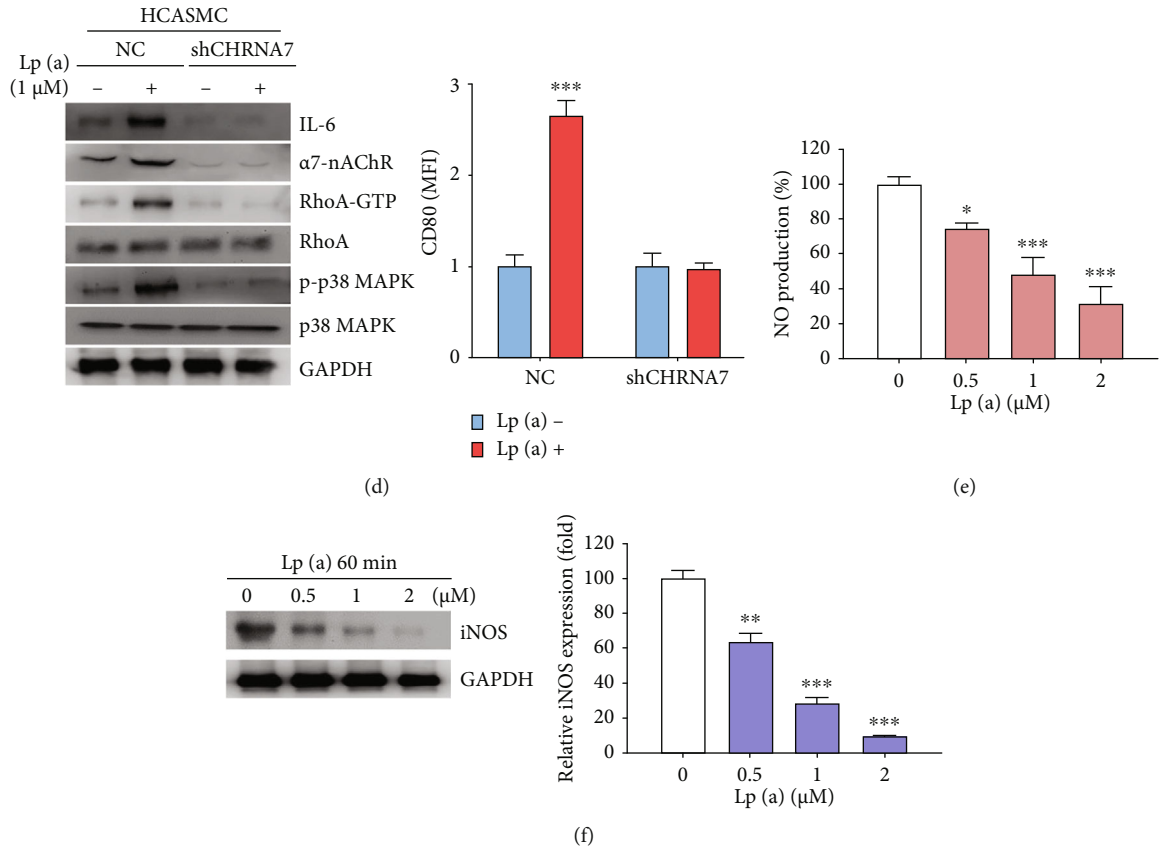

Figure 4: Lp(a) promotes inflammation in PMDMs and HCASMCs by inducing α7-nAChR-dependent activation of p38 MAPK signaling. (a) Representative western blot photo images showing the effect of treating patient monocyte-derived macrophages with 0.5 μM–2 μM Lp(a) for 60 min (*upper panel*) or with 1 μM Lp(a) at 15, 30, and 60 min time points (*lower panel*), on α7-nAChR, IL-6, p-p38 MAPK, and p38 MAPK protein expression levels. (b) Representative western blot photo images showing the effect of treating HCASMCs with 0.5 μM–2 μM Lp(a) for 60 min (*upper panel*) or with 1 μM Lp(a) at 15, 30, and 60 min time points (*lower panel*), on RhoA-GTP, RhoA, p-p38 MAPK, and p38 MAPK protein expression levels. (c) Representative western blot photo images showing that treating HCASMCs with 0.5 μM–2 μM Lp(a) for 60 min increases ROCK activity dose dependently. (d) Representative western blot images showing how shCHRNA7 affects the expression of RhoA-GTP, RhoA, α7-nAChR, p-p38 MAPK, and p38 MAPK in HCASMCs in the presence or absence of 1 μM Lp(a). Histograms show the effect of shCHRNA7 on CD80 MFI in HCASMCs in the presence or absence of 1 μM Lp(a). (e) PMDMs were treated with different concentrations of Lp(a) (0-2 μM) and the nitric oxide production was measured. (f) Lp(a) treatment dose dependently reduced the iNOS expression level in PMDMs. HCASMC: human coronary artery smooth muscle cell; MFI: median fluorescence intensity; PMDM: patient monocyte-derived macrophage; RhoA: Ras-homologous A; ROCK: Rho-kinase; shCHRNA7: α7-nAChR-targeting short hairpin RNA. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; GAPDH served as loading control.

binding in humans. In transgenic mouse aorta, elevated plasma level of apolipoprotein (a) or Lp(a) alone does not cause endothelial dysfunction [88], suggesting that either the plasma levels were too low or, more importantly, other factors are needed to observe the phenomenon, which seems to be supported by studies in adult humans [88] and children with familial hypercholesterolemia [89], where impaired endothelium-dependent vasodilatation was observed in the presence of multiple risk factors in addition to elevated plasma levels of Lp(a). Indeed, Lp(a) only exists in monkeys, apes, and humans [86]. While some species lack KV, human apolipoprotein (a) kringles are specialized domains to mediate ligand interactions [90], often with lysine-containing substrates, as it contains both KV and an intact lysine binding site in KIV_{10}. Therefore, human Lp(a) is exceptionally pathogenic. Even though a cognate Lp(a) receptor has not been identified, several other receptors interact with L(a) either via its apolipoprotein B, apolipoprotein (a), or oxidized phospholipid components [91]. The roles of these receptors, including lipoprotein receptors, toll-like and scavenger receptors, lectins, and plasminogen receptors, remain unclear [91]. While the uptake of oxidized low-density lipoprotein in macrophages is mediated through α7-nAChR [92], a similar interaction of Lp(a) with α7-nAChR in CAS patient monocyte-derived macrophages is observed. Obviously, mechanistic studies are required to determine the role of apolipoprotein (a)/Lp(a) in the interaction with α7-nAChR and the molecular basis for resultant increased risk in CAS development.

The provocative testing nowadays involves the use of ergonovine or acetylcholine [70]. In many countries, including Taiwan and the United States, only ergonovine is available for the diagnosis of CAS. Ergonovine, which is used to control postpartum uterine bleeding, was discovered in 1949 to provoke angina and was proposed in 1963 as a

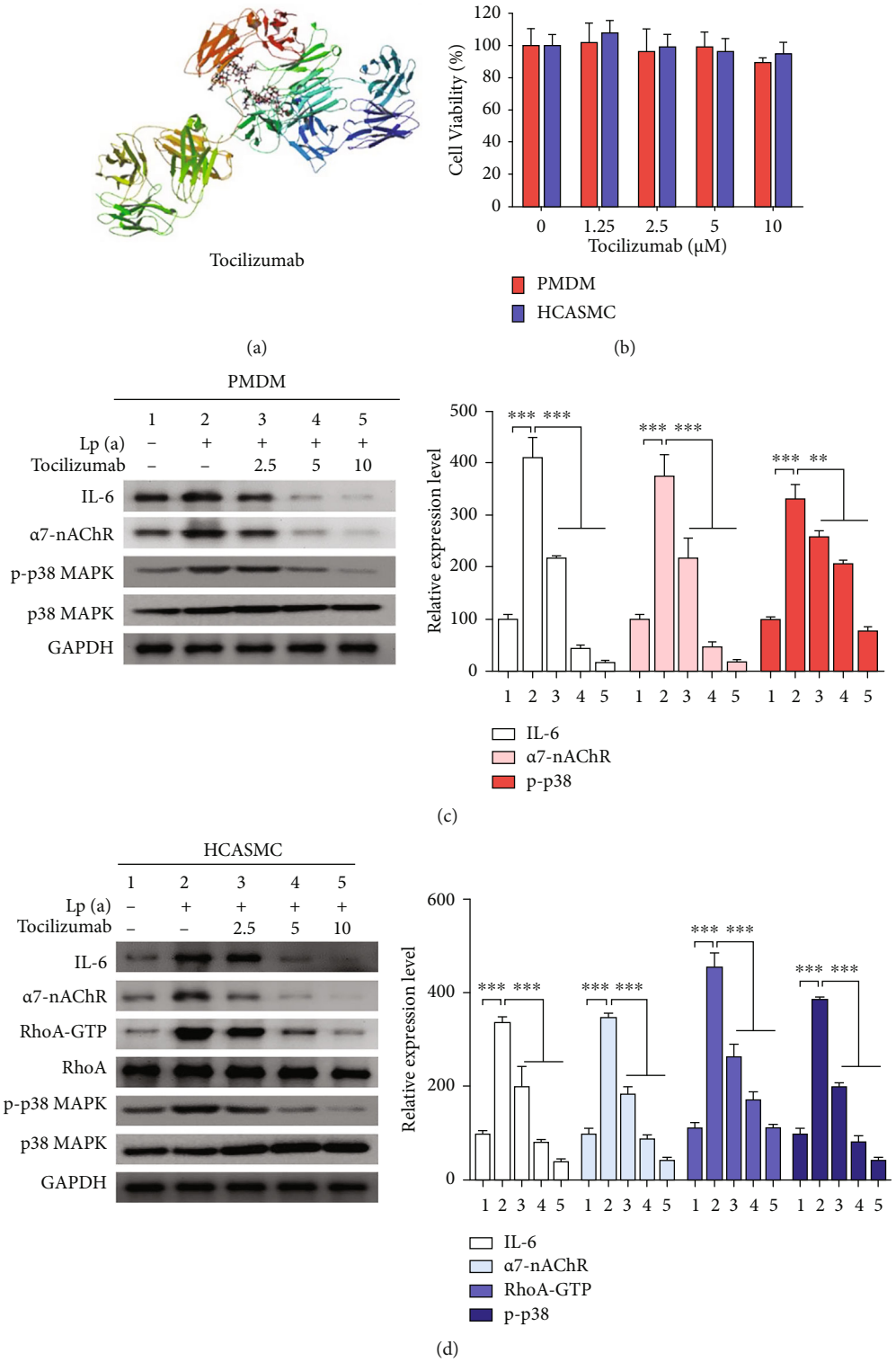

Figure 5: The human monoclonal antibody, tocilizumab, disrupts Lp(a)-induced α7-nAChR/p38 MAPK signaling by attenuating inflammation in patient monocyte-derived macrophages and HCASMCs. (a) 3D chemical structures of tocilizumab with molecular formula $C_{6428}H_{9976}N_{1720}O_{2018}S_{42}$ and molar mass 144987.06 g/mol. (b) Graphical representation of the effect of 1.25 μM–10 μM tocilizumab on the viability of HCASMCs or PMDMs. Representative western blot photo images and histograms showing how treatment with 1 μM Lp(a) and/or 2.5 μM–10 μM tocilizumab affects the expression of α7-nAChR, p-p38, and p38 proteins in (c) patient monocyte-derived macrophages or in (d) HCASMCs. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; GAPDH served as loading control. PMDM: patient monocyte-derived macrophage.

diagnostic test for coronary disease [93]. In normal coronary arteries, only mild widespread vasoconstriction (<20% diameter reduction) would be induced [94]. Ergonovine testing in the catheterization laboratory was used in the late1970s and early 1980s to identify the mechanism of chest pain when nonobstructive coronary artery disease was found by angiography. CAS is diagnosed when any one of the following conditions is present such as (1) spontaneous attacks, (2) positive non-drug-induced CAS provocation test (e.g., hyperventilation test and exercise test), or (3) positive drug-induced CAS provocation test (e.g., acetylcholine and ergonovine provocation test) [95]. While the frequency of provoked CAS by the intracoronary administration is about 2.5-fold higher than that by the intravenous administration of ergonovine and acetylcholine [96, 97], there is no difference regarding the incidence of provoked CAS between ergonovine and acetylcholine [98]. Provoked CAS by ergonovine tends to be proximal and focal, whereas CAS provoked by acetylcholine is distal and diffuse [99–101]. Although the intracoronary injection of ergonovine and acetylcholine provoked CAS in 65% and 80% in a previous study [100], respectively, no differences existed regarding the provoked CAS between intracoronary ergonovine and acetylcholine in a later study [101]. The efficacy of intracoronary administration of acetylcholine in doses of 10 to 100 μg is comparable to methylergonovine [70, 102, 103]. Of note, besides invasive diagnosis of CAS, ergonovine echocardiography has been used in Korea for noninvasive diagnosis of CAS [104]. Further studies are needed to evaluate the differences of coronary response between the ergonovine and acetylcholine examinations.

Acetylcholine, ergonovine, serotonin, and histamine cause endothelium-dependent vasodilation by stimulating NO release from the normal endothelium, and they can induce CAS in the presence of endothelial dysfunction [105]. Dysfunctional endothelial NO synthase and therefore deficient release of NO have been shown to be significantly associated with CAS [10, 106]. Furthermore, NO deficiency has been shown in the nonspastic coronary arteries as well as in the peripheral arteries, indicating that NO deficiency may occur in the entire vascular system in patients with CAS [107]. Remarkably, while neither stimulated NO synthesis nor basal NO production and release in endothelium seems to be impaired by elevated Lp(a) concentrations, the endothelium-dependent vasoconstrictive response to N-monomethyl L-arginine is enhanced in patients with high Lp(a) plasma levels [108]. Although oxidized Lp(a), but not native Lp(a), inhibits inducible NO synthesis in lipopolysaccharide/interferon stimulated mouse macrophages in a dose-dependent manner [109], we demonstrated for the first time that the inducible NO synthesis and the subsequent NO production in our CAS patient monocyte-derived macrophages were dose-dependently inhibited by Lp(a), suggesting a role of inducible NO synthase in CAS development and the effects on inducible NO synthesis by Lp(a) may be cell type selective. While NO is produced by different cell types and important in regulating smooth muscle relaxation [110], the activation of inducible NO synthase varies depending on cell types and species [111]. Furthermore, NO plays critical roles in immune suppression [112]. Inducible NO synthase-deficient mice than wild-type mice are more susceptible to the development of inflammatory diseases such as experimental allergic encephalitis [113]. Although endothelial NO synthase is the only NOS expressed in normal vascular endothelium, during inflammation, blood vessels express both inducible and endothelial NO synthase [114]. Moreover, inducible NO synthase produced in rabbit carotid arteries counteracts VSMC contraction by activation of soluble guanylate cyclase [115]. In pigs, NO produced by inducible NO synthase in the coronary VSMCs exerts an inhibitory and vasculoprotective effect against the cytokine-induced proliferative/vasospastic changes of the coronary artery in vivo [116]. In addition, inducible NO synthase is a signature molecule for M1 macrophages [117]. Thus, a complete understanding of the molecular mechanisms involved in the regulation of M1 innate immune responses should provide insights into the pathogenesis and treatment of CAS.

