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Saturated fatty acids promote chondrocyte matrix remodeling through re-programming of autophagy pathways

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HIGHLIGHTS

- Stimulation of chondrocytes with Palmitic acid (PA), and Stearic acid (SA), irrespective of presence or absence of IL-1β, resulted in increased activation of autophagy and NF-κB.
- Lauric acid (LA) stimulation resulted in decreased autophagy activation shown by decreased expressions of LC3-II, ATG5, and BEC1, suggesting decreased cellular stress.
- SFA may activate the NF-κB pathway, which in turn increases the expression of autophagy-related proteins and stimulate autophagocytosis.
- Pharmacological activation of autophagy can be used as a targeted therapeutic approach for OA.

Abstract

Objective: Obesity is known to be a strong risk factor for the onset of knee osteoarthritis (OA) and is often accompanied by dysregulated lipid metabolism with elevated levels of free fatty acids (FFA) such as saturated fatty acids (SFA). The purpose of this study was to determine how autophagy varies in chondrocytes in response to predominant saturated fatty acids (SFA) such as lauric acid (LA), myristic acid (MA), palmitic acid (PA) and stearic acid (SA).

Research Methods & Procedures: Normal human articular cartilage chondrocytes and C28/I2 chondrocyte cell lines were stimulated with the different SFA both in the absence and presence of IL-1β to study the effects of SFA and inflammatory cytokines respectively in mediating activation of autophagy. Furthermore, effects of rapamycin and LY290042 on autophagy in response to different SFA were also assessed.

Results: Our results showed that palmitic and stearic acid stimulation of chondrocytes resulted in increased activation of both autophagy and the canonical NFκB pathway as evidenced by increased expressions of key autophagy markers - LC3-II (Microtubule-associated protein-1 light chain 3), ATG5 (Autophagy related 5), and BEC1 (Beclin-1) and NFκB p65 respectively. In contrast, lauric acid stimulation resulted in decreased autophagy activation shown by decreased expressions of LC3-II, ATG5, and BEC1, suggesting decreased cellular stress.

Conclusions: The results presented in this study represent a novel mechanism by which various SFA activate autophagy and simultaneously modulate NFκB signaling pathways and the expression of chondrocyte regulatory genes.

Keywords: Saturated fatty acids, Obesity, Osteoarthritis, Autophagy, NFκB pathway

Abbreviations

OA Osteoarthritis

FFA Free Fatty Acids

SFA Saturated Fatty Acids

LA Lauric Acid

MA Myristic Acid

PA Palmitic Acid

SA Stearic Acid

BSA Bovine Serum Albumin

DMSO Dimethyl Sulfoxide

MMP Matrix Metalloproteinase

ECM Extra Cellular Matrix

ACC Articular Chondrocyte

RIPA Radioimmunoprecipitation Assay

PFA Paraformaldehyde

PBS Phosphate Buffer Saline

NFκB Nuclear Factor Kappa-light chain enhancer of activated B cells

Introduction

Obesity is the most commonly cited cause of osteoarthritis OA (1). Excess plasma levels of circulating free fatty acids (FFA), such as saturated fatty acids (SFA), during obesogenic conditions, may cause an increase in the expression of matrix-metalloproteinases (MMPs) in the extracellular matrix (ECM), triggering cartilage degradation (2). In our previous study, we demonstrated that the risk of OA increased with increase in SFA chain length, where PA and SA increased degradation of the chondrocytes (3). This observation led us to explore the mechanism behind these differential responses on to the chondrocytes. To date, the molecular mechanisms that govern the effects of obesity on chondrocyte metabolism are still not clear. Autophagy is a highly conserved degradation process that is involved in the clearance of proteins and damaged organelles to maintain intracellular homeostasis and cell integrity. Deregulation of autophagy is associated with a variety of disorders such as metabolic syndrome, aging, degenerative diseases and cancer. Autophagy is also known to play a vital role in the pathogenesis of obesity (4, 5). During obesity, the circulating SFA results in considerable cellular stress, which interferes with the cells ability properly sense and maintain the nutrient and energy status. When exposed to a continuous and high level of energy and nutrients, the cells fail to adapt to the prevailing conditions to maintain its homeostasis. This often leads to metabolic and oxidative stress which causes inflammation

and dysfunction of these cells (6, 7). In order to survive, cells in such tissues depend on a mechanism called autophagy to remove the damaged organelles and macromolecules (8). In addition to autophagy being a cell survival mechanism, it is also known to regulate lipid homeostasis (5). NF-κB is a well-characterized transcription factor that regulates inflammation and proliferation of the cells. Once activated, the degradation of Iκβα takes place, accompanied by the NF-κB nuclear translocation which triggers the downstream gene transcription. However, little is known on the how the SFA modulate autophagic and NF-κB activity in chondrocytes. Therefore, we wanted to determine if autophagy could be involved in SFA-mediated effects in chondrocytes which could further trigger the NF-κB pathway.

Methods

Human cartilage procurement and processing

Biopsies of articular cartilage were procured from five male donors aged between 60–65 years, who were primary OA patients undergoing knee replacement surgery at The Prince Charles Hospital, Brisbane. Informed consent was obtained in writing from all the participants and the study was approved by the Prince Charles Hospital Human Ethics Committee. The cartilage biopsies were sourced from the region of the femoral condyle that the surgeon considered to be intact and a healthy cartilage.. The chondrocytes were cultured according to our published protocols (9).

