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HMG-CoA reductase inhibitors activate caspase-1 in human monocytes depending on ATP release and P2X7 activation

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ABSTRACT

Recent studies have demonstrated the stimulatory effects of HMG-CoA reductase inhibitors, statins, on IL-1β secretion in monocytes and suggest a crucial role for isoprenoids in the inhibition of caspase-1 activity. In this study, we further elucidated the molecular mechanisms underlying the stimulatory effects of statins on caspase-1. Three commonly recognized mechanistic models for NLRP3 inflammasome activation (i.e., ATP/P2X7/K^+ efflux, ROS production, and lysosomal rupture) were investigated in statin-stimulated human THP-1 monocytes. We found that fluvastatin and lovastatin can synergize with LPS to trigger inflammasome activation. Moreover, statin-induced caspase-1 activation and IL-1β production in LPS-primed THP-1 cells are dependent on GGPP deficiency and P2X7 activation. In particular, increased ATP release accounts for the action of statins in P2X7 activation. We also provide evidence that statin-induced moderate ROS elevation is involved in this event. Moreover, the cathepsin B inhibitor was shown to reduce statin-induced IL-1β secretion. Consistently statins can induce cathepsin B activation and lysosomal rupture, as evidenced by LysoTracker staining. Statins also increase intracellular ATP secretion and IL-1β release in primary human monocytes and murine macrophages. Notably, exogenous ATP-elicited P2X7 activation and consequent IL-1β release, an index of direct NLRP3 inflammasome activation, were not altered by statins. Taken together, statin-induced enhancement of inflammasome activation in monocytes and macrophages covers multiple mechanisms, including increases in ATP release, ROS production, and lysosomal rupture. These data not only shed new insight into isoprenylation-dependent regulation of caspase-1 but also unmask mechanisms for statin-elicited inflammasome activation. *J. Leukoc. Biol.* 93: 289–299; 2013.

Introduction

IL-1β is a proinflammatory factor involving in many kinds of inflammatory diseases [1]. Currently, two signal steps are considered to be required for IL-1β production: a NF-κB-dependent signal that induces the synthesis of pro-IL-1β and a second signal that triggers the proteolytic processing of pro-IL-1β to mature IL-1β. Activation of cytosolic procaspase-1-containing multiprotein complexes called inflammasomes initiates autocatalytic activation of caspase-1 and results in cleavage of the p20 subunit, which subsequently mediates the processing and secretion of mature IL-1β [2]. Currently, NLRP3 is the best well-known sensor to detect pathogen-derived factors as well as danger-associated molecules [1, 3]. The latter includes ATP, monosodium urate, β-amyloid, calcium pyrophosphate dehydrate, cholesterol, silica, and asbestos [4].

Currently, three models, which are not necessarily exclusive, have been proposed for NLRP3 inflammasome activation [3, 4]. In the first channel model, exogenous ATP induces K^+ efflux via P2X7 receptor-gated ion channels and recruitment of pannexin 1 to form a large, nonselective pore, allowing the entry of NLRP3 agonists into the cell. In the second model, lysosomal instability induced by phagocytosed large particles or crystals can release the lysosomal proteinase, such as cathepsin B, into the cytosol and cause inflammasome activation. In the third model, ROS generated by NLRP3 activators are suggested to induce the NLRP3 inflammasome.

Statins are inhibitors of HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis, and are therapeutically used

Abbreviations: Ac-WEHD-AFC=acetyl-Trp-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin, BMDM=bone marrow-derived macrophage, $DCFH_2DA$=2′,7′-dichlorodihydrofluorescein diacetate, FPP=farnesyl pyrophosphate, GGPP=geranylgeranyl pyrophosphate, HMG-CoA reductase=3-hydroxy-3-methylglutaryl CoA, MKD=mevalonate kinase deficiency, MR=Magic Red, NAC=N-acetyl-L-cysteine, NLRP3=nucleotide-binding oligomerization-like receptor family, pyrin domain-containing 3, NTPDase1=nucleoside triphosphate diphosphohydrolase 1, oxATP=oxidized ATP, p17/p20=proenzyme into 17/20 kDa, PI=propidium iodide, ROCK=Rho-associated coiled-coil-containing protein kinase, TXNIP=thioredoxin-interacting protein

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in hypercholesterolemia. Evidence showed that statins also have beneficial effects in thrombosis, stroke, inflammation, cancer, bone fractures, glucose tolerance, and Alzheimer's disease [5]. These pleiotropic effects of statins are independent of cholesterol synthesis but are ascribed to the reduction of mevalonate, GGPP, and FPP, three intermediate products in the cholesterol synthesis pathway [6]. Recently, simvastatin, atorvastatin, and fluvastatin were found to synergize with LPS to activate caspase-1 and increase IL-1β secretion in monocytes and PBMCs, and this effect was suggested to be caused by an impairment of GGPP biosynthesis [7–11]. In agreement with this, defective synthesis of isoprenoids associated with aberrant caspase-1 activation and elevated IL-1β release is evidenced by the recurring inflammatory phenotype of MKD [9, 12, 13], which is a kind of autosomal-recessive disease caused by a genetic defect in the mevalonate pathway and isoprenoid biosynthesis and is characterized by IL-1β overproduction symptoms and increased IL-1β production in PBMCs [14, 15]. Therefore, although the crucial role played by isoprenoids in the inhibition of caspase-1 activation is highlighted, the detailed molecular basis leading to inflammasome activation is still poorly understood. As HMG-CoA reductase inhibitors, such as statins, can mimic the impairment of the isoprenoid synthesis pathway, in this study, we used statin-treated THP-1 monocytes as the major cell model to elucidate molecular mechanisms by which isoprenoids regulate NLRP3 inflammasome activation.

MATERIALS AND METHODS

Reagents

Antibodies for caspase-1 and active IL-1β were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies recognizing the pro-IL-1β and IL-1β ELISA kit were purchased from R&D Systems (Minneapolis, MN, USA). The P2X7 antibody was purchased from Proteintech Group (Chicago, IL, USA). The antibody against β-actin was from Upstate Biotechnology (Charlottesville, VA, USA). Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA). LysoTracker Red DND-99, TXNIP antibody, $DCFH_2DA$, DMEM, trypsin-EDTA, and penicillin/streptomycin were from Invitrogen (Rockville, MD, USA). The NTPDase1 antibody was purchased from Abcam (Cambridge, UK). The ECL reagent was from Perkin Elmer (Wellesley, MA, USA). The FastStart SYBR Green Master was from Roche Applied Science (Nutley, NJ, USA). Fluvastatin was provided by Novartis (Basel, Switzerland). Ac-WEHD-AFC was purchased from AnaSpec (Fremont, CA, USA). Y27632 was purchased from Tocris (Gloucestershire, UK). Other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Human THP-1 cells were cultured in RPMI-1640 complete media supplemented with 10% (v/v) heated-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Primary monocytes were prepared from the blood of healthy human donors under approval from the Institutional Review Boards of National Taiwan University Hospital (201111055RIC; Taipei, Taiwan). BMDMs were isolated from C57BL/6 mice (8–12 weeks old), as we described previously [13]. Animal experiments were conducted according to institute regulations and were approved by the ethics committee of the National Taiwan University College of Medicine.

Measurement of caspase-1 activity

The activity of caspase-1 was measured in cell lysates using the fluorometric substrate Ac-WEHD-AFC. THP-1 cells treated with the indicated agents were washed with ice-cold PBS and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 0.3% Nonidet P-40, 0.1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Lysates were then centrifuged at 14,000 × rpm for 10 min. The supernatants were collected, and protein concentration was measured. Equal amounts of protein in 50 μl lysis buffer were mixed with 50 μl reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10% sucrose, 10 mM DTT, and 100 μM Ac-WEHD-AFC) and then incubated at 37°C for 1 h. Samples were read at 405 nm in a 96-well microtiter plate. The fold increase of caspase-1 activity was determined by comparing the results of treated samples with those of the untreated control.