To differentiate Kounis syndrome from nonallergic CAS, the understanding of individual hypersensitivity is vitally important. While a relation exists between white blood cell counts and the incidence of coronary heart disease in epidemiologic studies [118], elevated peripheral white blood cell and monocyte counts, hs-CRP, interleukin-6, and adhesion molecules have been demonstrated in CAS patients [119, 120]. Although the eosinophil counts predict the severity of CAS, CAS can also result in an increase in eosinophil counts during follow-up in patients with CAS [118]. In our previous nationwide population-based cohort study showing the important role of CAS, regardless of sex, as a risk factor for incident diabetes, peripheral monocyte and eosinophil counts were borderline insignificant and significantly higher in nondiabetic CAS patients than nondiabetic non-CAS patients, respectively, in a single hospital substudy [121]. In addition, aspirin-induced eosinophilia-associated coronary artery vasospasm (EACAV) is a generalized terminology associated with various eosinophilic disorders such as aspirin-exacerbated respiratory disease that varies in presentation [122]. Of most EACAV patients, all were middle-aged, refractory to traditional antianginal therapy, and responsive to oral steroids [122]. While the allergic inflammatory response starts when an allergen activates the tissue resident mast cell, triggering the release of various granule-stored and newly formed mediators, as the inflammation progresses, a chronic allergic inflammation always features prominent tissue eosinophilia [123]. The interactions due to such "allergic effector unit" may modulate the severity and/or duration of the allergic inflammatory reaction [123]. Taken together, while monocytes play an important role in CAS, eosinophils and mast cells appear to be more important than monocytes in mediating nondiabetic CAS and allergic CAS, respectively. Future studies have to better delineate which patients benefit most from a measurement of differential cell counts to assess the development of CAS.

While the expression data related to macrophage polarization have previously highlighted interspecies discrepancy [124], few data are available regarding human monocyte-to-macrophage differentiation and polarization to M1 and

M2 upon exposure to Lp(a). Although in atherosclerotic lesions, cytokines can modify macrophage phenotypes, such as M1 and M2 [125], in disease contexts, M1 macrophages are implicated in initiating and sustaining inflammation [126] and can therefore be detrimental to health. Lp(a) elicits the proinflammatory response in healthy monocytes in vitro, an effect markedly attenuated by inactivating oxidized phospholipids present on apolipoprotein (a) [127]. Furthermore, in CAD, elevated Lp(a) levels compared with normal Lp(a) levels increase the expression levels of the scavenger receptors CD36 on monocytes, which is correlating to Lp(a) levels, whereas the expression of other receptors such as CD163 and CD206 was not different [127]. A similar phenomenon was observed that Lp(a) increases the expression of phenotypical M1 marker CD80 via α7-nAChR in our CAS monocyte-derived macrophages, indicating that 2 different vascular pathologies may exist in CAS and CAD. On the other hand, in human monocytes and monocyte-derived dendritic cells, the upregulation of α7-nAChRs and M1 marker CD40/CD86 enhances adaptive immunity in atherosclerosis, including T cell proliferation and cytokine production [128]. The similar upregulation of α7-nAChRs and M1 marker CD80 in our CAS patient monocyte-derived macrophages is of functional relevance for eicosanoid production and may contribute to pathophysiological reactions in CAS. Moreover, a hallmark of M1 polarization is the synthesis of IL-6 [129]. Similar to oxidized LDL [130], but not native LDL, Lp(a) diminished apoptosis of the activated macrophages. Hence, the upregulation of α7-nAChRs and M1 marker is important in both adaptive and innate immunity. In response to inflammatory stimuli, an afferent signal via the vagus nerve is triggered, activating efferent responses to attenuate tissue-specific cytokine production by the activation of the α7-nAChR in macrophages. Notably, spontaneous episodes of CAS in patients are often preceded by a decrease of vagal activity [131]. In addition, many studies using murine atherosclerotic models have either described an anti- or proatherogenic role of the α7-nAChR, which is still an area of debate in the literature [41]. The role of α7-nAChR in distinct immune cells may differ depending on cell type and function. In macrophages, besides decreasing the release of inflammatory cytokines, α7-nAChR stimulates the survival and polarization of the anti-inflammatory M2 phenotype [132], supporting the notion that immune cells have their own cholinergic system. However, we observed that Lp(a) polarized macrophages toward the M1 phenotype and subsequently increased IL-6 production. Because M1 macrophages are involved in inflammatory responses by producing chemokine ligands and proinflammatory cytokines, such as tumor necrosis factor-α and IL-6 for immune stimulation [133], our findings suggest that Lp(a)/M1 macrophage/IL-6 pathway contributes to the development of CAS. Hence, Lp(a) may modulate the acetylcholine-related cellular environment in an autocrine/paracrine way via α7-nAChR expressed by macrophages. Furthermore, activation of VSMC α7-nAChR has been reported to increase IL-6 following prior nicotine exposure [134]. This finding is insightful not only because it links increased IL-6 expression with α7-nAChR activation but also because it suggests the role of α7-nAChRs in vascular immunogenicity. Based on our findings, it seems reasonable to suggest that the interaction between Lp(a) and α7-nAChR significantly increases IL-6 levels, which ultimately prove critical during acute coronary syndrome. Although IL-6 has been implicated in the pathogenesis of atherosclerosis [135], in vitro studies have demonstrated that the activation of α7-nAChRs attenuates the release of IL-6 by macrophages [136], and the level of IL-6 is increased in patients with CAS [119]. Because different doses of nicotine can lead to activation or desensitization of nAChR function [137], complex interactions may exist between α7-nAChRs and smoking in atherosclerosis, while the role of these interactions in CAS development is currently unknown. Therefore, Lp(a) exposure α7-nAChR activation may increase IL-6 levels through undetermined mechanisms, which requires further exploration. Collectively, these observations suggest the different effects of Lp(a) and α7-nAChR on IL-6 production in CAS from their effects on atherosclerotic CAD. These properties of cells of the monocyte–macrophage lineage may represent a target for therapeutic exploitation.

The pathophysiological role of Lp(a) in humans is still not fully elucidated. While plasma concentrations of Lp(a) are observed to rise acutely under pathological challenge, for example, after myocardial infarction and percutaneous coronary intervention [138], it has been demonstrated that prolonged exposure to high-circulating apolipoprotein (a) levels would render the VSMCs more stationary and contractile [138]. Taken together, it appears that Lp(a), acting as an acute phase reactant, induces the activation of RhoA-GTP and ROCK, potentially leading to the development of CAS. Our finding that the downstream effector pathway by which Lp(a) activated monocyte-derived macrophages and HCASMC relied on the α7nAChR-dependent activation of p38 MAPK is consistent with the effect of α7-nAChR in dendritic cells [128]. Furthermore, Lp(a) has been demonstrated to activate endothelial cells through activation of intracellular p38 MAPK signaling pathway [138]. In human apolipoprotein (a) transgenic rabbits, the atherosclerotic lesions are predominantly enriched in VSMCs, suggesting that Lp(a) promotes the proliferation of immature or activated VSMCs [139]. Notably, we found that α7-nAChR was involved in the activation of the RhoA-GTP and downstream effectors ROCKs in HCASMCs, which facilitates VSMC dysfunction [17]. Consistent with our prior study [15], increased levels of ROCK activity in HCASMCs are associated with CAS. Furthermore, in a cellular study, using a small interfering RNA approach, selective suppression of ROCK2 expression significantly attenuated VSMC contraction by modulating myosin phosphatase activity [140], suggesting increased expression of ROCK2 could lead to CAS. Our findings complement and extend these previous studies that Lp(a) signals through α7-nAChR/p38 MAPK to activate CAS patient monocyte-derived macrophages and HCASMCs. Further studies are needed to clarify the role of these relationships in mediating the development of CAS.

Although aspirin at low doses decreases Lp(a) levels slightly, there are currently no pharmaceutical treatments, including lipid-lowering strategies, available for the reduction

of the effects of Lp(a) and hence a greater understanding of the mechanisms underlying its functional effects on monocyte-macrophage and VSMC may provide alternative therapeutic targets. Recently, although the inflammatory hypothesis of cardiovascular disease was demonstrated in 2 large-scale multicenter randomized clinical trials using either a selective IL-1β antagonist [141] or low-dose colchicine [142], its clinical application using other affordable mainstream anti-inflammatory therapies remains challenging [143]. Emerging clinical and translational data suggest a synergism between the effects of Lp(a) and systemic inflammation [127]. Tocilizumab, a monoclonal antibody against IL-6 receptor approved for the treatment of rheumatoid arthritis in 2009 in Europe [90], lowers Lp(a) serum levels in rheumatoid arthritis patients by up to 50% [144]. The up- and downregulation of α7-nAChR expression on immune T cells has been found to be under the influence of interleukin cytokines in inflammatory bowel disease, which is nicotine-mediated and smoking-related [145]. In our study, tocilizumab reduced Lp(a)-associated expression of α7nAChR and the receptor-dependent activation of p38 MAPK, IL-6, and additionally RhoA-GTP in HCASMCs, suggesting a direct and specific pathogenic effect of Lp(a). Collectively, these observations implicate that interleukin cytokines themselves can alter the function of α7-nAChR. RhoA-GTP and its downstream effector, Rho-kinase/ROCK, inhibit myosin light chain phosphatase, leading to augmentation of *myosin light chain* phosphorylation and the subsequent VSMC contraction in response to vasoconstrictor stimuli. Hence, one may consider anticytokine IL-6 as a new promising treatment of elevated Lp(a) levels in affected patients. However, despite these recent advances, it needs further studies to examine which patient populations would benefit the most from Lp(a) reduction and what degree of Lp(a) lowering would be required to demonstrate incremental clinical benefit despite the use of established medical therapies [146, 147].

In our study, all 64 patients, who had chest pain and suspected ischemic heart disease on noninvasive tests and no angiographic evidence of obstructive CAD, were subjected to intracoronary methylergonovine testing. In the Asymptomatic Cardiac Ischemia Pilot (ACIP) study, asymptomatic patients with CAS or CAD were those without symptoms to indicate myocardial ischemia [148]. While screening asymptomatic patients for the presence of CAS or CAD may potentially impact therapeutic management and outcome, the approach to asymptomatic patients with suspected CAS or CAD is based on the history and/or electrocardiographic (ECG) evidence of myocardial ischemia or an abnormal noninvasive test [149]. It is recognized that when tested, a subgroup of these asymptomatic patients will have transient abnormalities consistent with myocardial ischemia, which is termed silent ischemia, and the abnormalities detected may consist of reversible ECG ST-segment shifts on exercise testing or ambulatory monitoring, perfusion abnormalities on radionuclide scans (i.e., stress 201Tl, sestamibi, and PET) or regional wall motion abnormalities during left ventricular imaging (i.e., stress echocardiography or radionuclide ventriculography). Thus, the absence of symptoms does not necessarily mean the absence of either ischemia or an adverse prognosis. Diabetes, old age, females, hypertension, polyneuropathy, and cardiac transplantation, when accompanied by significant CAD, are all associated with a high frequency of myocardial ischemia without symptoms [70]. However, multiple guidelines and scientific statements have discouraged the use of ambulatory monitoring, treadmill testing, stress echocardiography, stress myocardial perfusion imaging, and electron-beam computed tomography as routine screening tests in asymptomatic individuals [149]. Furthermore, because a diagnosis of CAS cannot be directly established based on symptoms [97], standard 12-lead electrocardiography results [150], ambulatory monitoring of electrocardiography [27], or exercise testing [151], and invasive coronary angiography with provocative testing are the gold standard method of diagnosing CAS [70], direct referral for diagnostic coronary angiography may be indicated in symptomatic patients with chest pain possibly attributable to myocardial ischemia when noninvasive testing is contraindicated or unlikely to be adequate due to illness, disability, or physical characteristics [152]. The diagnosis of angina associated with diabetes can be particularly difficult because of the paucity of symptoms of myocardial ischemia due to autonomic and sensory neuropathy, and a lowered threshold for coronary angiography is appropriate [152]. Therefore, with only a few exceptions, coronary angiography is not indicated in asymptomatic patients with suspected CAS or CAD, unless noninvasive testing reveals findings that suggest a high risk for adverse outcome [70]. While our study was not a randomized controlled trial and the inclusion of a group of gender- and age-matched asymptomatic individuals referred for diagnostic coronary angiography was not approved by the Taipei Medical University Joint Institutional Review Board, future studies including gender- and age-matched asymptomatic individuals will help elucidate the role of Lp(a) in defining the severity and susceptibility of inflammation-associated CAS. Further investigation is required to better delineate these relationships. In our study, CAS patients were typically middle-aged men, often smokers, which is typical in East Asia, especially in Japan [153]. However, there are not enough data on the prevalence of CAS both in the Eastern and Western countries, probably because it is difficult to examine CAS systematically at each time of coronary angiography [27]. CAS appears to be more common in Caucasian than Japanese women [154], and there is lower incidence of smoking among the whole of CAS females than among males [155]. Hence, among Caucasians, CAS is not rare among youngish women, usually nonsmokers [156]. Recently, the presence of CAS is more frequent in Caucasians when invasive coronary angiography with provocative testing is aggressively performed [157]. Thus, the aggressive effort of making a diagnosis of CAS may help clarify the real prevalence of CAS worldwide.