Primary culture of human chondrocytes and pellet culture

The chondrocytes were cultured with DMEM high glucose medium with 10% FBS and 10000 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher, 15140122) at 37^{o}C with 5% CO_2 (complete medium). The ACCs were grown to confluence before they were used for 3D pellet cultures according to our published protocols (9).

C28/I2 human chondrocyte cell culture

The C-28/I2 human chondrocyte cell line was used as a reproducible '*in vitro*' model to determine chondrocyte pathophysiological changes in response to different experimental conditions in our study. The cells were grown in complete DMEM at 37^{o}C in 5% CO_2.

Preparation of SFA-BSA complexes

Analytical grade lauric acid (LA) (Sigma-Aldrich, L4250), myristic acid (MA) (Sigma-Aldrich, M3128), palmitic acid (PA), (Sigma-Aldrich, P0500) and stearic acid (SA) (Sigma-Aldrich, S4751) were purchased from Sigma-Aldrich. Bovine serum albumin (BSA) (fatty-acid free) (Sigma-Aldrich, A7030) was used as the vehicle. The SFA-BSA complex solutions were made using a protocol described earlier (3). The chondrocyte pellets and the C-28/I2 cell line were stimulated with the different SFA at a concentration derived from the MTT assay. In certain experiments, the cells were treated with IL-1β (10ng/mL) (Sigma-Aldrich, SRP3083) in the presence or absence of SFA, to study the effects of SFA and inflammatory cytokines. Also in certain experiments, rapamycin (Sigma, R8781 - 10µM) and PI3K inhibitor - LY290042 (Sigma, L9908 - 10µM) were used to respectively activate and inhibit autophagy respectively.

MTT Assay

The primary human chondrocytes were seeded in a 96-well tissue culture plate. The cells were stimulated with SFA at final concentrations of 30, 60 and 90 µM for 24 and 48 hours respectively. The assay was performed to assess the toxicity of SFA on human chondrocytes, according to the manufacturer's protocol (Sigma-Aldrich, TOX1).

Western Blotting

The chondrocyte cells were seeded at 30,000 cells/well in a 6 well plate. After reaching confluency, the cells were stimulated with 30μM of different SFA (derived from MTT) for a period of 24 hours. The procedure was performed according to our published protocols (9). The membranes were incubated with primary Rabbit monoclonal antibodies (LC3,1:1000; ATG5, 1:1000; BEC1, 1:1000, p-p65, 1:1000, p-65, 1:1000 and α-tubulin,1:5000, (Cell Signalling Technologies, 4445T) overnight at 4^{o}C. A Goat anti-Rabbit secondary antibody (IRDye 800CW, 1:10000, Li-cor, 926-32111) was used. A semi-quantitative assessment of the protein bands was performed using the Li-cor's Image Studio Lite software (version 5.0).

GFP-LC3 plasmid transfection

The human chondrocytes were seeded on coverslips in a 24 well plate at 2 x 10^4 cells/well and cultured for 24 h before being transfected with a pEGFP-LC3 plasmid (Addgene 21073) using Lipofectamine 2000 (Thermo Fisher, 11668019) following the manufacturer's protocol. Four hours after transfecting, the cells were treated with the different SFA for a period of 24 hours. After 24 hours, the cells were fixed in PFA and the coverslips mounted on a frosted glass slide with Prolong Gold-DAPI (Thermo Fisher, P36935) and observed by confocal microscopy (Leica TCS SP8, Germany) to determine the rate of autophagosome formation between the different treatment groups.

Immunofluorescence

Human chondrocytes were seeded at 2 x 10^4 cells/well and cultured in a 24 well plate to confluency. The procedure was performed according to our published protocols (10). The cells were then incubated with the primary NF-κB p65 antibody (1:500) (Cell Signalling Technologies, 8242T) overnight at 4^{o}C and the following day was incubated with an Alexa Fluor 488 conjugated secondary antibody and the nuclei stained with DAPI. Images were acquired with a confocal microscope (Leica TCS SP8, Germany).

Alcian Blue staining

The 3D chondrocyte pellets paraffin sections were stained with 1% Alcian blue solution (Sigma-Aldrich, B8438) cultures according to our published protocols (11).

Transmission electron microscopy (TEM)

Transmission electron microscopy was used to identify the presence of autophagosomes in the chondrocytes according to our published protocols (12).

RNA extraction and real-time PCR

Total RNA was extracted from the chondrocytes using TRIzol reagent (Thermo Fisher, 15596018), treated with DNase and purified according to manufacturer's protocol using an RNeasy Mini Kit (Qiagen, 74104). The cDNA was extracted and the Real-time quantitative PCR was performed as described previously by our group (9, 11, 13, 14).

Statistical analysis

Statistical analyses were performed using GraphPad Prism. The data are presented as the mean ± standard deviation (SD) for all variables. The normal distribution of the data was first verified then the data analyzed by ANOVA. Repeated-measures analysis of variance with *post hoc* tests (Dunnett's/Bonferroni) was used to assess statistical significance. All experiments were performed in triplicate using different human chondrocytes collected from 5 different donors (5 independent experiments), and the C-28/I2 cell culture was performed in triplicate from 5 independent experiments. The level of significance was set at $P<0.05$.