Measurement of IL-1β by ELISA

After drug treatment, the supernatants of cell medium were collected and stored at −80°C or measured immediately using the IL-1β ELISA kit, according to the manufacturer's instructions.

Intracellular ROS detection

After drug treatment, cells were collected and washed with ice-cold PBS twice and then incubated in PBS containing the reagent $DCFH_2DA$ (5 μM) in the dark for 30 min at 37°C. After incubation, cells were centrifuged at 3000 × rpm for 5 min, and pellets were collected, washed with PBS twice, resuspended in 500 μl PBS, and immediately submitted to flow analysis using the FACScan flow cytometer.

Measurement of ATP content in the culture medium

THP-1 cells (2×10^6 /ml), BMDMs (5×10^5 /ml), and human primary monocytes (2×10^5 /ml), plated in 3.5 cm dishes, were incubated with the indicated drugs at 37°C for the indicated periods. Media were collected, and the ATP content was analyzed with the CellTiter-Glo luminescent kit (Promega, Madison, WI, USA), following the manufacturer's instruction. The ATP quantified in control supernatant media was represented as 100%.

YO-PRO-1 uptake assay

YO-PRO-1, a 629-Da molecule, is a fluorescent nucleic acid dye, which is able to pass through P2X7 to enter and interact with DNA or RNA, and the emitted fluorescence after dye uptake is an index of P2X7 activation of live cells. After drug treatment, cells were treated with YO-PRO-1 (5 μM) for 15 min. Cells were then collected and resuspended in 500 μl PBS and immediately submitted to flow analysis using a FACScan flow cytometer.

Cathepsin B activity assay

Supernatants and total lysates were collected, and the cathepsin B activity was measured with the Cathepsin B Activity Assay Kit (BioVision, Milpitas, CA, USA), according to the manufacturer's instructions. In some experiments, cathepsin B detection was performed using the MR cathepsin detection kit (Part #937; ImmunoChemistry Technologies, Bloomington, MN, USA). The lyophilized MR-cathepsin substrate was reconstituted with DMSO to make a 260× reagent solution and was diluted further with PBS to a 26× reagent solution. After drug treatment, THP-1 cells were centrifuged at 2000 × rpm for 3 min, resuspended in 1 ml fresh medium, and treated with 40 μl 26× reagent solution. After incubating at 37°C for 2 h, cells were washed twice with PBS and then immediately submitted to flow analysis using a FACScan flow cytometer.

Lysosomal integrity assay

THP-1 cells were primed with LPS (1 μg/ml) for 6 h, washed, and pretreated with GGPP (10 μM) for 30 min, followed by fluvastatin (10 μM) for 18 h. To evaluate lysosomal rupture, cells were incubated with the lysoso-

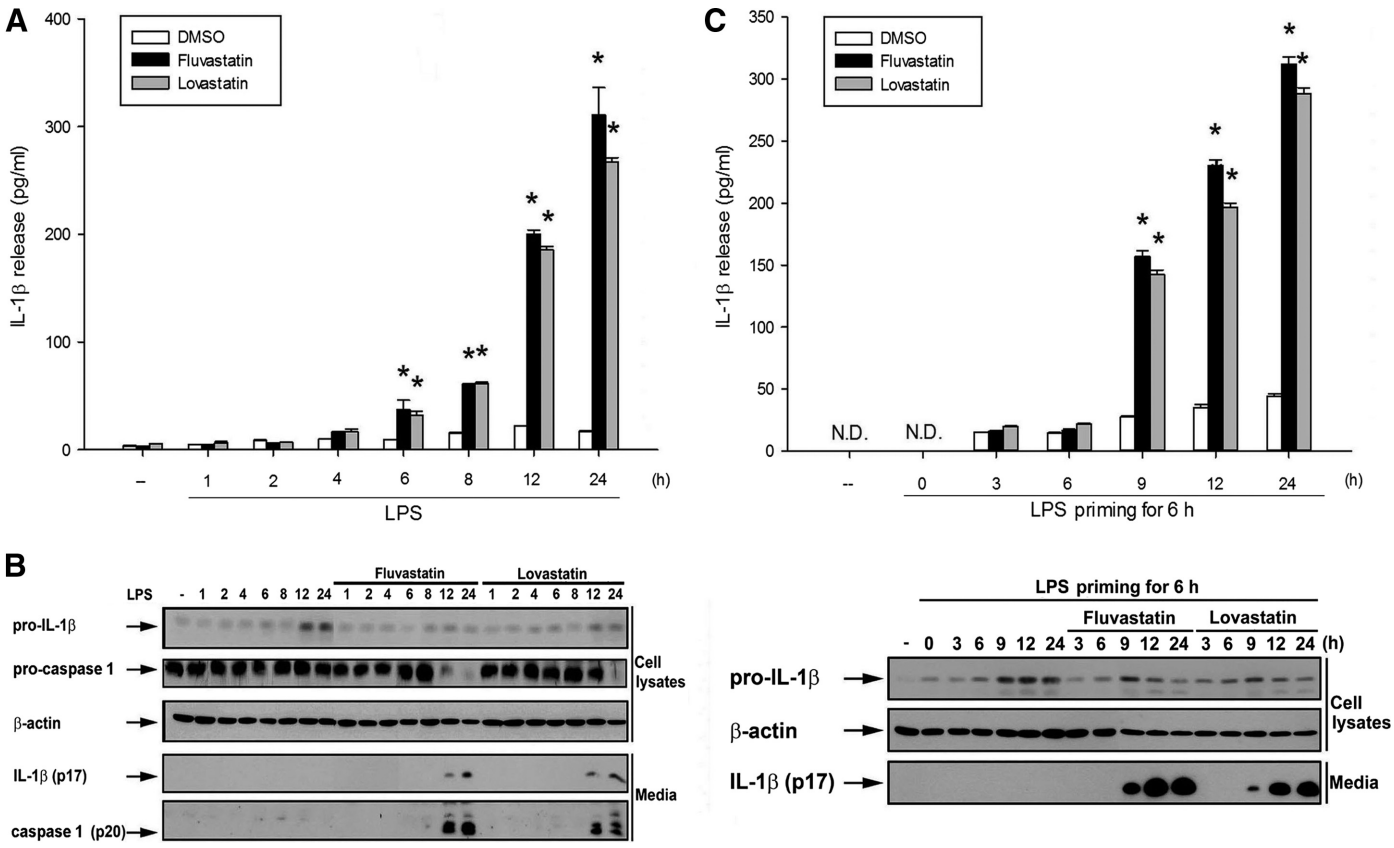

Figure 1. Statins induce IL-1β secretion in THP-1 cells cotreated or primed with LPS. THP-1 cells were cotreated with LPS (1 μg/ml) and statins (10 μM) with the indicated time course (A and B). In some experiments, THP-1 cells were primed with LPS (1 μg/ml) for 6 h and washed out, followed by stimulation with statins (10 μM) for different time periods (C and D). After treatment, the total cell lysates and supernatants were collected. The IL-1β levels of supernatants were measured by an ELISA kit (A and C). The total cell lysates and media supernatants were subjected to immunoblotting (B and D). (A and C) Data were the mean ± SEM from three independent experiments. *$P < 0.05$, indicating significant enhancement of the IL-1β response by statins; ND, nondetectable.

motropic probe LysoTracker Red DND-99 (50 nM) for 15 min at 37°C. After incubation, cells were centrifuged, and pellets were collected, washed with PBS, resuspended in PBS, and immediately submitted to flow analysis.

MTT assay

THP-1 cells (2×10^6/ml), plated in 24-well plates, were incubated with the indicated agents at 37°C for 24 h. MTT (5 mg/ml) was added for 1 h. Samples were centrifuged at 3000 × rpm, and the pellets were collected. The formazan granules generated by live cells were dissolved in DMSO and shaken for 10 min. The OD values at 550 nm and 630 nm were measured using a microplate reader. The net absorbance (OD_{550}–OD_{630}) indicated the enzymatic activity of mitochondria and represented the cell viability.