CAS is a multifactorial disease involving the contribution of both vascular wall- and blood-related factors in pathogenesis. Precipitating factors may trigger the onset of CAS and cause angina in the same patient under different conditions [153]. Moreover, VSMC hyperreactivity can cause CAS through various pathways [6]. Considering endothelial cell-smooth muscle cell coculture systems, they are sufficiently

developed such that they are mainly employed for high-throughput screening applications in atherosclerotic vascular wall remodeling [158]. Several different approaches of the coculture systems are available to identify drugs and targets for angiogenesis [158]. Although direct contact coculture systems provide several distinct advantages, they still need more development so that a normal intima can be produced, and the cells can be exposed to both stretch and fluid flow [158]. Microplate and microfluidic systems can be utilized to produce high-throughput identification formats of lead candidates [158]. Vascular endothelium responds specifically to arterial fluid shear stress but less so to pressure or cyclic stretch [159]. Steady or pulsatile laminar shear stresses cause the endothelium to align in the direction of flow, release vasodilators, reduce their growth rate, increase their elastic modulus, and increase expression of anti-inflammatory genes. In contrast, low and oscillating shear stresses promote the release of vasoconstrictors and the expression of proinflammatory and oxidative stress genes [159, 160]. Laminar shear stress applied to endothelial cells exert atheroprotective functions by modulating the underlying VSMCs from synthetic to contractile phenotype [161]; however, contractile rather than synthetic phenotype VSMCs play a main role in the pathogenesis of CAS [6]. Furthermore, laminar shear stress has anti-inflammatory effects by inhibiting VSMC-induced proinflammatory responses in endothelial cells. To date, no information is available concerning the effects of Lp(a) on monocyte-derived macrophages in patients with CAS and HCASMCs. We, therefore, analyzed the protein expression levels of Lp(a) and α7-nAChR in the monocytes of patients with CAS. Furthermore, we investigated the effects of Lp(a) on monocyte-to-macrophage differentiation and polarization based on CD80 or CD206 positivity and α7-nAChR-dependent activation of the p38 MAPK signaling in monocyte-derived macrophages and primary HCASMCs. In addition, we previously demonstrated the negative effects of diabetes mellitus and hypertension on CAS development in patients with high CRP levels, indicating 2 different vascular pathologies exist in CAS and atherosclerotic cardiovascular disease [6]. Collectively, more development is needed before applying endothelial cell-smooth muscle cell coculture systems to research in CAS. Nonetheless, endothelial cell-smooth muscle cell coculture systems should be utilized in the future studies of aberrant endothelial cell-smooth muscle cell communication in CAS. In our ongoing studies we aim to identify the nature of endothelial cell–VSMC crosstalk, which may provide the key cellular and molecular mechanisms of CAS-related vascular wall remodeling.

In the study of a *cause* of *disease*, Koch's postulates were invaluable when they were developed and remain largely valid for a few defined circumstances. Koch's postulates were initially developed in the 19th century to establish microorganism function and were modified in the 20th century to include methods to establish molecular causality [162]. Although isolation of the pathogen from the diseased host is the gold standard of the postulates, rigorously applying Koch's postulates to the etiology of CAS has several limitations. First, smoking that may not induce CAS in some people with low CRP levels can become a risk factor and potentially pathogenic for CAS in other people with high CRP levels [163]. Second, there are experimental animal models evaluating the causal role of Lp(a) in atherosclerosis and aortic stenosis, but not in CAS. Finally, something that may be useful in proving causality is whether eradication of the pathogen results in cure, which is not described in Koch's postulates. Although Lp(a) is not expressed in commonly studied laboratory animals, mouse and rabbit models transgenic for Lp(a) and apo(a) have been developed to study their pathogenicity in vivo, which have provided significant insights into the pathophysiology of Lp(a) in mediating atherosclerosis [164]. While apo(a) is retained in atheromas in mouse models and suggests that it promotes fatty streak formation and Lp(a) promotes atherosclerosis and vascular calcification in rabbit models, many of these models have limitations [164]. Because apo(a) is not covalently linked to mouse apoB to form Lp(a), mouse models need to be transgenic for both apo(a) and human apolipoprotein B-100 [164]. In established mouse and rabbit atherosclerotic models, Lp(a) levels are low, usually <20 mg/dL, which is within the normal range in humans [164]. Furthermore, only one apo(a) isoform can be expressed in a given model whereas more than 40 isoforms exist in humans [164]. It is ideal for mouse models to be studied for atherosclerosis in an LDL receptor negative background, as mice do not develop sufficiently elevated plasma cholesterol to form atherosclerosis [164]. As such, the development of optimized Lp(a) transgenic animal models will advance the understanding of the mechanistic role of Lp(a) in atherosclerosis and aortic stenosis [164], as well as in CAS, and provide a platform to examine novel therapies for cardiovascular disease. On the other hand, controversy exists regarding whether CRP is only a clinically useful determinant of disease or whether it also may play a causal role in the atherothrombotic process [165, 166]. Although much information has been provided by past studies, CRP cardiovascular biology remains largely observational, with few studies showing cause and effect relationships, which note that CRP induces endothelial cell activation and dysfunction, has substantive effects on VSMCs and neointimal formation, and directly affects monocyte and macrophage activity as well as matrix metalloproteinase function [167]. Furthermore, human CRP infusion studies show both proinflammatory and prothrombotic effects [168], whereas in transgenic mouse models, CRP seems to increase thrombosis rates only after vascular injury [169]. In a recent prospective study, Ridker and colleagues report on a monoclonal antibody targeting anti-interleukin 1β (anti-IL-1β) on cardiovascular events in humans [141]. The authors argue that this study [141] fulfills Koch's postulates for ASCVD since inflammation, including the proinflammatory cytokine, IL-1β, has been shown in animal models to contribute to atherosclerosis, and now, this study shows that blocking IL-1 with the 150 mg dose in humans results in a significant decrease in cardiovascular events [170]. Collectively, the "marker versus mechanism" debate remains open and is an area with a need for more research, including a need to develop novel Lp(a) and CRP inhibitors that can be used to test directly whether

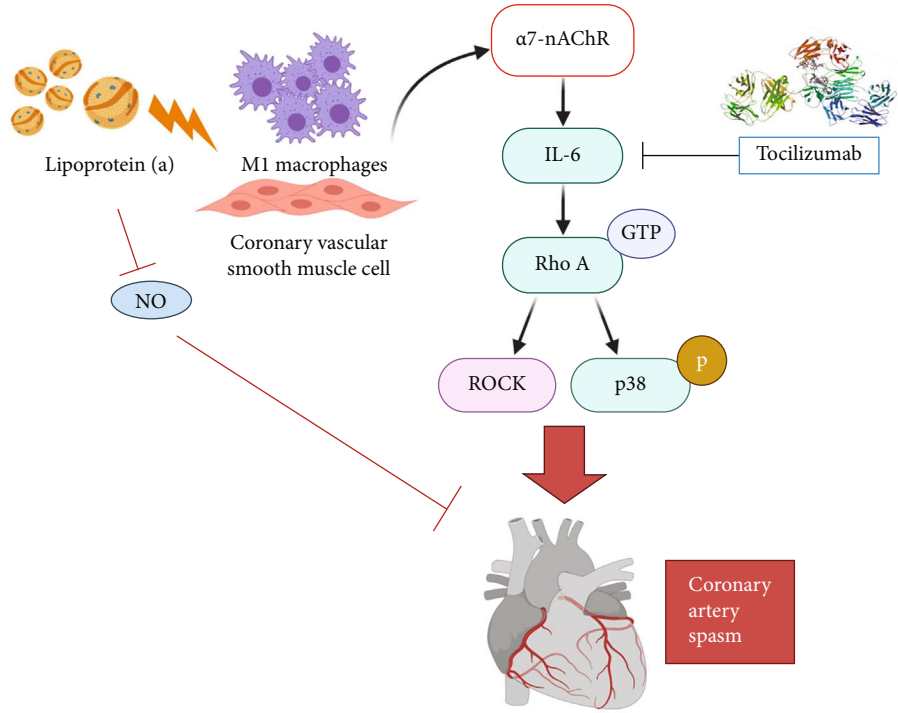

Figure 6: Graphical abstract depicting how Lp(a)-triggered inflammation drives CAS through macrophage M1 polarization, activation of coronary VSMC, and α7-nAChR/p38 MAPK signal induction. Tocilizumab disrupts Lp(a)-induced α7-nAChR/p38 signaling by attenuating the inflammation in coronary VSMCs and patient monocyte-derived macrophages.

Lp(a) and CRP reduction results in reduced event rates [167]. Debate concerning mechanistic properties of Lp(a) and CRP to fulfill the Koch's postulates before being useful in a clinical setting should have little bearing on their utilities as clinically effective biomarkers for risk detection [167]. In our study, we provided a framework of investigating Lp(a) in association with CAS to ensure that scientific rigor is applied when proposing a mechanistic role of Lp(a) in the development of CAS.

Our study has some limitations. Firstly, the relatively small cohort size ($n = 64$) might be difficult to establish causality. Secondly, the use of certain medications, including beta blockers [171], statins [172], Ca^{2+}-channel blockers [173], or nitrates [174], which are known to affect IL-6 or α7-nAChR expression and/or activity to varying extents, is a putative limitation. Thirdly, the presence of confounders that could have affected the accurate measurement of patients' cytokine, Lp(a), or α7-nAChR level is probable. Finally, translating findings to subjects without cigarette exposure history but who used other nicotine-containing products, e.g., Swedish SNUS, might have constituted some limitation.

5. Conclusion

Serum Lp(a) levels are positively correlated with the levels of CRP, IL-6, and monocytic α7-nAChR in CAS. Lp(a) induces macrophage M1 polarization and, through its apolipoprotein (a) chain, the expression of α7-nAChR/p38 MAPK/IL-6 and dose dependently inhibited the NO production and the expression of inducible NO synthase in the monocyte-derived macrophages of patients with CAS. Lp(a) activates HCASMCs via α7-nAChR/p38 MAPK/IL-6/RhoA-GTP signal induction. Tocilizumab reduces the interaction of Lp(a)/monocyte/HCASMC and the subsequent expression of α7-nAChR/p38 MAPK/IL-6/RhoA-GTP (Figure 6), suggesting α7-nAChR partly under the influence of IL-6 and anti-cytokine IL-6 as a promising treatment of CAS. Our study provides a new avenue in understanding the process of α7-nAChR-induced VSMC dysfunction and shows promise in the development of potential therapeutic agents for CAS.

Data Availability

The datasets used and analyzed in the current study are publicly accessible as indicated in the manuscript.

Additional Points

Highlights. (1) Apolipoprotein (a)/lipoprotein(a) induces inflammatory α7-nAChR/p38 MAPK/IL-6/RhoA-GTP signaling axis in coronary artery spasm. (2) Apolipoprotein (a)/lipoprotein(a)-triggered inflammation modulates coronary artery spasm through M1 macrophage polarization. (3) The α7-nAChR on macrophages could represent a promising new therapeutic target in coronary artery spasm.

Ethical Approval

This prospective cohort study was approved by the Taipei Medical University Joint Institutional Review Board (approval number: TMU-JIRB N201903036). The study was compliant with the recommendations of the Declaration of Helsinki for biomedical research involving human subjects.

Consent

All patients provided signed informed consent regarding use of their blood in scientific research.

Conflicts of Interest

The authors declare that they have no potential financial competing interests that may in any way gain or lose financially from the publication of this manuscript at present or in the future. Additionally, no nonfinancial competing interests are involved in the manuscript.

Authors' Contributions

Chi-Tai Yeh was responsible for data acquisition and collation and manuscript writing; Yen-Kuang Lin was responsible for study conception, experimental design, data collation, and manuscript writing; Kuang-Tai Kuo, Vijesh Kumar Yadav, and Iat-Hang Fong were responsible for data collation and interpretation, bioinformatics, manuscript writing, and critical review of initial draft; Nicholas G. Kounis and Patrick Hu were responsible for experimental design, critical review of initial draft, and provision of research materials; Ming-Yow Hung was responsible for study conception, experimental design, data collation, manuscript writing, and provision of research materials. All authors read and approved the submitted version of the manuscript. Yen-Kuang Lin and Chi-Tai Yeh contributed equally to this work.

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Supplementary Materials

Supplementary 1. Supplementary Table S1: western blot antibody sheet.

Supplementary 2. Supplementary Figure S1: full-size blots of Figures 2(a) and 2(b).

Supplementary 3. Supplementary Figure S2: full-size blots of Figures 2(c), 2(d), and 2(f).

Supplementary 4. Supplementary Figure S3: full-size blots of Figures 4(c) and 4(d).

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Review Article

The Role and Mechanism of Oxidative Stress and Nuclear Receptors in the Development of NAFLD

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The overproduction of reactive oxygen species (ROS) and consequent oxidative stress contribute to the pathogenesis of acute and chronic liver diseases. It is now acknowledged that nonalcoholic fatty liver disease (NAFLD) is characterized as a redox-centered disease due to the role of ROS in hepatic metabolism. However, the underlying mechanisms accounting for these alternations are not completely understood. Several nuclear receptors (NRs) are dysregulated in NAFLD, and have a direct influence on the expression of a set of genes relating to the progress of hepatic lipid homeostasis and ROS generation. Meanwhile, the NRs act as redox sensors in response to metabolic stress. Therefore, targeting NRs may represent a promising strategy for improving oxidation damage and treating NAFLD. This review summarizes the link between impaired lipid metabolism and oxidative stress and highlights some NRs involved in regulating oxidant/antioxidant turnover in the context of NAFLD, shedding light on potential therapies based on NR-mediated modulation of ROS generation and lipid accumulation.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), a pandemic disease, is predicted to be the most common indication for liver transplantation in the next decade. NAFLD refers to the state when hepatic lipid accounts for more than 5% of the liver weight without excessive alcohol consumption or other known causes of liver diseases (viruses, drugs, toxins, autoimmune disease, etc.). Regarding the clinical course, the full spectrum of NAFLD includes simple steatosis, steatohepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Recently, based on the finding of concomitant liver disease and the heterogeneous pathology, a new definition of metabolic dysfunction-associated fatty liver disease (MAFLD) has recently been put forward [1].

According to recent data, the overall global prevalence of NAFLD is estimated to be 24% among adults. The highest prevalence was reported to be 31.79% in the Middle East, followed by 30.45% in South America, 27.37% in Asia, 24.13% in North America, 23.71% in Europe, and 13.48% in Africa [2]. From 2012 to 2017, cirrhosis due to NAFLD and NAFLD-related death increased globally, especially in Australia, Latin America, and Asia [3]. NAFLD has replaced viral hepatitis to be the most common liver disease in China. The prevalence of NAFLD is higher in younger generations and lean people, in addition to the elderly population [4, 5]. Importantly, although there are no typical symptoms or signs of NAFLD, its potential harm to the liver and extrahepatic complications in cardiovascular and other systems cannot be neglected. NAFLD may start with the insulin resistance and dysfunction of adipocytes, providing a pathogenic milieu rich in lipid metabolites, and then, proinflammatory cytokines may be released into the peripheral circulation, leading to mitochondrial dysfunction, activation of apoptosis, and a chronic inflammatory state. The whole process includes crosstalk among the liver, muscles, adipose tissues, and a systemic disturbance of cytokines and hormones, which eventually causes systemic effects such as metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), and hypertension. Due to

inadequate awareness, unavailability of diagnostic tools, and a lack of effective medication, the vast majority of potential NAFLD patients are undiagnosed and untreated [6].