Results

Effects of SFA on chondrocyte proliferation

The rate of chondrocyte proliferation in response to various concentrations of SFA was determined at 24 and 48 hours. After both 24 and 48 hours of SFA challenge, we observed a gradual decrease in the chondrocyte proliferation commensurate with the increase in the concentration of SFA. In comparison to the BSA-treated controls, the LA-treated chondrocytes exhibited a very similar rate of proliferation irrespective of the concentration and time point, whereas the MA, PA, and SA treated chondrocytes showed a decrease in the rate of proliferation, however, was not significant **(Figure 1)**.

Effects of SFA on chondrocyte differentiation

We next studied the effects of different SFA on chondrocyte differentiation. Normal ACC pellets maintained in the chondrogenic differentiating medium were challenged with 30 µM of the various SFA for a period of 14 days. There was a significant decrease in Alcian blue staining indicative of loss of proteoglycans in chondrocytes exposed to PA and SA compared to the controls. In contrast, LA and MA-treated pellets showed increased Alcian blue staining compared to PA and SA **(Figure 2A)**.

The effect of the SFA on chondrogenesis was further evaluated by the expression of the chondrogenic markers *ACAN*, *COL2*, *SOX9* and the degenerative markers *MMP13*, *ADAMTS4* and *ADAMTS5* respectively **(Figure 2B)**. In comparison with the C treated chondrocytes, PA and SA treatment showed a significant downregulation in the gene expression of *ACAN*, *COL2* and *SOX9* and significant upregulation of the gene expression of *MMP13*, *ADAMTS4*, and *ADAMTS5*. However, in comparison with the C treated

chondrocytes, LA and MA treatment exhibited similar gene expression levels of *ACAN*, *COL2* and *SOX9, MMP13, ADAMTS4* and *ADAMTS5* and hence were not significant.

Differential effects of SFA on autophagy in chondrocytes

Western blot analysis was performed to determine the protein expression of the autophagy markers LC3-II, ATG5 and BEC-1 **(Figure 3A)**. Protein expression levels were normalized against alpha-tubulin. The expression of LC3-II, a key regulator of autophagy, was significantly increased in MA, PA and SA treated chondrocytes compared to control treated chondrocytes. However, we observed LA treated chondrocytes to exhibit similar expressions as that of the control **(Figure 3B)**. The expression patterns of both ATG5 and Beclin-1 were in cohesion with LC3-II, as MA, PA, and SA once again showed increased expression of Beclin-1 levels, whereas, LA treated chondrocytes showed similar expression levels as that of the control **(Figure 3C, D)**.

The use of GFP-LC3 constructs is a well-characterized method to monitor the accumulation of LC3 puncta, formation and stabilization of autophagosomes (15). The efficacy of the transfections was assessed by confocal microscopy 24 hours after transfection and SFA treatments. The presence of perinuclear GFP-LC3 puncta was an indication of successful transfections and also autophagy activation by the SFA. There was a significant increase in the level of perinuclear GFP-LC3 expression in the PA and SA treated chondrocytes compared with the LA and MA-treated cells, an indication of greater autophagosome formation. The LA treatment, in particular, showed GFP-LC3 expression levels which were quite comparable to the controls suggesting autophagosome stabilization **(Figure 4A)**.

Role of SFA in IL-1β mediated autophagy activation in chondrocytes

IL-1β stimulation has been reported to mimic OA-like gene expression changes in chondrocytes (16). The link between SFA and the inflammatory marker IL-1β in mediating activation of autophagy was explored. Cells treated with IL-1β (10 ng/ml) for 24 hours resulted in significant increase in GFP-LC3 puncta formation as well as ATG5 and BEC-1 gene expression, implying that the autophagy activity and stabilization was induced by catabolic stress **(Figure 4A)**.

IL-1β treatment significantly increased autophagosome formation compared to controls and there was significantly greater GFP-LC3 expression in PA and SA treated cells compared with LA and MA-treated cells, suggesting greater catabolic stress in the former. Interestingly, LA+IL1β treatment did not elicit a significant increase in autophagosome formation compared with LA treatment, suggesting LA had the capacity to attenuate the effect of IL-1β. Cells treated with MA, PA and SA showed a significant increase in the gene expression of *ATG5* both in the absence and the presence of IL-1β. By contrast, LA treatment resulted in a markedly reduced gene expression of *ATG5* both in the absence and the presence of IL-1β **(Figure 4B)**. A similar result was also observed in the case with *BEC-1*, where MA, PA, and SA treatment led to a significantly increased gene expression, whereas in LA-treated cells the *BEC-1* expression was comparable to the controls, both in the absence and presence of IL-1 β **(Figure 4C)**.

The gold standard of measuring the activation of autophagy is by using a TEM. We observed PA and SA treated chondrocytes to exhibit a significant increase in the presence of autophagosomes and autolysosomes with a double membrane in comparison with the C treated chondrocytes. However, in contrast, we noticed LA treated chondrocyte cells to show the similarly reduced activity of autophagy as compared to C treated chondrocytes, also evidenced by the percentage of autophagosomes **(Figure 4B and 4E)**.