PI uptake assay

Cell membrane integrity was assessed by determining the ability of the cells to take up PI. After drug treatment, cells were collected by centrifugation, washed once with PBS, and resuspended in PBS containing 25 μg/ml PI. The cells were incubated for 15 min at 37°C. After incubation, the cells were analyzed on a FACScan flow cytometer.

Immunoblotting analysis

After reagent treatment, intracellular protein expression was determined by electrophoresis and immunobloting, as we described previously [16].

Reconstituted caspase-1 activation in HeLa

HeLa cells were transfected with 100 ng procaspase-1 and 250 ng pro-IL-1β DNA, which were kindly provided by Dr. John Reed (Sanford Burnham Medical Research Institute, La Jolla, CA, USA). Six hours later, cells were changed to complete medium and treated with statin (10 μM) for another 6 h. Then, the supernatants were collected to assay IL-1β by ELISA.

Statistical evaluation

Values were expressed as the mean ± SEM of at least three independent experiments, which were performed in duplicate. ANOVA was used to assess the statistical significance of the differences, and a P value < 0.05 was considered statistically significant.

RESULTS

Statins increase LPS-induced IL-1β secretion in THP-1 cells

First, we showed that fluvastatin or lovastatin alone at 10 μM cannot induce IL-1β release after 24 h incubation but dramatically potentiated LPS (1 μg/ml)-induced IL-1β production in a time-dependent manner (**Fig. 1A**). Accordingly, each statin alone did not change pro-IL-1β or procaspase-1 expression

after 24 h incubation (data not shown). In contrast, LPS caused a time-dependent induction of pro-IL-1β protein expression, whereas neither mature IL-1β (p17) nor active caspase-1 (p20) was significantly detected in media supernatants of LPS-treated cells (Fig. 1B). These results confirm previous findings that LPS itself can induce signaling pathways leading to gene transcription of pro-IL-1β but is not effective to activate the inflammasome. However, coincubation of statins (10 μM) with LPS (1 μg/ml) for 24 h led to dramatic decreases of pro-IL-1β and procaspase-1 proteins in cell lysates and corresponding increases of active caspase-1 and mature IL-1β in culture media (Fig. 1B).

To accurately analyze the action of statins on the inflammasome and to avoid the interference from their action on LPS-induced pro-IL-1β expression, THP-1 cells that had been pretreated with LPS (1 μg/ml) for 6 h were washed with fresh culture medium and then treated with either statin (10 μM) for 3–24 h. Results revealed that after pro-IL-1β expression has been induced following LPS treatment for 6 h, subsequent treatment with fluvastatin or lovastatin markedly increased IL-1β secretion (Fig. 1C). The minimal time period required for achieving this action (i.e., 9 h incubation with statins) closely correlated with the onset time when statins were cotreated at the same time with LPS (i.e., 6–8 h incubation; Fig. 1A). This consistency suggests that a time lag is required for statin-induced caspase-1 activation. In agreement with the time course for IL-1β release, reduction of pro-IL-1β in the cell lysates and production of IL-1β (p17) in the culture media were concomitantly detected (Fig. 1D). These results obtained in LPS-primed THP-1 cells confirm that fluvastatin and lovastatin can activate caspase-1 to induce pro-IL-1β processing into mature IL-1β.

GGPP reverses statin-induced caspase-1 activation and IL-1β secretion in LPS-primed cells

To understand whether statin's action results from GGPP deficiency and/or interruption of small G protein functions, LPS (1 μg/ml)-primed THP-1 cells were pretreated with GGPP (10 μM), FPP (10 μM), Rac 1 inhibitor (10 μM), or ROCK inhibitor Y27632 (10 μM) for 30 min, followed by statin (10 μM) treatment for 18 h. As shown in **Fig. 2A**, statin-induced, mature IL-1β secretion was abolished by GGPP but was unaffected by FPP, Rac 1 inhibitor, or Y27632. To understand whether statin's action is relevant to reduced cholesterol biosynthesis, we also tested YM53601, which is a squalene synthase inhibitor and can block cholesterol synthesis [17]. In contrast to the stimulating effect of statins, YM53601 failed to affect IL-1β secretion in LPS-primed cells (Fig. 2A). Meanwhile, decreases of pro-IL-1β in the cell lysates as well as increases of mature IL-1β and active caspase-1 in the media caused by statins were prevented by GGPP but were unaffected by Y27632 (Fig. 2B). Directly assessing caspase-1 activity using Ac-WEHD-AFC as a substrate, we found that statin treatment for 18 h can induce caspase-1 activation, and this effect was unaltered by LPS priming or Y27632 treatment but was attenuated by the cotreatment with GGPP (Fig. 2C). Moreover, neither statins nor GGPP altered the mRNA level of procaspase-1 (data not shown). These results suggest that reduced GGPP synthesis, but not altered activities of Rac and ROCK or intracellular content of cholesterol, is crucial for statin-induced caspase-1 activation and pro-IL-1β processing.

To understand whether statins and GGPP have direct action on caspase-1, we used a reconstituted enzymatic assay. In HeLa cells ectopically expressing mouse pro-IL-1β and procaspase-1, we found that fluvastatin, FPP, and GGPP did not inhibit mouse IL-1β production, whereas zVAD (a cell-permeant pan caspase inhibitor) exhibited a dramatic inhibition (Fig. 2D). These results suggest that statins and GGPP can regulate caspase-1 activity through signaling pathways upstream of the inflammasome rather than through the direct action on its enzymatic activity.

Statins induce ATP release and P2X7 activation

Extracellular ATP binding to P2X7 ion channels, leading to K^+ efflux, is one of the currently proposed models for NLRP3 inflammasome activation. To understand a possible role of P2X7 in the action of statins, we first examined the effects of oxATP (an antagonist of P2X7) and high KCl, which can block intracellular K^+ efflux caused by P2X7. Treatment of THP-1 cells with oxATP (300 μM) or high extracellular potassium (130 mM) blocked the stimulatory effects of fluvastatin and lovastatin on IL-1β secretion (**Fig. 3A** and **B**) and caspase-1 activation (Fig. 3B). Data from the enzymatic assay of caspase-1 activity also confirmed the abilities of oxATP and high KCl to diminish the stimulating effect of statins (Fig. 3C).

To further understand whether P2X7 is involved in the action of statins, we determined the ATP level in the culture medium. The level of extracellular ATP was increased in a time-dependent manner during treatment with statins (**Fig. 4A**), whereas LPS treatment showed no effect (data not shown). Moreover, GGPP was able to block the effects of statins to increase ATP release (Fig. 4A). Considering that P2X7 might be activated via the autocrine effect of ATP upon statin treatment, we conducted a YO-PRO-1 uptake assay. YO-PRO-1, a 629-Da molecule, is able to pass through P2X7 and interact with DNA or RNA, and the emitted fluorescence after dye uptake is an activity index of P2X7 [18, 19]. Results shown in Fig. 4B indicate the ability of statins (10 μM) to increase the uptake of YO-PRO-1 by THP-1 cells. This action of statins was not altered by LPS priming but was attenuated by oxATP, supporting P2X7 as a statin-stimulated permeability pore. To further rule out that YO-PRO-1 uptake is due to nonspecific membrane permeabilization resulting from cell necrosis, we determined PI uptake in nonfixed cells. We found that after statin treatment for 18 h, PI uptake was only increased slightly from 10% to 15% of the cell population (Fig. 4C). Treatment with oxATP alone also slightly increased PI uptake, and this action was nonadditive to statin. Based on the lack of parallel effects of oxATP on PI and YO-PRO-1 uptake in conditions of statin treatment, we ruled out that the statin-induced YO-PRO-1 uptake is ascribed to cell damage. To investigate whether the increased extracellular ATP level following statin treatment resulted from the lower degradation of ATP, we measured the protein level of NTPDase1, which is the dominant ectonucleotidase expressed in macrophages and governs the P2X7-dependent functions [16]. We found that fluvastatin