According to the classical "two-hit" theory, NAFLD is characterized by two steps of liver injury: intrahepatic lipid accumulation (hepatic steatosis) and inflammatory progression to nonalcoholic steatohepatitis (NASH) [7]. This classical hypothesis has been modified to indicate that NAFLD may be a consequence of parallel "multihits" [8]. Lipotoxicity primes the liver for injury arising from "multiple and parallel hits" (oxidative stress and the activation of proinflammatory and fibrogenic pathways) [9]. Oxidative stress leads to cellular dysfunction and is considered a causative factor in the pathophysiology of NAFLD. When the generation of reactive oxygen species (ROS) exceeds the capacity of antioxidants to detoxify them, these highly toxic molecules induce damage to the normal lipid metabolism [10]. Moreover, increased ROS levels are responsible for insulin resistance in numerous settings [11], which indicates that redox-dependent molecular alterations also play an important role in the early stage of NAFLD.

Considering the vital role played by several nuclear receptors (NRs) and transcription factors in the development of NAFLD [12], this review focuses on the role played by ROS in the regulation of the transcriptional network that modulates hepatic lipid metabolism, suggesting a redox-centered pathogenic hypothesis. Moreover, the impact of endogenous hormones as environmental factors on NR expression in the development of NAFLD is discussed.

2. ROS Production and Oxidative Stress in the Development of NAFLD

The liver serves as the distribution center of nutrients, smoothing out blood glucose and lipid fluctuations between intermittent food intake. The content of triglycerides in the liver varies with the metabolic states. During fasting, fatty acids released from adipose tissues oxidize in hepatic mitochondria to generate energy. On the other hand, when fatty acids and chylomicrons are redundant in the circulation after a meal, the liver packages them in the form of lipid droplets for further use. As shown in Figure 1, increased uptake of free fatty acids and lipogenesis, defects in fatty acids oxidation, and decreased lipids export contribute to the impaired hepatic lipid metabolism. It is worth mentioning that ROS appears necessary in those processes that lead to the dysfunction of lipid metabolism and the development of NAFLD. The imbalance between ROS generation and antioxidant defenses causes oxidative stress and tissue damage [13]. Clinically, increased mitochondrial levels of ROS and mitochondrial dysfunction are observed in liver tissues from patients with NAFLD [14, 15]. The results from mouse models also indicate that impaired mitochondrial dynamics leads to metabolic abnormalities such as NASH phenotypes [16]. This section outlines the knowledge on ROS generation and highlights the role of oxidative stress in the NAFLD pathology.

2.1. Mechanism of Excessive ROS Production in NAFLD. ROS or oxidants can be classified as free radicals and major physiologically relevant ROS, including superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals (•OH), and hydrogen peroxide (H_2O_2). The imbalance between oxidants and antioxidants induces the oxidative stress [17]. Under conditions of normal antioxidant homeostasis, cells can effectively remove physiological ROS through protection systems consisting of enzymatic and nonenzymatic components. Some of the most relevant enzymes that detoxify ROS are superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidase and reductase (GSH-Px) [18]. The nonenzymatic components including some small molecules such as vitamin A/C/E and glutathione act as cell structures or electron receptors against the damage from free radicals [19].

Mitochondria have been considered a major site of ROS production, where molecular O_2 is reduced to $O_2^{\bullet-}$ through complexes I and III by nicotinamide adenine dinucleotide/-flavin adenine dinucleotide (NADH/FADH2). Monoamine oxidase, α-ketoglutarate dehydrogenase, and glycerol phosphatase dehydrogenase further contribute to generating $O_2^{\bullet-}$ [20]. Mitochondrial dysfunction seems to be a common mediator triggering oxidative stress. Under conditions of normal mitochondrial homeostasis, a cell can eliminate physiological ROS and make metabolic adaptations. In NAFLD, however, increased mitochondrial fatty acid oxidation and tricarboxylic acid (TCA) cycle activity persistently supply reducing equivalents to the electron transport chain (ETC) [21]. This prolonged dysfunction in the respiratory complex promotes the generation of superoxide anion ($O_2^{\bullet-}$). Notably, the uncoupling between β-oxidation, the TCA cycle, and ETC frequently results in inefficient lipid metabolism and ROS overproduction in the liver [22, 23]. In addition, the capability of mitochondria to reduce ROS levels is reduced in NAFLD, as indicated by decreased GSH metabolism [24], Mn superoxide dismutase (MnSOD) activity [25], and catalase activity [26]. Hence, either an increased production of prooxidant products or the dysfunction of the antioxidant system may induce oxidative stress. Accompanied by ROS accumulation, free fatty acid-induced hepatic lipotoxicity also promotes mitochondrial outer membrane permeabilization (MOMP) and alters the release of mitochondrial proteins and mitochondrial bioenergetics in NAFLD [27, 28].

Additionally, due to lack of histone protection, mitochondrial DNA (mtDNA) is highly sensitive to ROS. It is prone to damage and mutation, resulting in respiratory chain defects and decreased mitochondrial biogenesis under oxidative stress [29]. Oxidative damage to nuclear DNA impairs mitochondrial function and hinders the transcription of nuclear-encoded mitochondrial genes. For example, nuclear factor erythroid 2-related factor 2 (Nrf2), an essential modulator of antioxidant signaling that serves as a primary cellular defense against the cytotoxic effects of oxidative stress, has been reported to be decreased in NAFLD [30]. In the process of hepatic metabolism, specific polyunsaturated fatty acids (PUFAs) trigger lipid peroxidation, accompanied by increases in highly reactive aldehyde products such as malondialdehyde (MDA) and 4-hydroxy-2-non-enal (4-HNE) [31]. Thus, these mechanisms may eventually lead to a harmful cycle of mitochondrial damage and mitochondria-derived oxygen radicals.

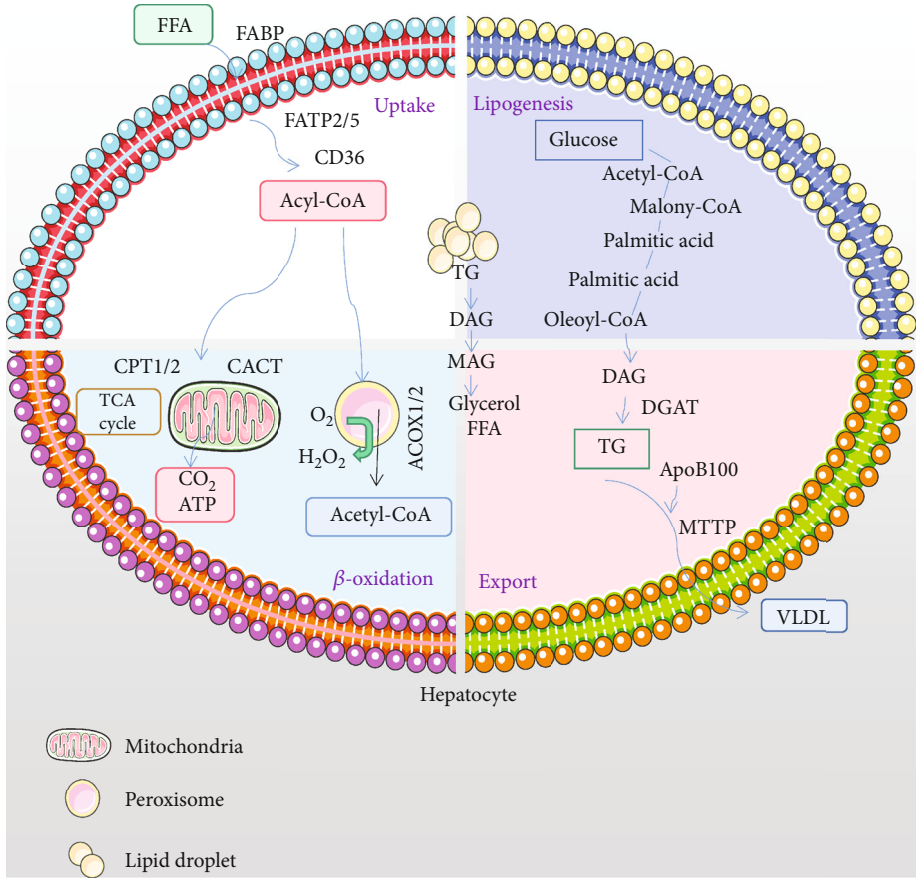

FIGURE 1: Hepatic lipid metabolism. Increased uptake of circulating free fatty acids (FFA) and *de novo* lipogenesis, impaired oxidation of fatty acids in league with decreased lipids export in the liver all contribute to the development of fatty liver.

In addition, the endoplasmic reticulum (ER) and peroxisomes are able to produce various kinds of ROS in liver tissues. Highly reactive molecules such as •OH, perhydroxyl radical (HO_2•), H_2O_2, and $^1O_2$ are produced from the reaction between O_2•− and other molecules [32]. ER stress in the development of steatosis and subsequent generation of ROS aggravate the liver injury and promote the progression of NAFLD [33]. Moreover, excess of long-chain fatty acids (LCFAs) promotes the generation of H_2O_2 through increasing peroxisomal β-oxidation [34]. Similarly, very long-chain fatty acids (VLCFAs) enhance ROS production by cytochromes P4504A- and P4502E1-mediated microsomal oxidation [35]. In addition, several enzymes in the plasma membrane and cytosol are the producer of free radicals. For example, cytochrome P450 (CYP) enzymes play vital roles in the metabolism of drugs and other xenobiotics and regulate the generation of ROS and bioactivated intermediates [36]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), xanthine oxidase, cyclooxygenases, and lipoxygenases also act as important regulators in the reactions of xenobiotic metabolism [37].

It has been proposed that the gut microbiota acts as a vital role in developing NAFLD [38]. In patients with NAFLD, the abundance and composition of the microbiome are altered (dysbiosis) [39], accompanied by enhanced intestinal permeability [40]. As a result, bacterial lipopolysaccharides (LPS) are derived from the overgrowth of Gram-negative bacteria. Evidence has shown that the serum level of LPS increases 38–40% in patients with NAFLD compared with that in controls [41]. High liver exposure to LPS induces the excessive release of ROS due to impaired antioxidant system [42]. Moreover, in patients with NAFLD, endogenous ethanol also caused by some microbial species increases ROS formation in hepatic stellate cells (HSCs) and stimulates intestinal bacteria to release LPS [38]. Besides the role in increasing hepatic inflammation and oxidation, LPS acts on Kupffer cells (KCs) to upregulate cytokine receptors such as tumor necrosis factor-α (TNF-α) receptor, which may also be involved in ROS overgeneration [43].

Overall, diverse sources of ROS and redox regulation may explain the pathogenesis of various liver diseases. In NAFLD, the increased formation of reducing equivalents results in an overflow of electrons from the mitochondrial respiratory chain, which induces higher ROS generation. ROS overproduction suppresses the capacity of antioxidant defense systems and causes further oxidative damage (Figure 2).

2.2. Implication of ROS and Oxidative Stress in the Development of NAFLD. Oxidative stress and imbalance of the redox state are distinctive characteristics of NAFLD [44]. Under physiological and pathological conditions,

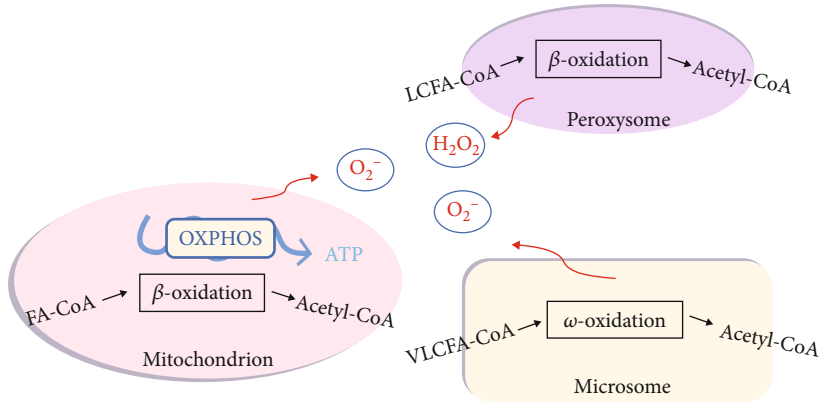

Figure 2: Main sources of ROS during the development of the fatty liver. In NAFLD, lipid oxidation induces the formation of reducing equivalents and causes an overflow of electrons through the mitochondrial respiratory chain (OXPHOS). Accumulation of long-chain fatty acids (LCFAs) increases peroxisomal β-oxidation, with consequent production of hydrogen peroxide. Excess of very long-chain fatty acids (VLCFAs) enhances microsomal oxidation with consequent generation of free radicals.

redox-dependent molecular alterations participate in the development of steatosis, providing new insights into the role of ROS as core regulators of liver lipid metabolism. Furthermore, increased ROS output and oxidative stress are identified as underlying mechanisms of insulin resistance, profibrogenic processes, and chronic inflammatory responses in NAFLD [10, 45]. This section outlines the recent knowledge on the regulators of ROS and oxidative stress in lipid metabolism and NAFLD progression.

2.2.1. Redox Regulation of Crucial Enzyme Activity in Lipid Metabolism. The increased lipid uptake and synthesis and impaired lipid oxidation and removal lead to hepatic steatosis. The redox status modulates the activity of some key enzymes involved in hepatic lipid metabolism [10].