Effect of SFA on activation or inhibition of autophagy

Activation and inhibition assays were conducted to further investigate the effects of autophagy flux in chondrocytes. Rapamycin is a potent activator of autophagy whereas the PI3K inhibitor LY290042 is a well know autophagy inhibitor. Upon treatment with rapamycin, we observed PA and SA treated chondrocytes to show increased autophagy activity compared to other treatment groups. These results suggest that PA and SA treatment coupled with rapamycin significantly increases the activation of autophagy. On the other hand, when the chondrocytes were treated with LY290042, we noticed a significant reduction in the activation of autophagy compared to rapamycin treatment. However, in line with our previous results, we observed PA and SA treatment to exhibit an increase in the activation of autophagy compared to other treatment groups **(Figure 5A)**.

Gene expression analysis in untreated samples revealed LA to show similar expressions of *ACAN*, *COL2*, *SOX9*, *MMP13*, *ADAMTS4*, and *ADAMTS5* in comparison with control. However, MA, PA and SA treatment significantly decreased early chondrogenic markers such as *ACAN*, *COL2*, and *SOX9* and significantly increased degenerative markers such as *MMP13*, *ADAMTS4* and *ADAMTS5* **(Figure 5B)**. In comparison with control treated cells,

MA, PA, and SA showed a significant reduction in the gene expression of *ACAN*, *COL2* and *SOX9* and a significant upregulation of the gene expression of *MMP13*, *ADAMTS4* and *ADAMTS5* irrespective of rapamycin and LY290042 treatments. Whereas, LA treated cells showed decreased expression levels of *ACAN*, *COL2*, and *SOX9* and a significant downregulation of the gene expression of *MMP13*, *ADAMTS4* and *ADAMTS5* **(Figure 5B)**.

SFA-mediated activation of NF-κB Pathway

The impact of NF-κB pathway activation by the SFA was assessed by immunofluorescence and Western blot analysis of NF-κB p65. There was a significant nuclear translocation of NF-κB p65 in response to all SFA compared to controls, particularly in cells treated with LA, PA, and SA, suggesting a robust activation of the NF-κB pathway resulting from these SFA **(Figure 6A)**. In contrast, we noticed a decreased nuclear translocation in MA-treated cells. Consistent with the nuclear translocations, Western blots showed a commensurate increase in the protein levels of p-p65 **(Figure 6B and 6C)**.

Discussion

Lipotoxicity has been suggested as one of the most important players in the progression of conditions such as diabetes and obesity (17, 18), conditions that are generally thought to be initiated by an excessive amount of circulation of fatty acids which leads to lipid accumulation. The initiation and molecular mechanisms of autophagy are widely recognized as a means of cell survival which is triggered by cells to cope up with the physiological stresses. Currently, little is known about the role of autophagy in chondrocytes. In normal chondrocytes, autophagy is regarded as a protective mechanism and loss of autophagy due to aging has been linked with OA (19). However, autophagy also plays a dual role in chondrocyte metabolism, both protecting chondrocytes but also has a role in inducing

apoptosis in the pathogenesis of OA (20). Autophagy regulates the maturation of chondrocytes and aids the survival of terminally differentiated chondrocytes when these cells are challenged by stress such as hypoxia (21-23). The current view is that lipid metabolism is regulated by autophagy; however, it is still unclear how lipids such as SFA affect autophagy. Therefore, we sought to determine the effects of nutrient overload on chondrocytes, given this is a key consideration for obese patients.

Recent studies have shown a reduced rate of autophagy in the liver in mice fed with high-fat diet as well as genetically modified obese (ob/ob) mice and diabetic (DB/DB) mice, as evidenced by decreased expression of various autophagy markers (7). Palmitic acid treatment is known to induce autophagy in pancreatic – β cells (24, 25). However, it has also been found out reported that stimulation of PA inhibited autophagic vesicular fusion (26). In addition, PA stimulation did not cause activation of autophagy in hepatocytes (5). These inconsistencies may be attributable to factors such as the cell type used, the age of the animals, the concentration of FFA, and length of time of the FFA treatments (27). We, therefore, sought to understand the mechanisms behind the apparent effects of SFA both in the presence and absence of chondrocyte catabolism. We found evidence of differential effects of SFA on autophagy activation, by comparing protein expression of LC3-II, ATG5, BEC-1, GFP-LC3, autophagy activation, and inhibition assay using Rapamycin and LY290042.

In this study, there was evidence that suggested all four SFA tested were capable of inducing autophagy in chondrocytes; however, stimulation of PA and SA resulted in a significantly higher rate of activation when compared with MA and LA, as was evidenced by increased expression of LC3-II in the PA and SA groups. These findings are consistent with reports that showed that PA induces autophagy in osteoblasts (28) and pancreatic β cells (24). Other

studies have shown increased expression of the autophagy marker microtubule-associated protein-LC3 (MAP1LC3II) in chondrocytes in OA cartilage (29), a marker that is also increased in the superficial and the middle zones in a rat OA model (30). There is also evidence of a marked increase of LC3-II expression in late stage OA compared with normal cartilage (31). In the present study, this suggested that the increased expression of LC3 in chondrocytes, caused by the treatment of PA and SA, correlated to an OA cartilage. This notion was supported by the in vitro data that showed increased expression of the cartilage degenerative marker MMP13 and the hypertrophic markers *ADAMTS4* and *ADAMTS5*, as well as decreased expression of *ACAN*, *COL2*, and *SOX9* in MA, PA and SA treated pellets compared to LA treatment. The expression of LC3-I subunit has only been observed in normal healthy cartilage (31), which could explain the absence of LC3-I expression seen in the SFA treated chondrocytes since these cells could be in potentially be in a hypertrophic state. The response of the chondrocytes to IL-1β, irrespective of the SFA treatment, increased the activation of autophagy, as evidenced by increased expression of ATG5 and BEC-1 in MA, PA and SA treated cells, suggesting catabolism mediated by MA, PA, and SA treatment increases the rate of autophagy activation.