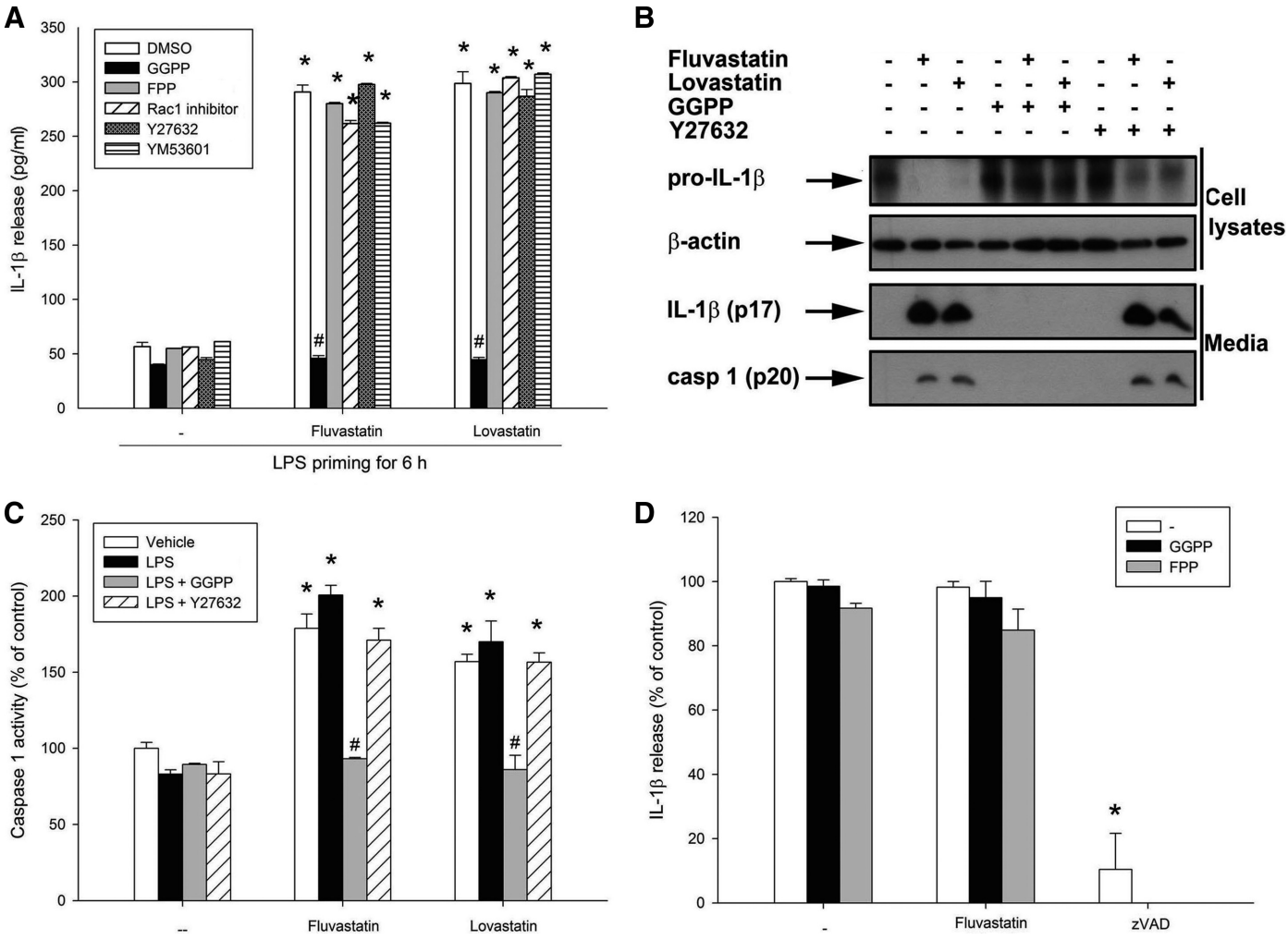

Figure 2. GGPP abolishes statin-induced IL-1β secretion. (A–C) THP-1 cells were primed with LPS (1 μg/ml) for 6 h, washed out, and pretreated with GGPP (10 μM), FPP (10 μM), Rac inhibitor (10 μM), Y27632 (10 μM), or YM53601 (10 μM) for 30 min, followed by statins (10 μM) for 18 h. The media supernatants and cell lysates were collected for measuring IL-1β (A) and protein expression (B). Caspase-1 activity in the cell lysates was determined (C). (D) HeLa cells ectopically expressing pro-IL-1β and procaspase-1 were treated with fluvastatin (10 μM) or zVAD (20 μM). IL-1β production in the media was measured. (A, C, and D) Data were the mean ± SEM from three independent experiments. $*P < 0.05$, indicating significant enhancement of IL-1β secretion or caspase-1 activation by fluvastatin and lovastatin, as well as inhibition of caspase-1 by zVAD; $\#P < 0.05$, indicating significant inhibition of statins' response by GGPP.

could not reduce NTPDase1 expression, with or without LPS priming (data not shown). Moreover, to understand whether statins exert a direct activation of P2X7, we determined the exogenous ATP-induced YO-PRO-1 uptake. Results showed that ATP-induced P2X7 permeability was not altered by statins but was abrogated by oxATP (Fig. 4D). Altogether, these results suggest statins enhance ATP secretion and P2X7 activation through an isoprenylation-dependent pathway, in turn, leading to inflammasome activation via an autocrine pathway.

Role of lysosomal instabilization in statin-induced IL-1β secretion

Next, to investigate whether phagocytosis or lysosomal rupture is involved in statin-induced caspase-1 activation, LPS-primed THP-1 cells were pretreated with cytochalasin D (an inhibitor of phagocytosis; 200 nM) or CA-074-Me (cathepsin B inhibitor; 10 μM), followed by stimulation with statins (10 μM) for another 18 h. As shown in **Fig. 5A**, CA-074-Me moderately attenuated the effects of statins on IL-1β secretion, whereas cytochalasin D had no effect. These results suggest that lysosome-dependent cathepsin B activation rather than phagocytosis is involved, at least in part, in statin-mediated IL-1β release.

To confirm this notion, we measured cathepsin B activity and lysosomal integrity after statin treatment of LPS-primed cells. Results indicated that fluvastatin incubation for 16 h is able to increase cathepsin B activity in total lysates and media supernatant (Fig. 5B). In addition to the enzymatic assay, we confirmed the ability of statins to activate cathepsin B using flow cytometry (Fig. 5C). With the use of LysoTracker staining as a reverse index of lysosome integrity and activity, our data