First, *de novo* lipogenesis (DNL) is activated when abundant glucose and insulin are in the plasma, usually in the postprandial state. However, under the selective insulin-resistant state in NAFLD, gluconeogenesis cannot be suppressed while DNL is promoted [46]. The human isotope-labeling studies showed that DNL is significantly elevated in patients with NAFLD, and the portion DNL accounts for intrahepatic triglyceride-palmitate increases as the severity of insulin resistance increases, about 11% in the lean group, 19% in the obese group, and 38% in the obese-NAFLD group [47, 48]. Saturated fatty acids (SFA) are the first product of DNL and can promote redox imbalance and the formation of reactive oxygen intermediates. In human HepG2 cells, SFAs were reported to increase ROS production by upregulating the expression levels of several components of the NADPH oxidase, including NOX3, NOX4, and p22phox [49]. Moreover, stearoyl-CoA desaturase -1 (SCD-1) can improve the toxic effects of SFAs [50]. While the downregulation of SCD-1 enhances delivery of FAs to mitochondria and oxidation in the fed state [51]. Once fatty acids reach the liver, they are bound to fatty acid-binding protein-1 (FABP-1) and then transport to the liver with the help of cell surface receptors such as fatty acid transport protein (FATP) family members and fatty acid translocase (CD36). In palmitic acid- (PA-) treated hepatocytes, H_2O_2 pretreatment abolished the effects of CD36 knockdown in attenuated oxidative stress [52]. Third, fatty acid oxidation usually takes place in mitochondria and peroxisome of energy-requiring tissues such as the liver and skeletal muscles. Hepatic β-oxidation mainly provides the fuel for hepatic basal energy requirements [53]. Liver-specific peroxisome proliferator-activated receptor α (PPARα) knockout mice with impaired β-oxidation spontaneously are prone to NAFLD in aging even under a standard diet [54]. Notably, increased lipid oxidation and the TCA cycle are increased in NAFLD, indicating that hepatocytes enhance oxidation when counteracting lipid overload [55]. β-Oxidation is the primary producer to generate reducing equivalents (NADH or FADH2). The excess reducing equivalents cannot be resolved in the mitochondrial respiratory chain, resulting in higher ROS generation. Peroxisomal β-oxidation and microsomal oxidation also contribute to the redox unbalance in NAFLD [56]. These changes increase hepatic reduction degree, as indicated by alterations in the NADH/NAD^+ ratio [57]. The increased ratio suppressed the activities of acyl-CoA dehydrogenase (LCAD) and β-hydroxyacyl-CoA dehydrogenase (β-HAD), which are involved in the pathway of fatty acid oxidation [58, 59]. Lastly, the export of lipids is another way for the liver to reduce lipid accumulation. Lipoproteins, such as chylomicrons (CM) and very-low-density lipoproteins (VLDL), contain core lipids like triglycerides and cholesterol esters. Hepatic endoplasmic reticulum synthesizes VLDL with apolipoprotein B (ApoB) and triglyceride with the help of microsomal triglyceride transfer protein (MTTP). This process enables the liver to alleviate endogenous triglycerides by secreting water-soluble VLDL into circulation [60]. However, a marked decline in VLDL secretion is observed in the insulin-resistant state of NAFLD. The unbalance between lipid droplets production with VLDL secretion leads to hepatic steatosis [61, 62].

In addition, the role of cholesterol metabolism in NAFLD is also an attractive topic. Cholesterol can further induce the alteration of cellular redox status and associates with the progression of liver damage [50]. Previous studies

reported that 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CR), the rate-limiting enzyme in the cholesterol synthesis pathway, may be modulated by its thiol redox status and induced by hepatic ROS [63, 64]. More investigations are needed to elucidate the role of ROS in cholesterol metabolism.

2.2.2. Oxidative Stress Involvement in NAFLD Progression. Simple steatosis may progress to NASH with apparent inflammation, advanced fibrosis, and cirrhosis [8]. In 1965, Comporti first reported that increased lipid peroxidation levels in carbon tetrachloride- (CCl_4-) treated rats and described the production of ROS in hepatic injury. Then, in 1972, slater and colleagues hypothesized that ROS plays a causative role in the progression of liver damage [65]. In the context of NAFLD, impaired redox status and ROS accumulation are the origins of hepatic maladaptive responses to fat accumulation, thereby leading to hepatic metabolic impairment and NASH progression [10]. Moreover, oxidative stress-related oxidized phospholipids accumulate and induce mitochondrial dysfunction in hepatocytes [66]. The mitochondrial GSH depletion is also induced by cholesterol accumulation in the progression of NAFL to NASH [67]. Mitochondrial DNA (mtDNA), released from fatty liver-damaged hepatocytes, causes liver inflammation by toll-like receptor 9 (TRL9) activation [68]. Koliaki et al. reported that the mtDNA levels are decreased in patients with more advanced forms of NAFLD [69]. Thus, increased oxidative stress triggers hepatic stress pathways, and maintaining cellular redox homeostasis is a promising strategy for NASH therapy [70].

The hepatocytes are the primary cells affected by lipotoxicity-induced oxidative stress in the liver. However, nonparenchymal cells (NPCs), including HSCs, liver sinusoidal endothelial cells (LSECs), and KCs, are also involved in oxidative stress-induced liver damage [71]. As we know, HSCs are responsible for extracellular matrix (ECM) deposition in the development of liver fibrosis. Cytochrome P4502E1- (CYP2E1-) induced free radicals can activate the transdifferentiating of HSCs. On the contrary, antioxidants could prevent the effect of ROS on increasing collagen production [72]. The NOX1- and NOX2- deficient mice exhibited improved ROS production and hepatic fibrosis in CCl_4 or bile duct ligation-treated models [73]. In addition, mice deficient in antioxidant cytoglobin (Cygb) are susceptible to oxidative stress, inflammation, and fibrosis under diethylnitrosamine (DEN) or a choline-deficient diet [74]. Specifically, LSECs govern the regenerative process initiation, but oxidative stress damages the typical phenotype of LSECs. Aberrant LSEC activation in chronic liver injury induces fibrosis [75, 76]. In addition, oxidative stress increases M1 polarization and promotes proinflammatory cytokines in Kupffer cells [77]. Therefore, it is intriguing to investigate oxidative stress-targeting, possibly even cell type-directed strategies for treating NASH progression.

During liver injury, oxidative stress induces the activation of redox-sensitive transcription factors, such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), leading to an inflammatory response and the activation of cell death pathways in hepatocytes. In NAFLD, ROS regulates NF-kB activation by increasing the expression of proinflammatory cytokine TNF-α [78]. NF-κB, a significant regulator of the inflammatory response, plays a vital role in regulating the transcription of genes involved in the establishment of the immune and inflammatory responses [79]. Reduced NF-κB activity by antioxidants has been proposed as a therapeutic target in NASH due to its anti-inflammatory properties [80, 81]. Moreover, in the development of steatohepatitis, E2-related factor 2 (Nrf2) acts as a significant regulator of the redox balance and mediates anti-inflammatory and antiapoptotic effects of antioxidants [82]. The release and activation of Nrf2 increase the expression levels of the antioxidant genes in hepatocytes with ROS accumulation [83], while Nrf2-knockout mice treated with methionine- and choline-deficient (MCD) diet show exacerbation of liver inflammation and steatosis compared to control mice [84]. Evidence has shown that the dysfunctional Nrf2 in patients with NASH is tightly involved in the grade of inflammation, but not steatosis [85]. In addition, upregulated Nrf2 in senescent hepatocytes is related to the activation of cocultured HSCs. The Nrf2 agonist sulforaphane remarkably inhibits the effect of lipid accumulation-induced hepatocyte senescence on activation of HSCs by the Nrf2-antioxidant response element (ARE) pathway [86]. A new study reported that the dysfunction of redox homeostasis induces hepatocytes to be highly susceptible to proteasome-associated metabolic stress. In comparison, insufficient PPARγ/Nrf2-driven antioxidative response is the main factor [87]. Moreover, the interaction between NF-κB and Nrf2 is also a noticeable target for NAFLD progression. Evidence showed that NF-κB p65 subunit represses the Nrf2/ARE system at transcriptional level by competitive interaction with the binding domain of CREB-binding protein (CBP) [88]. NF-κB dissociates from inhibitor kappa B (IκB) and then translocates to the nucleus. Nrf2 negatively controls the NF-κB signaling pathway by multiple mechanisms, including inhibiting nuclear translocation of NF-κB and blocking the degradation of IκB-α [89].

Overall, oxidative stress plays a central role in the pathogenesis of various liver diseases. Modulation of the antioxidant response emerges as a promising direction to prevent NAFLD progression. Moreover, monitoring oxidative markers can recognize liver dysfunction and observe the response to pharmacological therapies.

3. Transcriptional Regulation of Lipid Metabolism by NRs in NAFLD

Metabolic homeostasis is regulated through a network of programs, involving transcription factors, phosphatases, kinases, and NRs. NRs function directly on the genome to control gene transcription, often in response to small lipophilic ligands. Our group recently reported that nuclear receptor subfamily 2, group F, member 6 (NR2F6), acts as a causal factor in the development of NAFLD by binding directly to the CD36 promoter region in hepatocytes [90]. Moreover, several endogenous and exogenous lipids, including FAs and cholesterol, can serve as physiological NR ligands, and NRs also regulate the metabolism/catabolism

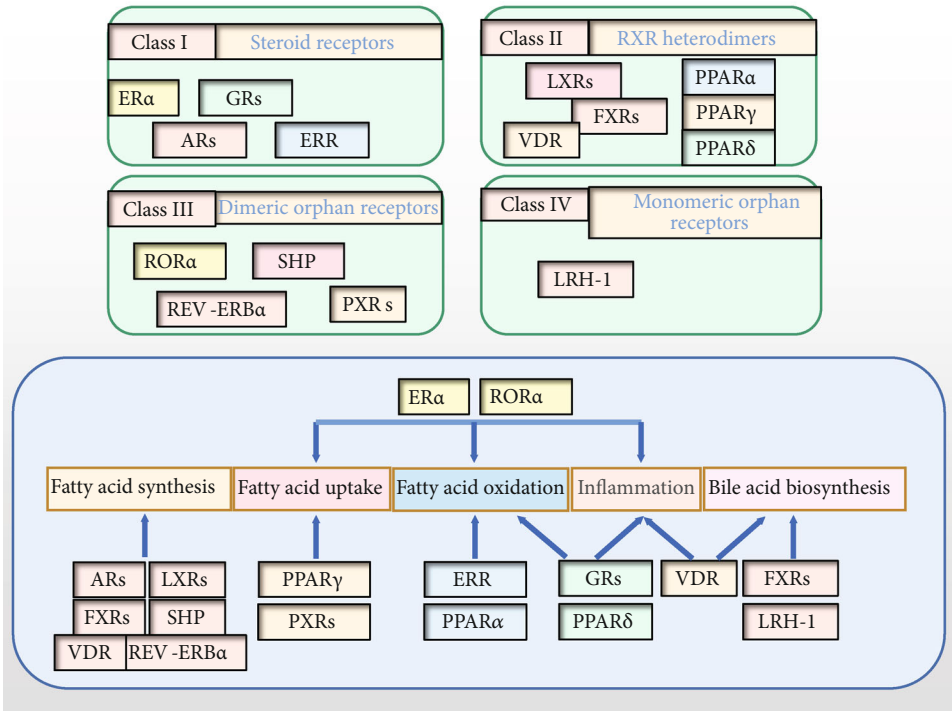

Figure 3: Nuclear receptor involved in hepatic lipid metabolism. Metabolic-related NRs can be classified into four classes according to their domains and ligands. Glucocorticoid receptors (GRs) coordinate energy requirements and mitochondrial oxidative phosphorylation enzyme biosynthesis, affecting lipid oxidation and the progression of inflammation. Androgen receptors (ARs), estrogen receptor α (ERα), and small heterodimer partner (SHP) contribute to the synthesis of fatty acids. ERα decreases fatty acid uptake and ROS generation. Fatty acid oxidation is favored by estrogen-related receptor (ERRs) and peroxisome proliferator-activated receptor α (PPARα). PPARγ regulates fatty acid uptake, and PPARδ is a dual regulator of lipid utilization and inflammatory signaling. Pregnane X receptors (PXRs) play an essential role in lipid uptake by regulating the expression of CD36 and PPARγ. Rev-erbα/β mainly modulates the activity of SREBPs to maintain lipid homeostasis, and it acts as a regulator in bile acid metabolism. Retinoic acid receptor-related orphan receptor α (RORα) regulates lipid metabolism by modulating PPARγ, AMPK, and liver-X-receptor α (LXRα) signaling. LXRs are vital for controlling lipid homeostasis by upregulating gene transcription involved in fatty acid and cholesterol metabolism. Vitamin D receptor (VDR) mainly acts as a regulator in lipogenesis and inflammation. Activation of VDR, farnesoid-X-receptor (FXR), and liver receptor homolog 1 (LRH-1) inhibit bile acid synthesis and prevent toxic accumulation.

of their respective ligands [91]. Notably, the cellular redox state may affect NR ligands or induce conformational changes in NRs to alter their DNA binding or nuclear import [91, 92]. The regulatory roles of some metabolic-related NRs in the development of NAFLD are specifically addressed below (Figure 3).

3.1. Introduction of NRs. NRs can be classified into four classes according to their domains and ligand: class I steroid receptors (e.g., glucocorticoid receptor (GR), androgen receptor (AR), estrogen receptor α (ERα), and vitamin D receptor (VDR)), class II retinoid X receptor (RXR) heterodimers (e.g., retinoic acid receptor (RAR), PPARs, liver-X-receptor (LXR), and farnesoid-X-receptor (FXR)), class III dimeric orphan receptors (e.g., pregnane X receptor (PXR), and class IV monomeric orphan receptors (e.g., liver receptor homolog 1 (LRH-1)). Class I classic nuclear receptors modulate lipid metabolism by reacting to traditional hormones including, but not limited to, thyroid hormone, glucocorticoids, estrogen, and testosterone. Class II nuclear receptors are linked to lipid metabolism and interact with metabolites as metabolic sensors. The third and fourth class of the nuclear receptor family is called orphan receptors, whose ligands have not been identified and functions remain elusive. A typical nuclear receptor has five regions in order: a variable N-terminal region (A/B) usually has a hormone-independent transactivation function, a conserved DNA binding domain (C) with two zinc-finger structures, a variable short hinge region (D), a conserved ligand binding-domain (E), and a variable C-terminal region (F). Regions C and E are signatures of nuclear receptors [93]. The human NR family can be classified into six evolutionary groups. In humans, all forty-eight NRs have these six domains except for 2 NRs in the subfamily NR0B lacking a DNA binding domain, but only half of the NRs are ligand-dependent. When ligands bind to these NRs, the ligand-binding domain (LBD) of the receptor changes conformationally to switch on the activity of the NRs. Notably, steroid receptors may modify enzymes and ion channels independent of transcriptional activation, namely, nongenomic effects [94]. Besides metabolic regulation through binding to multiple hormones, NRs also widely impact the embryonic development and maturation of several organ systems, signaling control in proliferation, and reproduction [95].

3.2. Metabolic-Related NRs

3.2.1. Glucocorticoid Receptor (GR). Chronic stress or excessive exposure to glucocorticoids (GCs) contributes to the pathogenesis of NAFLD [96]. GR mediates the action of GC and may act as a regulator on the effects of ROS in liver diseases. Mitochondrial GR coordinates the energy requirement with the mitochondrial oxidative phosphorylation enzyme biosynthesis, affecting the generation of free radicals [97]. In contrast, antioxidants can decrease the GR expression and increase the activity of the hypothalamus-pituitary-adrenal (HPA) axis in the pituitary [98]. Oversecretion of serum GCs induced by hyperactivity of HPA promotes ROS production in the brain tissues [99].