The NF-κB family consists of a group of transcription factors that are activated by pro-inflammatory cytokines in the course of OA pathogenesis. In the present study, the expression of p65 was localized to the cytoplasm in the chondrocytes. SFA treatment increased p65 expression, with LA, PA, and SA resulting not only in higher expression but also nuclear translocation. This evidence suggests that stimulation of chondrocytes by LA, PA, and SA significantly increased the rate of NF-κB pathway activation in chondrocytes. However, the link between NF-κB and autophagy is convoluted and by no means clear, as we also observed decreased expression in MA-treated chondrocytes. Nevertheless, we found a

strong correlation between autophagy activation and NF-κB activation, which suggests that excess circulating SFA may activate the NF-κB pathway, which in turn increases the expression of autophagy-related proteins and stimulate autophagocytosis. Further investigations are needed to determine how these two pathways are linked.

Conclusion

This study showed that chondrocytes exposed to SFA concentrations of up to 90 μM had no cytotoxic effects. Stimulation of chondrocytes with MA, PA, and SA, irrespective of presence or absence of IL-1β, resulted in increased activation of autophagy compared to LA. We also found increased activation of the NF-κB pathway in LA, PA and SA treated cells which strongly correlated with autophagy activation. These findings shed light on the molecular mechanisms by which certain SFA activate autophagy and the NF-κB pathway, and provide functional nutritional insights – they also open a path for novel treatment options for obesity-related OA, and as such pharmacological activation of autophagy can be used as a targeted therapeutic approach for OA.

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Statement of author's contribution

YX and IP and RC designed research, SS and XW conducted research, SS and XW and IP analyzed data and SS wrote the paper. IP and TF proof read the manuscript. IP had primary responsibility for final content. All authors read and approved the final manuscript.

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367 Disclosure of potential conflicts of interest

368 The authors have declared that no financial conflict of interest exists.

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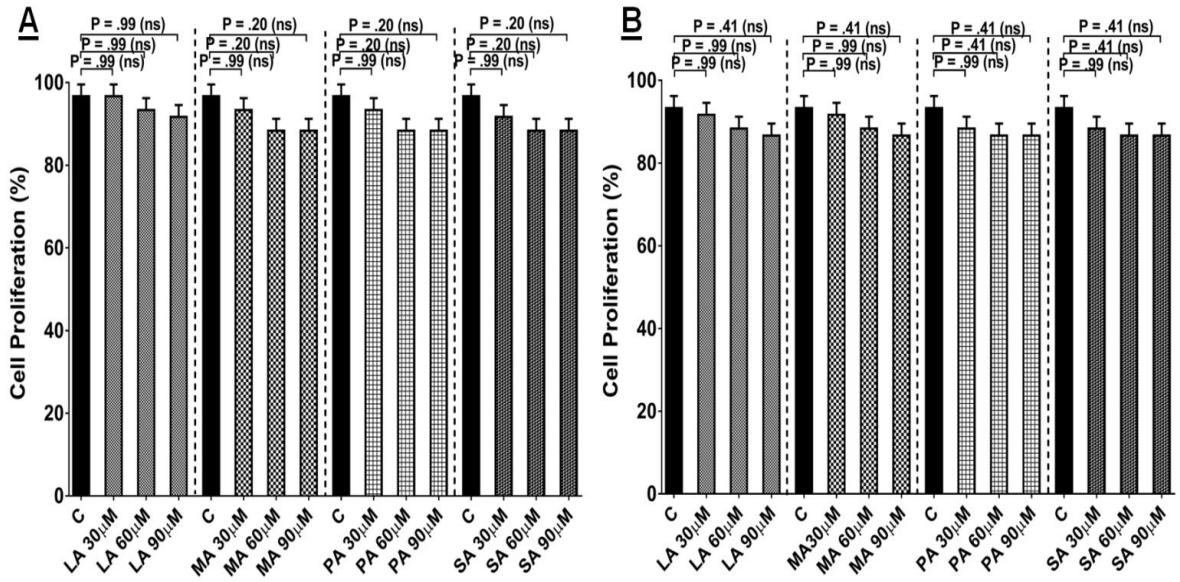

Figure 1: Effects of SFA on the viability of chondrocytes. Human chondrocytes were treated with 30–90 μM of the different SFA for **(A)** 24 and **(B)** 48 hours and analyzed by MTT assay. We observed a gradual decrease in the cell viability with the increase in the concentration of the SFA. All experiments were performed in triplicate. The values are presented as mean ± SD. ($P < 0.05$); (n=5).