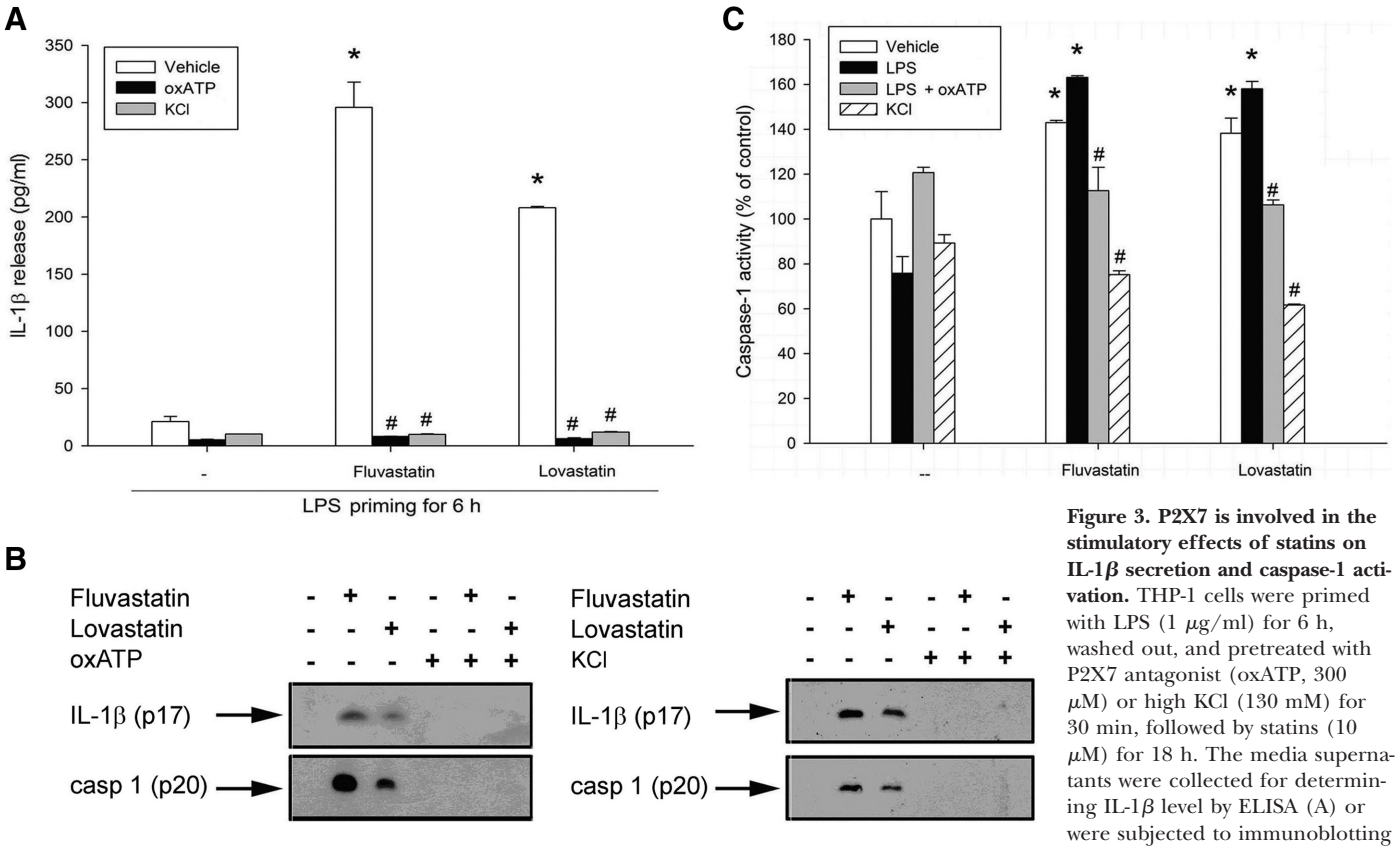

Figure 3. P2X7 is involved in the stimulatory effects of statins on IL-1β secretion and caspase-1 activation. THP-1 cells were primed with LPS (1 μg/ml) for 6 h, washed out, and pretreated with P2X7 antagonist (oxATP, 300 μM) or high KCl (130 mM) for 30 min, followed by statins (10 μM) for 18 h. The media supernatants were collected for determining IL-1β level by ELISA (A) or were subjected to immunoblotting (B). The caspase-1 activity in cell lysates was measured (C). (A and C) Data were the mean ± SEM from three independent experiments. *$P <$ 0.05, indicating significant enhancement of IL-1β secretion and caspase-1 activation by statins; #$P < 0.05$, indicating significant inhibition of IL-1β secretion and caspase-1 activation by oxATP and KCl.

revealed the stimulating effect of fluvastatin, but not LPS, on the lysosome. Moreover, this action of fluvastatin was prevented by the presence of GGPP (Fig. 5D).

ROS-dependent mechanism of statin-induced IL-1β secretion and caspase-1 activation

To determine whether ROS is involved in statin-induced IL-1β secretion, we tested the effect of an antioxidant, NAC. Results in **Fig. 6A–C** showed that in the presence of NAC (10 mM), statin-stimulated IL-1β secretion and caspase-1 activation were decreased. A recent study further showed that TXNIP might be involved in ROS-mediated IL-1β secretion [20]. Nevertheless, our results showed that fluvastatin and lovastatin treatment for 18 h led to a reduction of TXNIP expression in THP-1 cells, with or without LPS (Fig. 6D). With the use of a fluorescent dye, which specifically detects cytosolic hydroxyl-free radicals, we found that both statins can induce a slight increase in intracellular ROS production, by 10–20% and 45%, at 9 h and 12 h incubation, respectively (Fig. 6E). All of these results suggest that a moderate increase of intracellular ROS production is another pathway by which statins mediate caspase-1 activation.

Statins increase IL-1β secretion in primary monocytes

To further understand if statin-induced enhancement of IL-1β production seen in human THP-1 monocytes is cell type-specific, we assessed this event in human primary monocytes and murine BMDMs. In LPS-primed monocytes from PBMCs, fluvastatin indeed can increase mature IL-1β and ATP levels in culture media (**Fig. 7A** and **B**), and the increased IL-1β release was inhibited by the presence of oxATP (Fig. 7A). Fluvastatin also exerted similar actions in BMDMs, i.e. increased IL-1β production in a manner susceptible to oxATP (Fig. 7C), and a parallel increase of ATP release (Fig. 7D). Conducting the MTT and PI uptake assays, we did not observe any cytotoxicity in fluvastatin-treated human primary monocytes and BMDMs, with or without LPS priming (data not shown). Concerning whether statins' effect on increasing IL-1β production might result from affecting signals of NLRP3 ligands, we also determined the inflammasome-activating responses of ATP, alumn gel, and nigericin, which are three common NLRP3 ligands. As a result, in LPS-primed BMDMs, these ligand-induced IL-1β releases were not changed by the presence of fluvastatin (Fig. 7E).

DISCUSSION

Accumulating lines of evidence indicate that statins have cholesterol lowering-independent and pleiotropic effects. The most common molecular mechanism behind these events is the inhibition of isoprenoid synthesis and impairment of protein modification via isoprenylation. Although studies have shown the abilities of statins to inhibit the production of a va-