Lipid accumulation is a vital source of ROS production in the liver. Patients with Cushing's syndrome are inclined to develop hepatic steatosis [98]. GC receptors boost hepatic gluconeogenesis in response to oxidative stress and fasting. Long-term treatment with GCs usually leads to hyperglycemia and hepatic steatosis, partly because GCs can increase the expression of a set of circadian genes in the liver [100]. The detrimental metabolic actions of GCs can be mitigated by timed administration [101]. GRβ coordinates with GRα in GC signaling, inducing high blood triglyceride levels and fatty liver in mice. The activity of glycogen synthase kinase 3 β (GSK3β) increases in the liver of GRβ-Ad mice, in contrast to the decrease in PPARα and fibroblast growth factor 21 (FGF21) [102]. GRs, binding to its ligand corticosteroids, recruit histone deacetylases 2 (HDAC2) and then translocase to the nucleus to bind GC response elements (GREs). The complex promotes the expression of anti-inflammatory proteins by reversing their histone acetylation [103]. Furthermore, GR-dependent fat mass- and obesity-associated (FTO) transactivation and m6A demethylation on mRNA of lipogenic genes are involved in the pathogenesis of NAFLD [104]. Importantly, the investigation of GR signaling provides new strategies for NAFLD treatment. E47 is required to activate GR target genes, as evidenced by free of GC-induced hyperglycemia or hepatic lipid accumulation in E47-knockout mice. Targeting E47 acts as a potential approach to improve the side effects of GC treatment because E47 can selectively regulate a subset of target genes [105]. In the liver, SET domain bifurcated 2 (SETDB2) serves as a GC-induced putative epigenetic modifier to regulate the GR-mediated gene activation. GR-SETDB2 dependent induction of insulin-induced gene 2 (Insig2) inhibits SREBP-1c-driven lipogenesis [106]. Dexamethasone-induced lipid accumulation can be reversed by hairy and enhancer of split 1 (Hes1) reconstitution and subsequent restoration of lipase gene expression (PNL and PNLRP2), highlighting the role of Hes1 in GR-mediated lipolysis. The deficiency of Hes1 in response to GC action explains the steatotic phenotype under starvation, myotonic dystrophy, and Cushing's syndrome [107]. Kruppel-like factor 9- (klf9-) mediated GR activation induces hepatic gluconeogenesis and hyperglycemia. Thus, targeting Klf9 might be a therapeutic approach to GC therapy-induced diabetes [108]. The increased expression of periostin in white adipose tissues mediates the effect of dexamethasone on hepatic lipid accumulation [109]. Moreover, other nuclear receptors also play roles in GR signaling. LXR α/β double-knockout (DKO) mice are protected from dexamethasone-induced insulin resistance by suppressing the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK). While LXRβ is required for the metabolic role of GR, it does not facilitate anti-inflammatory effects. The LXRα/β DKO mice hint at an opportunity to use selective GC agonists to induce anti-inflammatory effects without negative metabolic effects [110]. The selective GR modulator CORT118335 mimics the physiological GC action, stimulating the secretion of VLDL to delay the onset of NAFLD [111].

Notably, the tissue-specific action of GC gives it potential value in the metabolic modification of the liver, adipose tissue, and other tissues. 11β-Hydroxysteroid dehydrogenase type 1 (11β-HSD1) is an enzyme that promotes local GC regeneration. Mice with hepatic overexpression of 11β-HSD1 present increased hepatic lipid flux and impaired hepatic lipid clearance [112]. Global 11beta-HSD1 knockout mice show reduced expression of lipolytic enzymes (HSL and ATGL) in adipose tissue. Impaired hepatic 11β-HSD1 expression in ob/ob mice contributes to the pathogenesis of obesity [108]. Elevated in NAFLD but reduced in NASH, 11β-HSD1 has versatile roles in lipid metabolism and GC-related anti-inflammatory effects [113]. The 11β-HSD1 inhibitor RO5093151 slightly reduces liver-fat content in comparison with placebo [87]. Numerous compounds targeting 11β-HSD1 are under investigation, including natural products such as glycyrrhetinic acid and resveratrol, in the search for a therapeutic approach to NAFLD. However, unselective inhibition of 11β-HSD1 accelerates the activation of HSCs in the liver [108], suggesting that suitable target tissues should be established to bring into full play its inhibitory potency and low toxicity [114].

Overall, glucocorticoids modulate mitochondrial calcium homeostasis, ROS overproduction, and lipolysis [115, 116]. Multiple stressors activate the HPA-axis, which stimulates the adrenal secretion of glucocorticoids, thereby participating in the modulation of immune responses and inflammation [117]. These mechanisms may contribute to the effect of glucocorticoids in treating NAFLD/NASH. Targeting hepatic GR signaling by the star strand miR-192-3p is promising for treating fatty liver and insulin resistance [118]. However, given their complex pharmacology and effects on the immune system, more investigations are needed to evaluate the applicability of GRs as therapeutic targets in NAFLD.

3.2.2. Androgen Receptor (AR) and Estrogen Receptor α (ERα). The prevalence of NAFLD differs in gender and age [119]. Premenopausal women are less likely to develop NAFLD than men of the same age. In the same BMI level (27 ± 3 Kg/m^2), postmenopausal women (60.2%) show a significantly higher prevalence of NAFLD than premenopausal women (42.9%), implying the protective effect of estrogen against hepatic steatosis [120]. Consistently, female mice receiving ovariectomy or tamoxifen treatment also suffer from TG accumulation [121].

Sex steroids are mainly inactivated in the liver. Both AR and ER are expressed in the human male and female livers.

Independent of insulin resistance and obesity, sex steroids play vital roles in lipid and glucose metabolism by regulating the transcription of hepatic metabolic genes including carboxylase (ACC), transcription factor forkhead box protein O1 (Foxo1), SREBP-1, and FGF21. Androgen promotes the progression of hepatic fibrosis and HCC while estrogen has the countereffect [122]. Moreover, estradiol modulates mitochondrial metabolism and activities, including bioenergetics, oxygen consumption rate (OCR), and extracellular acidification (ECAR). Activation of nuclear respiratory factor-1 (NRF-1) transcription may mediate the effect of estradiol on mitochondrial function [123]. Loss of estrogen signaling contributes to hepatic oxidative damage induced by low levels of PGC-1α, exacerbating steatohepatitis in mice with high fat-diet [124]. ERα, the most well-characterized isoform of ER in the liver, can upregulate the expression of miR-125b to decrease fatty acid uptake and synthesis, which protects female mice from NAFLD [125]. Hepatic ERα genetic deletion/mutation mice develop severe hepatosteatosis regardless of gender [122]. Moreover, the estrogen-ER axis also plays a protective role in improving fatty acid oxidation and insulin response in adipose tissue and skeletal [123, 126]. Since cardiovascular events are more frequent in men and postmenopausal women, estrogen replacement therapy may be used in postmenopausal women to prevent cardiometabolic consequences in NAFLD [127].

Estradiol has protective effects in males and females. Whereas androgen only reduces hepatic steatosis in the male group. Liver-targeted deletion of AR promotes fatty liver in male rodent models [128]. Since the incidence of obesity-related HCC is much higher in men than in women, androgen receptors may produce ontogenetic efficacy through alternative mechanisms, such as interaction with signal transducer and activator of transcription 3 (STAT3) [129]. AR plays a role in developing of neovascularization and liver cancer metastasis, which may participate in the progression from NASH to HCC [130].

The hepatic and whole-body metabolisms are improved in diabetic patients with estrogen treatment [48]. Consistently, hepatocyte ERα is considered a relevant molecular target for NAFLD prevention [131]. The effect of activation in ER signaling is complicated. At present, the clinical evidence for drugs that target ER is insufficient.

3.2.3. Vitamin D Receptor (VDR). VDR is highly expressed in gastrointestinal tract and endocrine tissues. Meanwhile, VDR is widely expressed in chronic liver disease patients' inflammatory cells and liver tissue [132, 133]. VDR mediates the genomic actions of vitamin D. It has been proposed that VDR may act as a druggable target for NAFLD in light of the discovery of vitamin D deficiency in NAFLD patients [134].

The primary active form of vitamin D is $1,25(OH)_2D_3$, and the VDR ligand alters DNA-bound VDR homodimers into VDR-RXR heterodimers [135]. Exposing obese mice to $1,25(OH)_2D_3$ prevents lipid accumulation and inflammation in developing NAFLD/NASH [136, 137]. However, vitamin D treatment has not consistently conferred expected therapeutic benefits. A new result of a meta-analysis indicated that vitamin D supplementation does not improve glucose metabolism parameters or lipid levels [138]. Moreover, plasma and hepatic ROS levels are decreased in the liver of VDR-deficiency mice compared to WT mice with acute hepatitis [139]. Several studies have shown that VDR-knockout mice are resistant to the development of liver steatosis and inflammation by decreasing lipid synthesis and promoting fatty acid oxidation [140]. In contrast, some long-term studies reported that VDR deficiency develops hepatic inflammation and fibrosis [141, 142]. Interestingly, nonparenchymal cells in the liver, including HSCs, KCs, and biliary epithelial cells, exhibit higher expression levels of VDR than in hepatocytes. Activation of VDR in hepatocytes promotes lipid accumulation [143], whereas inducing VDR in hepatic macrophages and HSCs attenuates hepatic inflammation and fibrosis [141, 142]. Moreover, ER stress induces increased VDR expression in hepatic macrophages. It has been proposed that VDR signaling regulates a shift between proinflammatory and anti-inflammatory activation during ER stress-induced inflammation to promote hepatic ER stress resolution [142]. Besides vitamin D, bile acids also act as ligands for VDR. It has been reported that hepatic VDR inhibits bile acid synthesis, thus preventing the liver injury in cholestasis [144].

Multiple genetic polymorphisms of the VDR gene or vitamin D-associated genes may explain these contradictory effects of vitamin D treatment in humans. Moreover, VDR-independent mechanisms or the binding ability of VDR to other endogenous ligands may play roles in mediating different effects of vitamin D. Thus, the diversity of VDR ligands and the cell type specificity of VDR activation would likely create difficulties in exploring VDR-targeted strategy for NASH treatment.

3.2.4. Peroxisome Proliferator-Activated Receptors (PPARs). PPARs are named for their interaction with peroxisome proliferators [145]. PPARs act as crucial regulators in lipid metabolism and determine synthesis rate of many enzymes involved in lipid, glucose, bile acid metabolism, adipocyte differentiation, and plasma apolipoprotein regulation. Three types of PPARs work in different organs [146].

PPARα is mainly expressed in the liver and brown adipose tissue. It promotes energy utilization during fasting by boosting fatty acid oxidation and hepatic ketogenesis in the liver. PPARα-knockout mice showed impaired fatty acid oxidation and a lower metabolic rate, resulting in hepatic steatosis, while the rate of VLDL secretion and gluconeogenesis remained unchanged [147]. PPARα modulates liver-derived FGF21 in diabetic ketotic states [148]. Diet-induced obesity leads to disruption of circadian metabolic rhythms in PPARα and SREBP-1. SREBP-1 regulates the production of endogenous PPARα ligands to affect fatty acid oxidation [149]. Krüppel-like factor 6 (KLF6) [150], fatty acids [151], nutrition status [152], miR-27 [153], and other factors all affect the activity of PPARα. Yoo et al. reported that fenofibrate, a PPARα agonist, decreases hepatic fat accumulation through increasing TFEB-mediated lipophagy [154]. Moreover, PPARα is also engaged in anti-inflammatory responses by interacting with NF-κB and activator protein-1 (AP-1) [155]. PPARα agonists reverse

steatohepatitis and improve fibrosis [156]. In the livers of patients with NAFLD, increased poly (ADP-ribose) polymerase 1 (PARP1) activity represses PPARα transactivation and may lead to weakened fatty acid oxidation [157]. PPARα may act as a modulator in the antioxidant response, given the evidence that PPARα expression is correlated with the $Cu2^{+}$, Zn^{2+}-superoxide dismutase (SOD) expression [158]. Moreover, the levels of PPARα and its target genes including acyl-CoA oxidase type 1 (ACOX1) and carnitine palmitoyl transferases 1 (CPT-1) are decreased by H_2O_2 exposure in hepatocytes [159]. Clinical data showed that the expression levels of PPARα negatively correlate with NASH severity [160].

PPARγ regulates lipid storage and insulin sensitivity in adipose tissue, macrophages, and skeletal muscle. Hyperinsulinemia accelerates the development of hepatosteatosis in a PPARγ-dependent manner [161]. PPARγ protein expression is significantly downregulated in NAFLD, and PPARγ transgene liver-knockout mice show a similar decrease in the expression levels of lipogenic genes such as fatty acid synthase (FAS) and SCD-1 [162, 163]. PPARγ in macrophages of adipose tissue regulates genes involved in fatty acid synthesis, β-oxidation, and insulin-stimulated glucose uptake [149]. PPARγ activators enhance cholesterol efflux in human macrophages [155] and suppress inflammatory cytokines in monocytes [164]. In fibrosis regulation, the reduced expression of PPARγ results in inhibited HSC activation and increased collagen production [165]. In addition, PPARγ agonists are insulin sensitizers that have been used to treat diabetes. Other studies have shown the prospects of nonagonist PPARγ ligands for their antidiabetic actions [166]. Notably, a new study showed that PPARγ2 translocates to the nucleus and activates signal transduction through a complex of PPARγ2 and transportin 1 (Tnpo1) that forms via redox-sensitive disulfide bonds. The increased DNA-bound PPARγ induces lipid accumulation in the liver. This evidence supports that a redox environment is a potential therapeutic target in the treatment of PPARγ-related diseases [167].

PPARδ is a dual regulator of lipid utilization and inflammatory signaling. Meanwhile, it can effectively improve insulin sensitivity and reduce atherogenic dyslipidemia [168]. PPARδ stimulates FFA breakdown, fat depletion, and weight loss. microRNA-122 regulates hepatic fatty acid and cholesterol metabolism by targeting various genes, including PPARδ [169]. The PPARδ agonist GW501516 increased fat oxidation in skeletal muscle [170] and decreased serum ApoC-III concentration to help hepatic VLDL secretion in a small clinical data sample [171]. In a diet-fed obese diabetic mouse model, the PPARδ agonist seladelpar (MBX-8025) reversed insulin insensitivity and improved NASH pathology independent of weight loss [172].

PPARβ/δ shares several similar functions to PPARα in inducing fatty acid oxidation and improving NAFLD by functioning in the liver and other tissues [173]. Moreover, the effects of PPARβ/δ on NAFLD, including its capacity to decrease lipogenesis, improve inflammation and endoplasmic reticulum stress, alleviate insulin resistance, and attenuate liver injury [174]. PPARβ/δ agonists have been employed to prevent fibrosis in preclinical animal studies [175]. Thus, synthetic or natural ligand-induced activation of hepatic PPARβ/δ provides a promising therapeutic strategy for NAFLD. Despite no PPARβ/δ activator being approved for patients with NAFLD/NASH, various compounds are under clinical development at different stages.