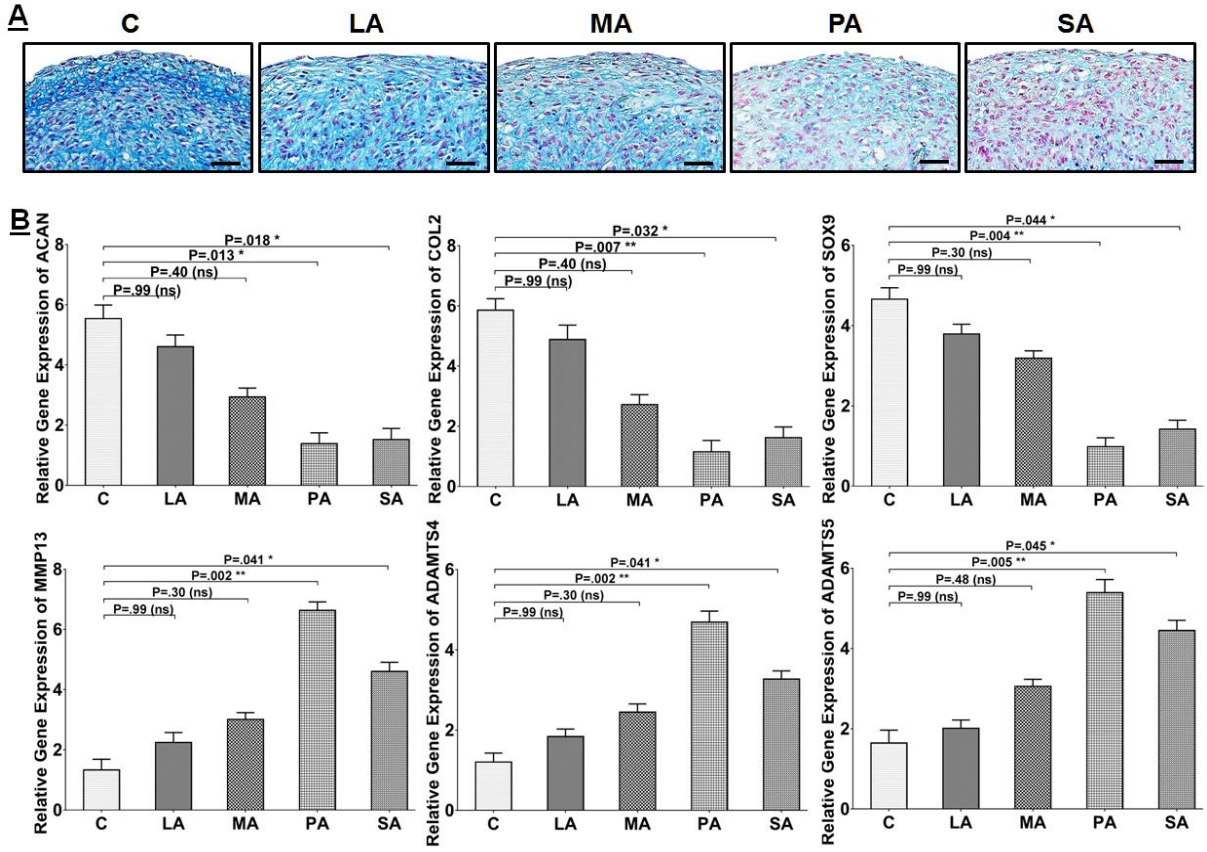

Figure 2: Effects of SFA on chondrogenic differentiation. (A) Alcian blue staining on 5 µm paraffin sections of human chondrocytes treated with the different SFA for 14 days in a 3-D chondrogenic pellet culture. Proteoglycans are shown in blue. Scale = 100 µm. **(B)** Relative gene expression of *ACAN*, *COL2*, *SOX9*, *MMP13*, *ADAMTS4*, and *ADAMTS5* in response to the treatment with different SFA as determined by RT-qPCR. All experiments were performed in triplicate. The values are presented as mean ± SD. ($P < 0.05$); (n=5).