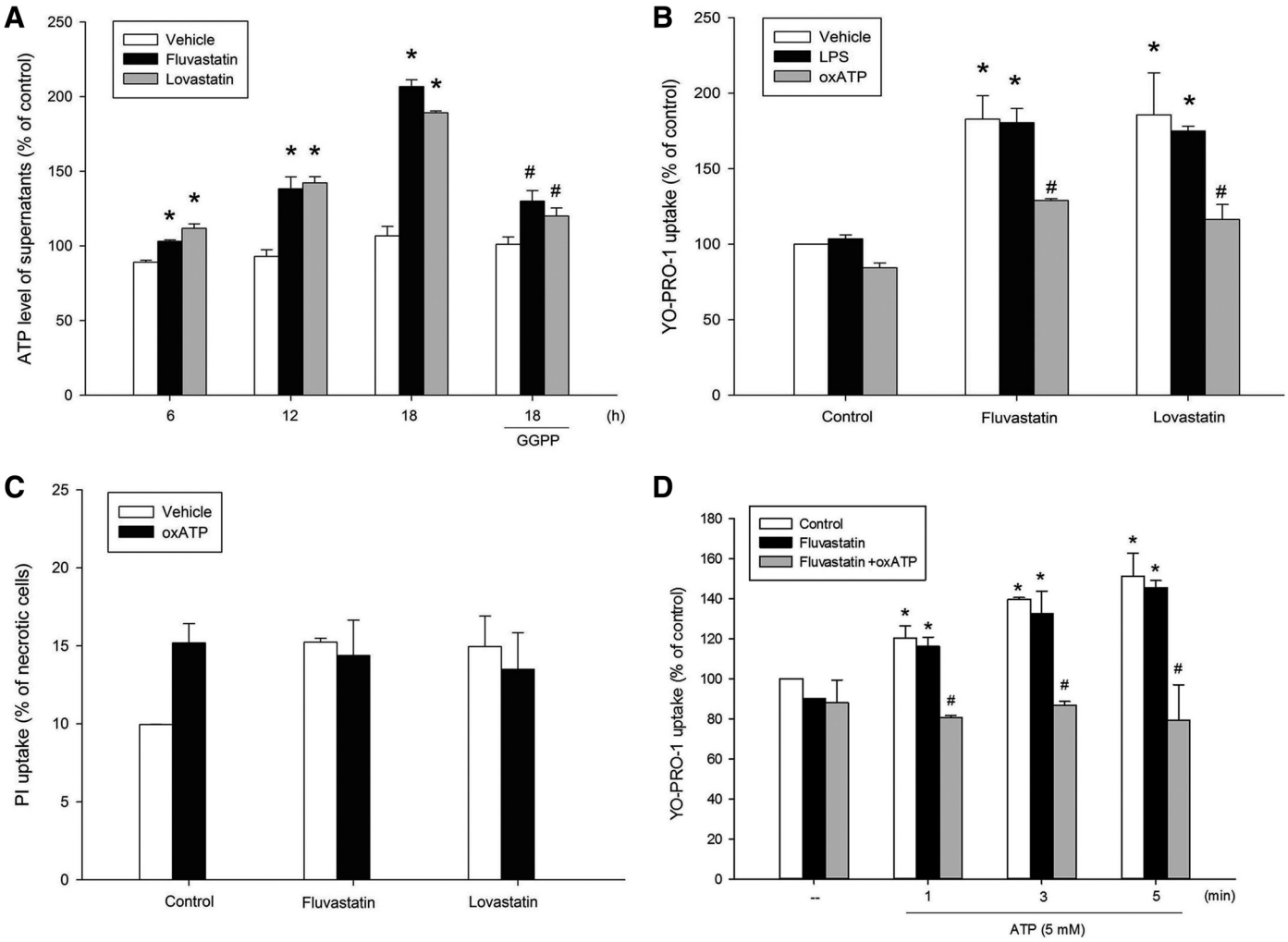

Figure 4. Statins induce ATP release and P2X7 activation. (A) THP-1 cells were treated with statins (10 μM) or GGPP (10 μM), as indicated for a different time course. The media supernatants were collected and measured with an ATP assay kit (A). (B and C) THP-1 cells were treated with statins or LPS at the concentrations indicated for 16 h, followed by adding vehicle or oxATP (300 μM) for another 2 h. After treatment, cells were collected and then incubated in PBS containing YO-PRO-1 (5 μM; B) or PI (5 μM; C) for 15 min at 37°C. After incubation, cells were centrifuged, and pellets were collected, washed with PBS twice, resuspended in 500 μl PBS, and immediately submitted to flow analysis using a FACScan flow cytometer. (D) THP-1 cells were treated with statins and/or oxATP for 1 h, followed by ATP (5 mM) for different time periods. After treatment, YO-PRO-1 uptake was determined as described above. Data were the mean ± SEM from three independent experiments. *$P < 0.05$, indicating significant enhancement of ATP release and YO-PRO-1 uptake; #$P < 0.05$, indicating significant inhibition of ATP release and YO-PRO-1 uptake by GGPP and oxATP, respectively.

riety of inflammatory mediators [21–24], their effect on IL-1β release is different. In this study, we found that statins can enhance IL-1β release in LPS-treated monocytes/macrophages and proved that there is a GGPP inhibitory action on inflammasome, whose activation is an essential and unique process for IL-1β production. Consistently, a latest study also demonstrated that statin use is associated with pulmonary fibrosis through a mechanism involving NLRP3 inflammasome activation [25]. Regarding whether this cytokine might be affected and modulate the therapeutic benefits of statins for antiatherosclerosis, we gave Apolipoprotein E-deficient mice (an atherosclerotic mice model) with fluvastatin at 10 mg/kg (i.p.) once/day. Within 3 months, the serum level of IL-1β was found not to be changed (data not shown). Thus, we suggest that the antiatherogenic effect of statins is unrelated to IL-1β.

Regarding the P2X7 channel model of inflammasome activation, we found that statin-induced caspase-1 activation and IL-1β secretion in LPS-primed THP-1 cells were abrogated in the presence of an irreversible P2X7 antagonist (oxATP) or high potassium, suggesting the involvement of P2X7 in statin-induced caspase-1 activation. Further evidence supporting this notion is the increased ATP release and YO-PRO-1 nucleic acid dye uptake after statin treatment. Measurement of this dye uptake has been previously used to reflect ATP-induced pore formation activity [18]. Thus, we speculate that autocrine action of ATP is involved in the stimulating action of statins. This notion highlighting the necessity of endogenous ATP release for caspase-1 activation has also been proposed in human monocytes [26]. However, as ATP concentration released in the culture medium by statins is consistently much less than that required to induce caspase-1 activation