3.2.5. Liver X Receptor (LXR). LXRs, including LXRα (NR1H3) and LXRβ (NR1H2), are mainly expressed by the digestive tract where lipids are digested and absorbed. They are nuclear receptors that regulate the metabolism of several vital lipids, including cholesterol and bile acids [176]. LXRs upregulates cholesterol 7alpha-hydroxylase 1 (CYP7A1) in the reverse cholesterol pathway. Moreover, LXRs act as glucose sensors and strengthen fatty acid synthesis by activating SREBP-1c and carbohydrate responsive element-binding protein (ChREBP) [177]. As reported, hepatic insulin resistance leads to an increase in the activity of SREBP-1a, 1c and -2, resulting in elevated fatty acid synthesis [178, 179]. LXRα plays a crucial role in the insulin-induced proteolytic process to activate SREBP-1c. However, LXR agonists cannot affect SREBP-2 or its downstream targets [180]. Target genes of LXRs include hepatic cholesterol efflux modulator ATP-binding cassette transporters (ABCA1) and apolipoproteins as well as mitochondrial metabolic regulator PARP1 in brown adipose tissue and skeletal muscles [181]. LXRα/β also plays a role in the dynamic modulation of membrane phospholipid composition through Lpcat3, indirectly regulating the ER stress and inflammation in the liver [182]. LXR-null mice show impaired reverse cholesterol transport and increased atherosclerosis [183]. LXRα/β-deficient-ob/ob (LOKO) mice exhibits improved insulin sensitivity and weaken SREBP-1c and ChREBP activity in the liver accompanied by impaired hepatic lipogenesis [184]. Although liver-specific activation of LXRs does not impact reverse cholesterol transport, intestinal-specific LXR activation suppresses the absorption of cholesterol and improves lipoprotein profile [185]. When in the state of hypercholesterolemia, LXRα maintains peripheral cholesterol homeostasis [186], and LXRβ can compensate for the antiatherosclerosis effect in the absence of LXRα [187]. Pharmacological activation of LXR by GW3965 and T0901317 increases transintestinal excretion of plasma cholesterol in different mouse models [185, 188]. Moreover, LXR functions are required for Kupffer cell identity and survival in response to NASH-induced environmental signals. These results show the regulator role of LXR in the development of NASH by controlling diversification in macrophage phenotypes [189].

The phosphorylation state of LXRα is associated with the progression of NAFLD [190]. LXR activity can be enhanced through deacetylation by sirtuin type 1 (SIRT1) [191]. Toll-like receptor- (TLR-) LXR signal crosstalk works under the regulation of transcription cofactor nuclear receptor coactivator 5 (NCOA5) [192]. Then, activation of adenosine monophosphate-activated protein kinase (AMPK) supports the S6 kinase 1- (S6K1-) mediated inhibition of LXR activity in lipogenic gene induction [193], while uncoordinated 51-like kinase 1 (ULK1) has the opposite function by reducing

NOCR1 nuclear uptake and its interaction with LXR, which ends in a decrease in SCD-1 expression [194]. Fatty acid intake may also impact the expression of LXRs and its downstream targets ABCA1 and SREBP-1c [195].

However, some studies reported inconsistent experimental results about the role of LXRα in patients with NASH [196, 197]. Besides improving lipid accumulation in the liver, LXRβ-selective and LXRα/β-dual antagonism may lead to hypercholesterolemia in nonhuman primates [198], which represents a barrier to the development of LXR antagonist as a therapy for NAFLD.

3.2.6. Farnesoid X Receptor (FXR). FXR is widely expressed in several tissues and has been demonstrated to be the primary sensor for modulating bile acids uptake and synthesis, gluconeogenesis, and fatty acid oxidation [199]. Gain of FXR function studies in nongastrointestinal tissues indicates that FXR signaling improves various experimentally induced metabolic and immune diseases [200].

Hepatic FXR expression can be upregulated by hyperglycemia and repressed by insulin. Our previous study showed that FXR downregulation accounts for the aging-induced fatty liver and ER stress represses FXR expression by inhibition of hepatocyte nuclear factor 1 alpha (HNF1α) transcriptional activity in old mice [201]. Moreover, we found that suppressing FXR expression by Yin Yang 1 (YY1) increases obesity-associated hepatosteatosis [202]. Interestingly, lean NAFLD patients have significantly higher FXR activity and a distinct microbiota profile, but their favorable metabolic profile not help resist hepatic lipid accumulation [203].

FXR agonists reduce lipogenesis by the interaction with LXR and small heterodimer partner (SHP) [204]. FXR-null mice show lower expression of SHP and higher serum and hepatic triglyceride levels [205]. FXR negatively regulates glycolysis and lipogenesis in the liver through inhibition of ChREBP [206]. FXR increases the expression and secretion of gene FGF21 [207]. Insulin sensitivity and glucose homeostasis are also impaired in mice with FXR depletion [208]. FXR-SHP-LRH1 pathway represses bile acid biosynthesis by targeting CYP7A1 [209]. Moreover, hepatic FXR mediates the protective effect of AMPK activators on oxidative injury and mitochondrial dysfunction induced by serum deprivation [210]. Intestinal reclamation of bile salts also works under the FXR-SHP-LRH1 pathway [211]. Interestingly, intestinal FXR takes charge of bile acid uptake [212] and changes hepatic lipidomics through the microbiome [213]. Hepatic FXR contributes to lipid accumulation under a cholesterol diet rather than intestinal FXR [214]. The pentose phosphate pathway regulates the expression of FXR in the liver, suggesting T2DM patients may suffer from lipid and bile acid dysregulation due to hyperglycemia [215].

FXR integrates the protein kinases A (PKA) and the forkhead box protein A2 signal in hepatic glucose production [216]. Src-mediated FXR phosphorylation after a meal maintains bile acid homeostasis [217]. The SUMOylation of FXR is higher in HSCs from NASH patients than healthy donors. Moreover, SUMOylation inhibitor can restore FXR activity, thus synergizing with FXR agonists when treating NASH [218]. FXR acetylation is regulated by SIRT1 and p300, which constitutively elevated in metabolic syndrome [219]. Besides the dysregulated acetyl/SUMO switch of FXR [220], the glucose-sensing O-GlcNAcylation pathway contributes to NAFLD in obesity [221].

Obeticholic acid, an FXR agonist, is approved by the FDA for biliary cholangitis therapy but not for NASH resolution. Nevertheless, FXR remains an attractive target for NAFLD/NASH. It is not clear whether redox states or ROS-derived compounds may directly regulate the FXR signaling pathway. This topic needs more investigation.

3.2.7. Pregnane X Receptor (PXR). PXR is abundantly expressed in the liver and gut, targeting metabolic enzymes and transcription factors such as CD36 and PPARγ [222]. PXR ablation alleviates steatohepatitis in high-fat diet-induced obesity mice and genetic obesity model ob/ob mice, suggesting the therapeutic potential of PXR antagonists in NAFLD [223]. PXR target gene Cyp3a11 was consistently increased 3-4-fold in addition to the increased microsomal Cyp3a enzymatic activity at all stages of NAFLD [224]. Further, Di (2-ethylhexyl) phthalate- (DEHP-) induced ROS production activates the Nrf2 and nuclear xenobiotic receptor (NXR) system including aryl hydrocarbon receptor (AHR), PXR, and constitutive androstane receptor (CAR) in the development of liver injury [225]. Despite the promotion of hepatic steatosis and insulin resistance, PXR also shows antifibrotic and antiproliferative efficacy. Rifampicin activates PXR in human hepatic stellate cell line LX-2 and decreases the expression of fibrosis-related gene TGF-β1 and reduces the secretion of proinflammatory cytokine IL-6 [226].

It is worth noting that the consequence of PXR activation on overall metabolic health has not yet been fully elucidated, and varying experimental results on the effect of PXR activation or deficiency on metabolic disturbance have been reported [227]. Moreover, obese levels of parental mice decrease the hepatic expression of PXR in offspring [228]. At present, PXR is not being targeted in clinical trials for NAFLD therapy due to its uncertain role in hepatic metabolism.

3.2.8. REV-ERBα/β and Retinoic Acid Receptor-Related Orphan Receptor α (RORα). Circadian rhythm, in other words, the sleep-wake cycle, regulates lipogenesis independent of the fasting-feeding process [229]. Circadian oscillations are observed in the expression of Rev-ERBα/β and ROR$\alpha/\beta/\gamma$ in the liver. REV-ERBα/β binds to RORE to recruit histone deacetylase 3 (HDAC3) and NCoR in rodent models to inhibit lipogenesis during daytime, while RORs bind to RORE instead of REV-ERBα/β at night [230].

REV-ERBα modulates the activity of SREBPs to maintain lipid homeostasis and regulates the expression of CYP7A1 to balance bile acid metabolism [231]. A large proportion of REV-ERBα target genes in hepatic lipid metabolism also requires the presence of HNF6 to work correctly [232]. Pharmacological activation of REV-ERBα by SR9009 attenuated hepatic steatosis, insulin resistance, inflammation, and fibrosis in mice with intestinal barrier dysfunction-related disorders and NASH [233]. Compared

with REV-ERBα knockout mice, REV-ERBα and REV-ERBβ double-knockout mice exhibit more severe hepatic steatosis, failing to recruit HDAC3 and NCoR in the liver, justifying the collaboration of REV-ERBs in hepatic lipid metabolism [234]. Hepatic REV-ERBα and Rev-ERBβ double-knockout impairs daily rhythms of a subset of liver genes and alters the diurnal rhythm of de novo lipogenesis in mice. Moreover, the loss of hepatic REV-ERBs also remodels the rhythmic transcriptomes and metabolomes of nonhepatocytic cells within the liver [235]. In the light of the loop feedback in Clock/BMAL1 and REV-ERBs, both agonists and antagonists of REV-ERBs could be a potential therapeutic approach to reestablish metabolic balance [236].

In contrast to REV-ERBs, RORα act as a transcriptional activator and coordinate the circadian rhythms of lipid metabolism and inflammation in the liver. RORα recruit HDAC3 to PPARγ promoters as a negative regulator of lipogenic genes [237]. Moreover, RORα attenuates hepatic steatosis through AMPK activation and LXRα repression [238]. Liver-specific knockout of RORα aggravates NASH development by impairing mitochondrial function. The expression level of PGC-α is positively related to RORα in patients with NASH [239]. In mice models, RORα decreases lipid peroxidation and inflammatory cytokine (TNFα, IL-1β) levels to prevent NASH. JC1-40, a RORα activator, controls M2 polarization and reduces oxidative stress to improve symptoms of NASH [240, 241]. Targeting RORα is an effective strategy for reducing ROS generation and increasing antioxidant capacity in endothelial cells and prepubertal cumulus cells [242, 243]. Moreover, RORα regulates polarization in liver macrophages, which plays a fundamental role in liver fibrosis. RORα agonist SR1078 validates that by suppressing HSC proliferation potently [244]. Whereas macrophage-specific knockout RORα does not prevent insulin resistance and NASH [245]. Thus, the roles of ROR in different cell types need consideration. In addition, RORα may increase its ligand maresin 1, which in return increases the expression and transcriptional activity of RORα. This autoregulatory circuit provides a new potential therapeutic target for the NASH treatment [246].

In the liver of patients with NASH, RORα expression is reduced [247]. The clinical application of targeting RORα remains to be further investigated for NAFLD pharmacological therapeutics.

3.2.9. Estrogen-Related Receptor (ERR). ERR family is comprised of ERRα, ERRβ, and ERRγ. Both in vitro and in vivo models, regulation of ERRα activity via genetic or pharmacological manipulation has been fundamental in delineating the vital roles of ERRα in lipid and carbohydrate metabolism, as well as in mitochondrial function under both physiological and pathological conditions [248]. The expression of fatty acid synthesis genes (Acly, Fasn, and Scd-1) shows a rise in ERRα-null mice, supporting the prominent role of ERRα in rapamycin-induced NAFLD [96]. Inhibition of ERRα decreases triglyceride biosynthesis and prevents hepatic steatosis. Targeting glycerophosphate acyltransferase 4 and glycerolipid synthesis is an important mechanism for ERRα-regulated NAFLD progression [249]. Moreover, ERRα participates in the weakened lipid oxidative catabolism after fasting-refeeding in mice [250].

In addition, liver-specific ablation of ERRγ normalizes blood glucose levels in db/db mice. GSK5182, an inverse agonist of ERRγ, may be a treatment option to inhibit hepatic gluconeogenesis [251]. ERRγ directly regulates the transcription of lipogenic gene srebp-1c via binding to an ERR-response element. Consistently, GSK5182 significantly improved NAFLD in chronically alcohol-fed mice by inhibiting SREBP-1c-mediated fat accumulation [252]. Moreover, the expression levels of ERRγ and fibrotic genes are elevated in liver tissue of obese patients. Overexpression of ERRγ increased fibrinogen expression in hepatocytes [253].

Given the experimental evidence, targeting hepatic ERRα activity may have therapeutic potential. The complex interplay of the three ERRs in the development of NAFLD and metabolic syndrome should be considered in future research and drug development.

3.2.10. Small Heterodimer Partner (SHP). In 1996, Seol and his colleagues reported that SHP is an orphan member of the NR superfamily that contains the dimerization and ligand-binding domain found in other family members. However, the conserved DNA binding domain is lacking in the SHP gene. In general, SHP is a negative regulator in receptor-dependent signaling pathways by inhibiting transactivation induced by the superfamily members with which it interacted [254]. In the liver, SHP involves the pathogenesis of steatosis by regulating the transcriptional activity of SREBP-1c [255]. SHP knockout mice show decreased expression of genes involved in lipogenesis (PPARγ and ACC) and increased expression of genes involved in lipid oxidation and export (PPARα and VLDL) [256]. A new study reported that SHP overexpression in mice inhibits lipogenesis in a DNA methyltransferase-3a- (DNMT3A-) dependent manner [257]. Moreover, SHP expression is regulated by other NRs in livers. Our previous study found that in obese mice, SHP deficiency blunted the effect of estrogen in improving hepatic steatosis [258]. FXR can bind to the SHP promotor region and induce its expression. FXR-SHP axis is closely associated with bile acid and lipid metabolism and represents a promising target for treating NAFLD. New evidence has shown that miR-802-mediated defective FXR-SHP regulation promotes insulin resistance and the development of fatty liver [204, 259]. However, the expression level of FXR, but not SHP, was decreased in the liver tissue of patients with NAFLD [260]. Notably, SHP may serve as a ROS-sensitive regulator in the effect of glycochenodeoxycholic acid (GCDCA) treatment on improving cell death and oxidation stress [261]. At present, the role of SHP in the diagnosis and treatment of NAFLD in humans remains unclear, and the data of related clinical trials are lacking.