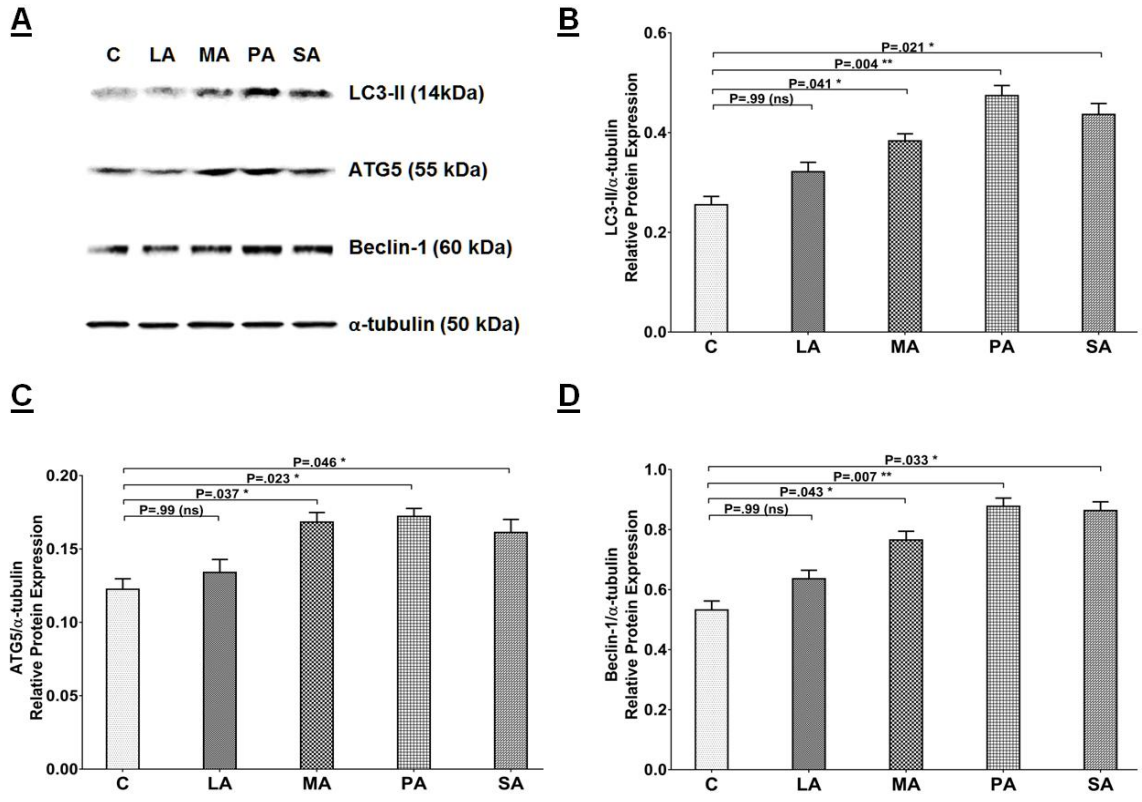

Figure 3: State of autophagy in human chondrocytes following SFA treatment. (A) Human chondrocytes were treated with SFA for 24 hours, and LC3-II, ATG5, and BEC-1 protein expression assessed by Western blot analysis. The relative expression of LC3-II **(B)**, ATG5 **(C)** and BEC-1 **(D)** were assessed by densitometry. All experiments were performed in triplicate. The values are presented as mean ± SD. ($P < 0.05$); (n=5).