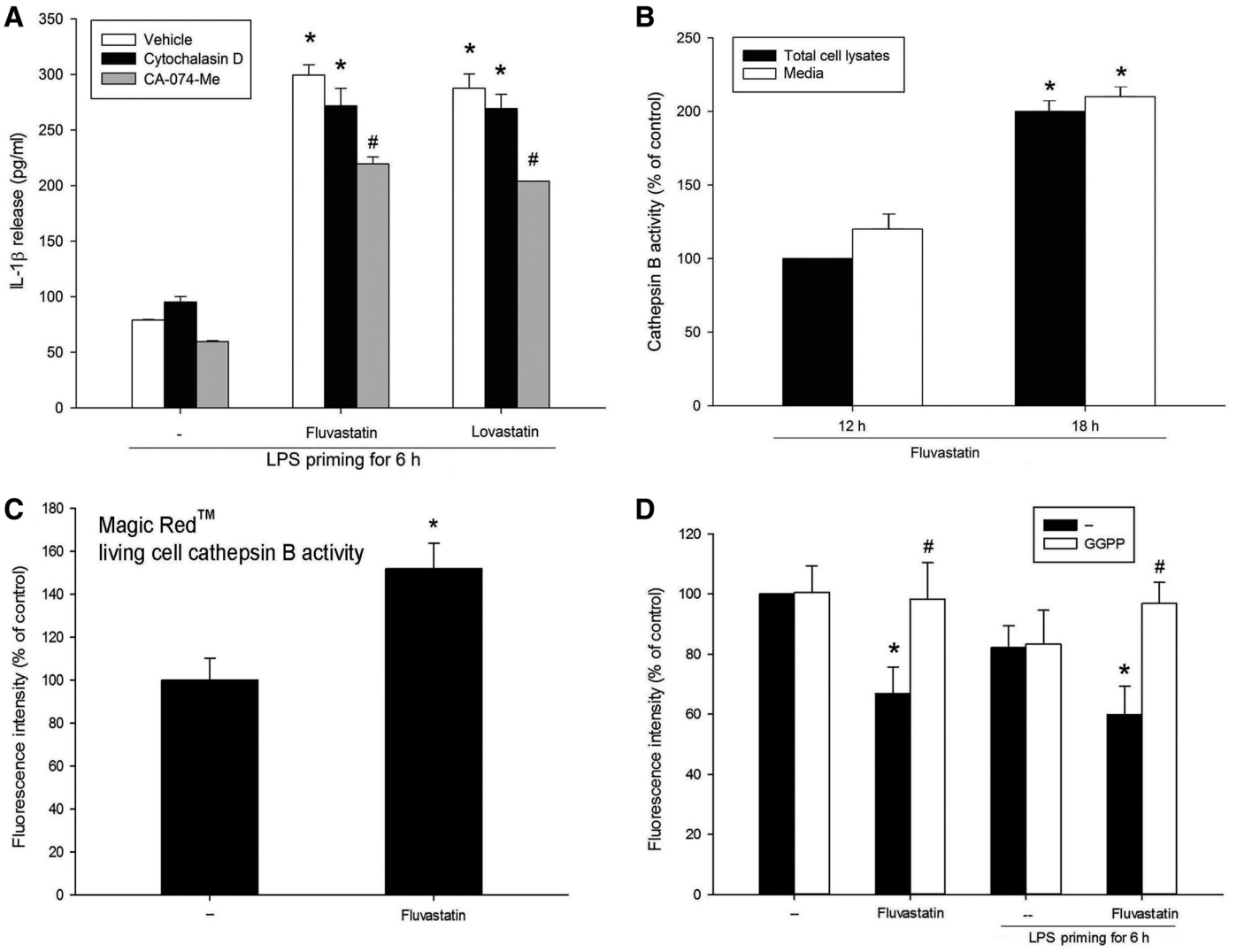

Figure 5. Cathepsin B is involved in statin-induced IL-1β secretion. (A) THP-1 cells were primed with LPS (1 μg/ml) for 6 h, washed out, and then pretreated with cytochalasin D (200 nM) or CA-074-Me (10 μM) for 30 min, followed by statins (10 μM) for 18 h. The media supernatants were collected and measured with an ELISA kit. (B) Cells were treated with fluvastatin (10 μM) for the indicated time periods, and then cathepsin B activities in cell lysates and media supernatants were measured. (C) After treatment with fluvastatin for 18 h, cathepsin B was detected with the MR cathepsin detection kit and by flow cytometry. (D) Cells with LPS priming or not were treated with fluvastatin or GGPP for 18 h. Then, LysoTracker Red staining was performed as described in Materials and Methods. *$P < 0.05$, indicating a significant increase of IL-1β secretion, cathepsin B activation, and inhibition of LysoTracker staining by statins; #$P < 0.05$, indicating significant inhibition of statins ' effects by CA-074-Me or GGPP.

by exogenous ATP (usually 5 mM), we suggest other molecular action mechanisms of statins, except that P2X7 activation might be involved and coordinate to evoke caspase-1 activation. Next, we excluded the possibility that the gradually increasing ATP level caused by statins is related to cell necrosis. Treatment with statins did not induce marked cell necrosis under the conditions tested nor did pro-IL-1β release into the culture medium. On the other hand, calcium-dependent exocytosis, active transporters (e.g., ATP-binding cassette family transports), and/or hemichannels that are composed of protein subunits, such as the connexin or pannexin family, have been shown as ATP release pathways [27–30]. In addition, P2X7 might provide an amplification device to spread the ATP wave to induce further nonlytic ATP release [31]. Therefore, exploring pathway candidates for statin-elicited ATP release and in turn, transferring danger signals from intact cells require investigation in the future.

ROS-modulated IL-1β secretion is another speculated model for inflammsome activation. Our study showed that statin-induced caspase-1 activation and IL-1β secretion were attenuated by the antioxidant NAC and that statin can induce weak ROS production. These results imply the involvement of an isoprenylation-dependent ROS production in the action of statins. A recent study demonstrating that lack of isoprenoids in MKD patients could cause oxidative stress as a result of decreasing endogenous antioxidant molecules also mimics our findings [32]. On the other hand, a recent report suggests that oxida-

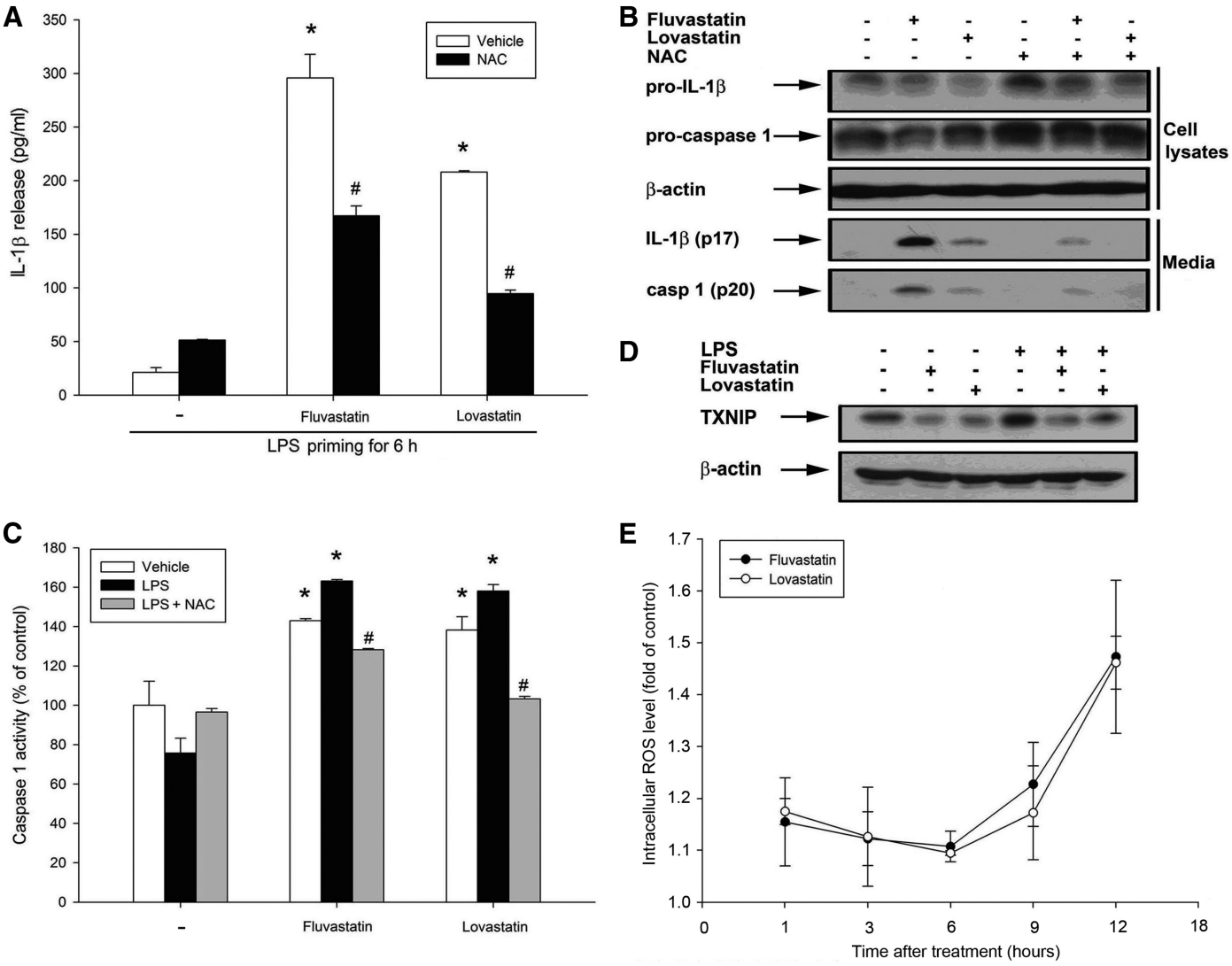

Figure 6. ROS dependency of statin-induced IL-1β secretion and caspase-1 activation in LPS-primed THP-1 cells. THP-1 cells were primed with LPS (1 μg/ml) for 6 h, washed out, and then pretreated with NAC (10 mM) for 30 min, followed by statins (10 μM) for 18 h. The media supernatants were collected and measured with an ELISA kit (A). Total cell lysates (B and D) and media supernatants (B) were collected for immunoblotting and caspae-1 enzymatic assay (C). (E) THP-1 cells were treated with statins for the indicated time periods, and cytosol ROS was measured by flow cytometry. Data were the mean ± SEM from three independent experiments. *$P < 0.05$, indicating significant enhancement of IL-1β secretion or caspase-1 activation by statins; #$P < 0.05$, indicating significant inhibition of statins' effects by NAC.

tive stress could dissociate TXNIP from thioredoxin to activate the NLRP3 inflammasome [20]. In this aspect, we ruled out this possibility in statin-treated monocytes, as TXNIP expression in THP-1 cells is reduced after statin treatment. Although the real function of TXNIP in caspase-1 activation in monocytes remains unknown, its decrease with statin treatment is consistent with that of monosodium urate, another activator of NLRP3 inflammasome [20].