3.2.11. Liver Receptor Homolog-1 (LRH-1). LRH-1 is expressed in the intestine, liver, pancreas, and ovary. In metabolic fields, LRH-1 regulates bile acid biosynthesis and reverses cholesterol transport [262]. SUMOylation, a kind of posttranslational modification, is primary for LRH-1 regulation. SUMO-deficient LRH-1 knock-in mice have better

lipid metabolism and are less likely to develop atherosclerosis because of the inhibition of a set of genes linked to reverse cholesterol transport [263]. LRH-1 mutant mice have defects in SUMOylation and represent enhanced SREBP-1 expression and promoted DNL in high-fat diet or high sucrose diet [264]. Hepatic LRH-1 deficient mice show reduced hepatic glucose fluxes followed by a reduction in DNL because of the direct inhibition of glucokinase in transcription level by LRH-1, indicating LRH-1 plays a role in glucose-sensing in postprandial glucose and lipid metabolism [265]. Besides the glucose sensor, LRH-1 also functions as a phospholipid sensor to maintain the hepatic arachidonoyl phospholipids pool [266]. Coimmunoprecipitation confirms the synergy of FXR and LRH-1 in the activation of Cyp7A1 and fasn promoters in mice liver [267]. LRH-1 ligand dilauroyl phosphatidylcholine (DLPC) activates phosphatidylcholine signaling pathway and displays antidiabetic and lipotropic effects in mice [268]. LRH-1 agonist BL001 impedes β cell apoptosis in T2DM while it favors insulin secretion [266]. Notably, in the livers of LRH-1-knockout mice, the NADPH/$NADP^+$ and GSH/GSSG ratios are decreased, supporting the role of LRH-1 in facilitating NADPH generation [269, 270]. In addition, evidence has shown that ROS production induced by a high concentration of palmitate in hepatocytes is reduced after LRH-1 agonist RJW101 intervention [271]. Thus, LRH-1 participates in metabolic processes to govern liver physiology and pathology. However, more clinical studies are needed to clarify the role of LRH-1 in treating NAFLD.

4. Clinical Research Findings Involving Metabolic Therapeutic Targets

We have briefly presented how NRs participate in modulating metabolic adaption and NAFLD/NASH progression. Given these findings, selecting transcription factors for the treatment of metabolic disorders is on the agenda. Here, we introduce the compounds ongoing in clinical trials.

4.1. PARs. PPARα has been proposed as a promising therapeutic target based on its function in lipid and apolipoprotein regulation and inflammation and fibrosis resolution [272]. PPARα agonist fibrates were introduced more than 35 years ago to improve the serum lipid profile and reverse atherogenic dyslipidemia [273]. In the obese animal models, fenofibrate treatment markedly improves hepatic oxidative stress and steatosis, ameliorates dyslipidemia, and improves insulin resistance [274, 275]. In addition, bezafibrate reduces plasma triglycerides (-49%) and hepatic triglycerides (-78%) in fructose-enriched diet- (FED-) treated rats [276]. However, the side effects of PPARα agonist, including hepatomegaly and aminotransferase abnormalities, were observed in the animal studies need to be emphasized.

PPARγ agonist pioglitazone belongs to thiazolidinediones. Thiazolidinediones improve insulin sensitivity by enhancing the differentiation of adipocytes. Pioglitazone 30 mg shows slight improvement in fibrosis in a 24 months clinical trial (NCT00063622)[277]. Thickened subcutaneous adipose tissue is frequently observed in thiazolidinediones, and pioglitazone is no exception. Heart failure, cardiogenic edema, and bone fractures in females [278] remain barriers for further clinical application.

PPARδ agonist seladelpar decreases liver enzyme levels, inflammation marker levels, insulin resistance, circulating, and atherogenic dyslipidemia. It also reduces hepatic TGs [279]. However, it has been recently reported that seladelpar fail to decrease liver fat as quantified by magnetic resonance imaging in a phase 2 trial (NCT03551522).

Dual PPARα/δ agonist elafibranor (GFT505) shows positive effects in glucose and lipid metabolism and reduces inflammation in NASH patients in a phase 2 clinical trial. Although elafibranor mildly increases serum creatinine, it is well-tolerated and does not exacerbate liver fibrosis [280]. A phase 3 clinical trial for patients with NASH is in progress (NCT02704403).

Dual PPARα/γ agonist saroglitazar was first launched to treat diabetic dyslipidemia, uncontrolled by statins [281]. In NASH mice models, saroglitazar dose a better job than pioglitazone and fenofibrate in improving liver histopathology and biochemistry [282]. A phase 2 clinical trial (NCT03061721) of saroglitazar magnesium was finished in April 2020, aiming at lowering the serum ALT level in NASH. Current data showed that saroglitazar magnesium also improves the histological appearance in NASH. The drug firm Zydus Cadila has filed a new drug application of saroglitazar magnesium in NASH.

Pan PPAR$\alpha/\delta/\gamma$ agonist lanifibranor shows positive effects on histology with a significant benefit over placebo for resolution of steatohepatitis, regression of fibrosis, and the combination of both [283]. A phase 2 clinical trial for patients with T2DM and NAFLD is in progress (NCT03459079).

4.2. FXR. FXR plays a critical role in maintaining bile acid and cholesterol homeostasis and regulating hepatic glycogen synthesis. FXR is a promising target for NAFLD/NASH [284]. FXR agonists targeting the gut-liver axis are promising for NAFLD/NASH for they not only relieve hepatic steatosis but also resolve fibrosis at histology level by antagonizing NFκB [285, 286].

FXR agonists GW4064, GSK2324, chenodeoxycholic acid (CDCA), and fexaramine (Fex) have been tested in rodent models. GW4064, a synthetic agonist of FXR, lowers blood glucose and improves hepatic glycogen storage in normal and db/db mice regardless of whether they are fasted or fed [287]. GW4064 suppresses hepatic apolipoprotein CIII and apolipoprotein A-I [288] expression to prevent mice from coronary heart disease. FXR activation with the FXR agonist GSK2324 controls hepatic lipids via reduced absorption and selective decreases in fatty acid synthesis. The results in tissue-specific FXR KO mice show that hepatic FXR controls lipogenic genes, whereas intestinal FXR controls lipid absorption [289]. FXR activation by chenodeoxycholic acid (CDCA) in Zucker (fa/fa) obese rats reverse insulin resistance and hepatic steatosis [290]. Intestine-selective FXR inhibition by glycine-β-muricholic acid (Gly-MCA) improves metabolic dysfunction by reducing intestinal-derived ceramides [291]. Gut-restricted FXR

agonist fexaramine (Fex) induces browning white adipose tissue, increases the metabolic rate in brown adipose tissue, alters bile acid composition, and improves hepatic steatosis and insulin sensitivity [213]. Fex improves FXR-gut microbiota-TGR5-GLP-1 signaling and increases FGF15 secretion without changing appetite in mice [292].

FXR agonist obeticholic acid successfully lowers serum markers representing hepatocellular injury (ALT, AST) and oxidative stress (GGT) in mice. Obeticholic acid also lowers serum LDL-C and increases liver LDLR expression [293]. In human patients, obeticholic acid (trade name Ocaliva) was first approved to treat primary biliary cholangitis for its function in reducing alkaline phosphatase and bilirubin levels to prevent cirrhosis [294]. Besides the anticholestatic and antifibrotic effects, obeticholic acid shows great potential in treating NAFLD. Obeticholic acid shows efficacy in improving the insulin sensitivity of NAFLD and T2DM patients. However, it also causes an increase in LDL and a reduction in HDL [162]. Biopsy proved the histologic improvement by obeticholic acid in parallel to the change of aminotransferases [295]. In the interim analysis from a phase 3 clinical trial, obeticholic acid 25 mg daily significantly improved histological endpoints in advanced fibrosis due to NASH compared to the 10 mg low dose group or placebo [296]. Side effects like pruritus can be conquered by symptomatic treatment, and elevated LDL cholesterol levels can be treated with lipid-lowering agents like statins. Obeticholic acid is the first drug application for NASH-related liver fibrosis accepted by the FDA.

Nonsteroidal FXR agonist cilofexor (GS-9674) (NCT02854605) [295], nonbile acid FXR agonist tropifexor (LJN452) (NCT02855164) [297], and nidufexor (LMB763) (NCT02913105) [298] are undergoing phase 2 clinical trial in NASH patients.

5. Hormones Affecting the Expression of NRs in the Hepatic Lipid Metabolism

5.1. Thyroid Hormones. Thyroid hormones 3,5,3′-triiodothyronine (T3) and 3,5,3′,5′-tetraiodothyronine (T4) play essential roles in developmental process, differentiation, growth, and metabolism in cells through the genomic or nongenomic pathways. The genomic action occurs through their interaction with nuclear receptors TRα and TRβ, together with coactivators or corepressors to modulate gene expression and protein synthesis [299]. Thyroid hormones are potent regulators in body weight, lipogenesis, lipid metabolism, and insulin resistance. Evidence confirmed that the liver is a significant target for thyroid hormones [300]. Moreover, TRβ is mainly expressed in the liver tissue, and TRα is more common in bone and cardiovascular organs. Mice with a dominant-negative mutation in TRβ (Thr$\beta^{PV/PV}$) develop hepatic steatosis and have larger livers. Moreover, these mutated mice exhibit upregulated activation of PPARγ signaling and reduced fatty acid β-oxidation, leading to the development of steatosis [301]. In addition, thyroid hormones also regulate the expression and activities of many NRs involved in lipogenesis, such as LXR [222]. HMG-CoA reductase, the limiting enzyme of cholesterol synthesis, is inhibited by thyroid hormones [302]. Meanwhile, liver fibrosis begins with injury and mitochondrial dysfunction in cells. The increased free fatty acids and ROS induce lipid peroxidation and activate HSCs. Under liver injury, the dominant hormone receptor becomes TRα instead of TRβ. TRα produces a more robust wound-healing response in the fibrogenic process [303].

5.2. Melatonin. The pineal hormone melatonin is synthesized from tryptophan via 5-hydroxytryptamine and is considered a potent regulator of oxidative damage in different vertebrates [304]. Melatonin acts through specific receptors, including melatonin 1 (MT1), MT (2), and MT (3) receptors as well as a nuclear receptor belonging to the orphan nuclear receptor family. M1 is the one mainly expressed in the liver tissue. Exciting, therapeutic effects of melatonin on improving fatty liver are observed in obese rats by inhibiting oxidative damage [305]. Moreover, in diabetes and obesity, melatonin supplementation has been found to protect liver function by recovering mitophagy via blockade of nuclear receptor 4 A1 (NR4A1) [108]. In hepatocytes exposed to H_2O_2, melatonin treatment reduces the levels of oxidative stress and ROS generation, thereby improving liver damage [306]. Meanwhile, melatonin induced a dose- and time-dependent inhibition on the proliferation of hepatocytes [307]. Chronic CCl_4 exposure induces collagen deposition and oxidative stress, while melatonin protects against liver fibrosis via increased mitophagy and mitochondrial biogenesis [308]. Therefore, melatonin is considered a potent antioxidant drug to improve fatty liver [309].

6. Summary and Outlook

The onset of NAFLD is characterized by changes in redox status in the hepatocellular system that lead to ROS generation and impaired hepatic metabolism. Oxidative stress is also a causative factor in the pathologies of the fatty liver. The molecular mechanisms accounting for these alterations are not entirely understood, but activation of NRs plays a vital role in regulating the redox status and the metabolic network. Antioxidant molecules favorably modulating the cellular redox environment may also regulate NRs that play a role in lipid metabolism. This autoregulatory circuit provides more potential therapeutic strategies for NAFLD/-NASH treatment.

Nuclear receptors have largely maintained their dominance of the drug target space for human use [310]. Based on the vital role of NRs in regulating hepatic metabolism and on the promising results observed in animal models with NAFLD, drugs which interfere with NRs are among the strongest candidates for NAFLD therapy. However, several clinical trials utilizing pharmacological manipulation of NRs have yielded conflicting results about the efficacy and safety of these drugs. Despite specific favorable metabolic effects, PPARα activator fibrates have failed to improve hepatic steatosis or NASH in humans [311, 312]. PPARγ agonist rosiglitazone has shown the impact of resolution on hepatic steatosis but not on NASH. It might increase in

bone fractures, fluid retention, and cardiac decompensation [313–315]. In practice, considering drug safety, pioglitazone is the only thiazolidinedione (TZD) in use clinically today for the treatment of T2DM. Although previous studies have indicated a limited efficacy of activating individual PPARs, ongoing clinical trials show that dual and pan-PPAR agonists might serve as promising strategies for NASH therapy. Moreover, the FXR agonist obeticholic acid shows significant benefit in phase 3 interim results and remains the candidate for first conditional approval as a NASH therapeutic [316]. Additional research is needed to confirm this promise and address concerns about tolerability and side effects.

Drug discovery programs targeting NRs have been greatly facilitated by the emergence of ligand-binding domains and the resulting opportunities to identify new chemical activators/inhibitors. NRs act directly on the genome to control transcription. Unlike targeting traditional drugs, targeting transcription factors and their cofactors results in less drug resistance but is more likely to have other side effects. For instance, PPARγ agonist rosiglitazone treatment-induced adverse events such as bladder cancer and heart failure have become highly aware in clinical application. Thus, selective inhibition/activation of a transcription factor may require a low dose with minor side effects. Moreover, it seems that the next-generation dual-PPAR or pan-PPAR agonists are presently the most promising strategies, addressing the therapeutic benefits of targeting more than one PPAR subtype in the treatment of NASH [146]. In addition, given the diverse actions of NRs in multiple organs and how they affect metabolic crosstalk with various layers of complexity, clarifying the tissue-specific and cell-type-specific roles of NRs is essential for precise pharmacological treatments. Nowadays, the advancements made to the development of "human-on-a-chip" models seem as effective strategies for testing novel drug candidates. The system provides a simple but unique platform to evaluate preclinical drug efficacy and reassess human dosing regimens [317, 318]. Moreover, by implementing in these chips, patient-derived stem cells carrying high-risk genetic backgrounds for developing NASH, the evaluation of personalized therapies might ever become a reality [319]. Furthermore, some natural compounds have been reported to treat NAFLD by acting on NR-targeted pathways with fewer adverse reactions, presenting a promising therapeutic prospect [320]. These drugs are naturally present in the human body and function by stimulating the physiological status. Notably, in addition to hepatocytes, targeting NRs should include anti-inflammation/fibrosis in nonparenchymal cells. NR ligand-based therapies are not the only strategy for NAFLD. Targeting posttranslational modifications such as acetylation of NRs and coregulators is also a promising direction for dealing with changes in the redox-microenvironment. Therefore, targeted redox-dysregulated NRs is a promising strategy for treating NAFLD.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ting Hong and Yiyan Chen contributed equally to this work.

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