The last pathway commonly recognized for NLRP3 inflammasome activation is lysosomal rupture model. Previous studies indicated that some exogenous particles could trigger NLRP3 inflammasome activation through a phagocytotic pathway, which leads to lysosomal rupture and release of the protease cathepsin B. Recently, P2X7 activation was also shown to induce lysosomal proteases release, including cathepsin S, via a phagocytotic pathway [33]. To assess the possible involvement of phagocytosis and lysosomal activity in the action of statins, we treated THP-1 cells with the phagocytosis inhibitor (cytochalasin D) or cathepsin B inhibitor (CA-074-Me) in statin-stimulated THP-1 cells. We found that IL-1β secretion was slightly but significantly attenuated by CA-074-Me but not by cytochalasin D. In addition, we observed the ability of statins to induce cathepsin B activation and LysoTracker Red staining. The latter is a cationic fluorescent dye in acidic cellular compartments and is a standard marker of lysosome contents. Thus, we suggest that lysosomal destabilization might also partially account for the activation of caspase-1 by statins.

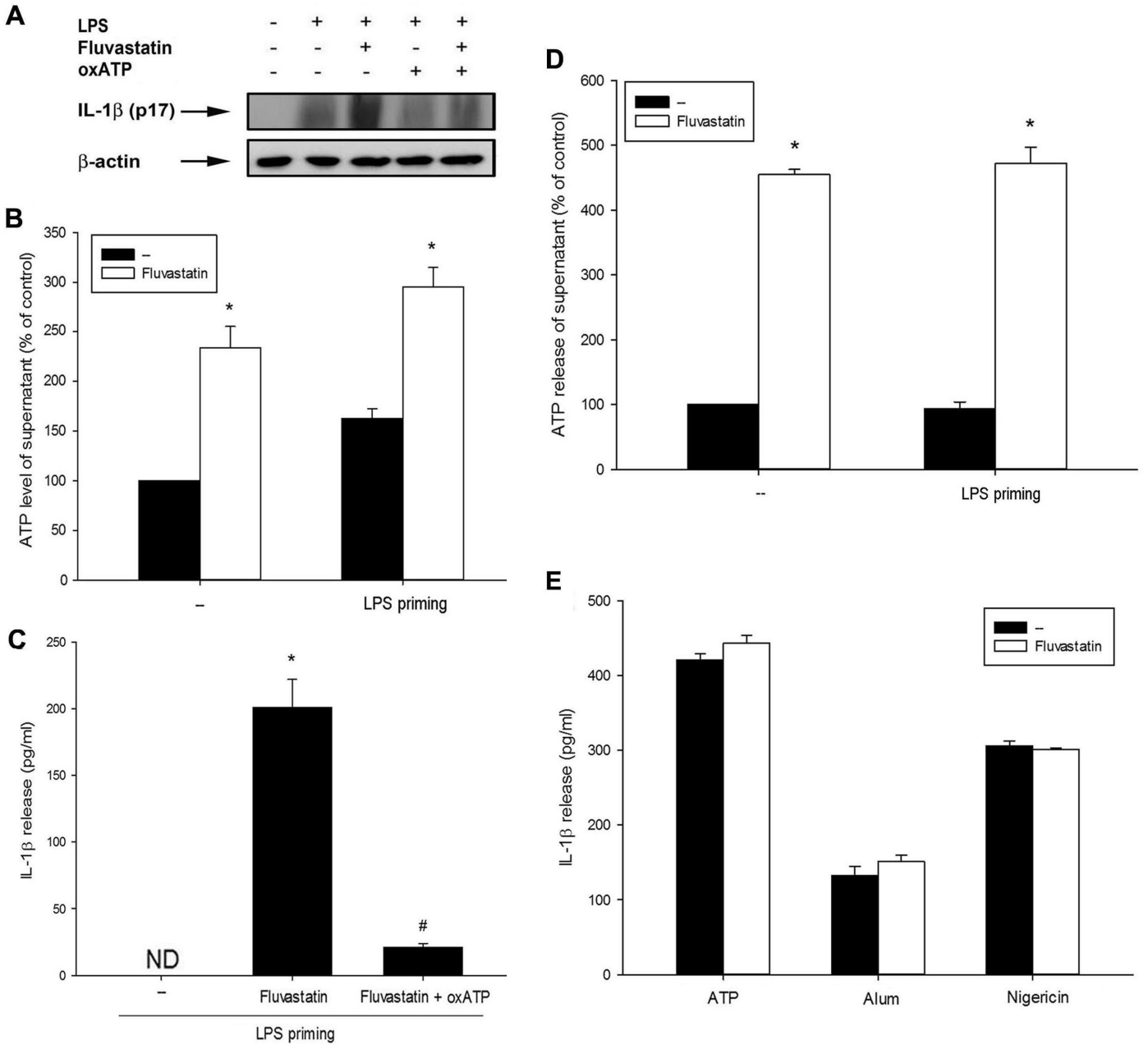

Figure 7. Statins induce intracellular ATP release in PBMC and BMDM. Primary monocytes (A and B) and BMDMs (C–E) were primed with LPS (1 μg/ml) for 6 h, followed by wash out and stimulation with statins (10 μM) and/or oxATP (300 μM) for 12 h. Mature IL-1β was determined by immunobloting with the cell lysate (A) or ELISA with media supernatants (C). ATP release in the media was also determined (B and D). (E) LPS-primed BMDM was washed with fresh culture medium, treated with fluvastatin (10 μM) for 1 h, and then stimulated with ATP (5 mM), alumn gel (Alum; 150 μg/ml), or nigericin (1 mM) for 20 min, 9 h, and 20 min, respectively. IL-1β release was then determined by ELISA. Data were the mean ± SEM from three independent experiments. *$P < 0.05$, indicating significant enhancement of IL-1β secretion and ATP release by statins; #$P < 0.05$, indicating significant inhibition of statins' effects by oxATP.

As mentioned above, several previous studies suggested that statins can induce IL-1β secretion via decreasing GGPP levels. Paradoxically, other studies have also indicated that the activation of small GTPase proteins, such as Rac1 and Cdc42, could be a novel pathway to activate caspase-1 [34–36]. Theoretically, reducing GGPP is supposed to induce Rac1 and Cdc42 inactivation via interfering with protein isoprenylation. Thus, the roles played by GTPases in caspase-1 activation remain poorly understood.

In conclusion, our current study demonstrates the molecular mechanisms for the activation of caspase-1 by fluvastatin and lovastatin in human monocytes and murine macrophages. Statin-induced stimulation of IL-1β secretion results from the decrease of GGPP, which negatively regulates caspase-1 activation. Increases in ATP release, P2X7 activation, ROS production, and lysosomal rupture are all involved in statin-induced caspase-1 activation. These data not only shed new insights into isoprenylation-depen-

dent molecular regulation of caspase-1, which improves our current understanding of the inflammatory disease of MKD, but also emphasize a novel action of statins in inducing the danger signal pathway for inflammation.

AUTHORSHIP

Y-H.L., Yi-Chieh Lin, S-T.T., Ying-Cing Lin, A-J. Y., and C-T.H performed the experiments and discussed the results. K-C.H. and W.W.L. designed the experiments, analyzed data, and wrote the manuscript.

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DISCLOSURES

The authors have no conflict of interests to declare.

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KEY WORDS:
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