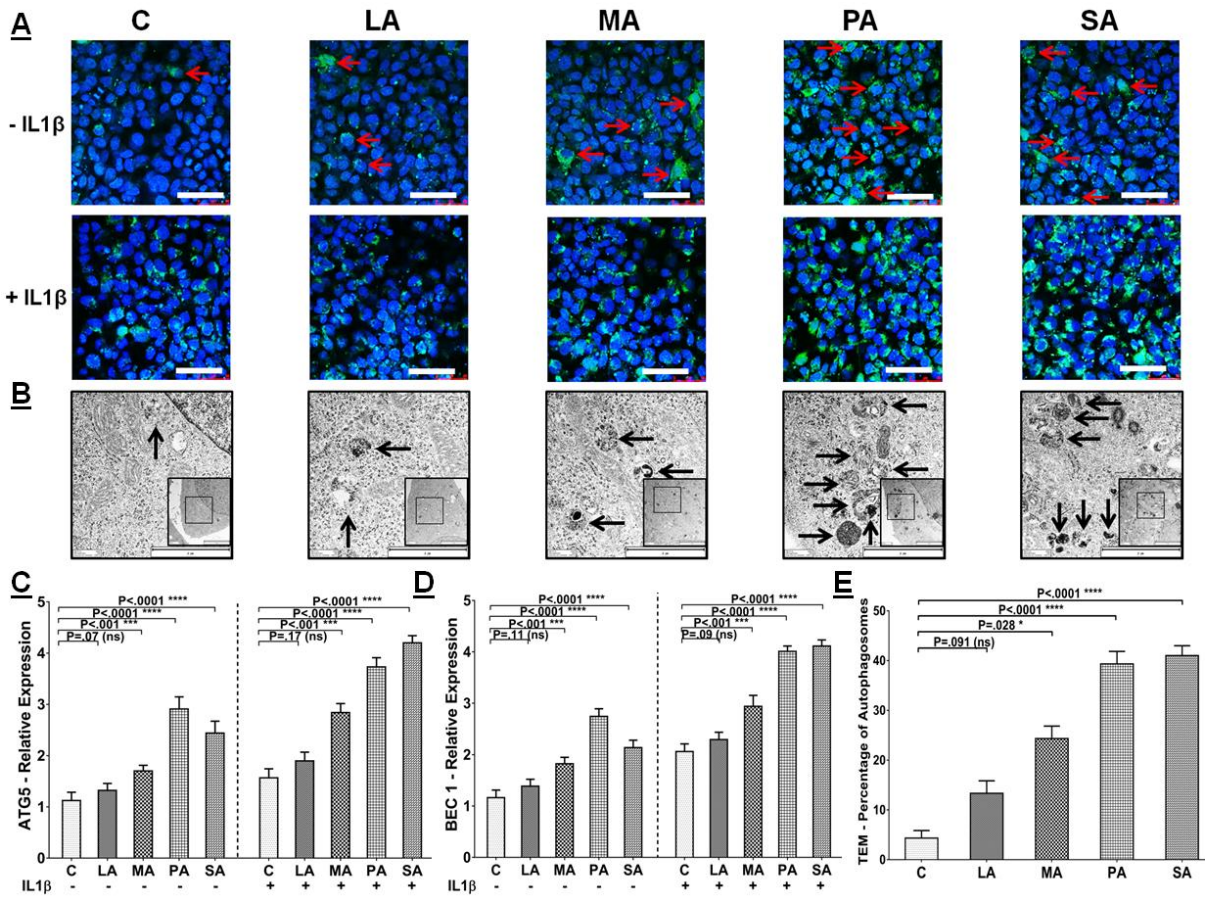

Figure 4: Autophagy activation by SFA under inflammatory conditions. (A) The differential rate of autophagy activation caused by the treatment with SFA under normal and inflammatory conditions (10ng/ml-IL1β) 24 hours after GFP-LC3 transfection and SFA treatment (Red arrows indicate autophagy positive cells) in C28/I2 cells. Scale = 50 µm. **(B)** Activation of autophagy was examined by TEM in C28/I2 cells. Increased presence of autophagosomes and autolysosomes can be observed in PA and SA treated groups (Black arrows indicate autophagosomes). Scale = 2 µm (Insert Image Scale = 5 µm). Relative gene expression of *ATG5* **(C)** and *BEC-1* **(D)** in response to the treatment with SFA ± IL1β was determined by RT-qPCR. **(E)** Graph showing percentage of autophagosomes in the different groups determined using Image J software. All experiments were performed in triplicate. The values are presented as mean ± SD. ($P < 0.05$); (n=5).

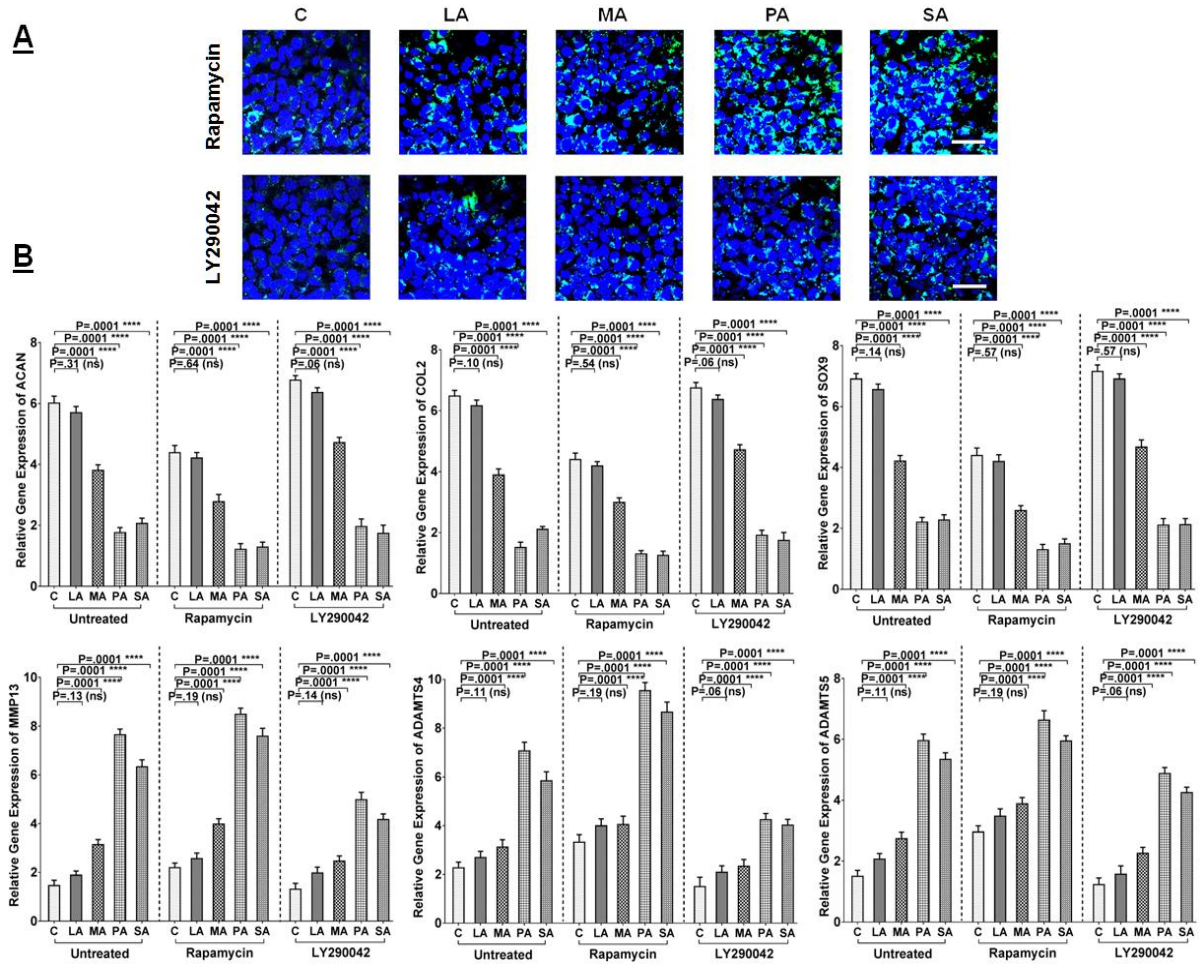

Figure 5: Role of SFA on autophagy activation and inhibition. (A) Effect of SFA on C28/I2 cells treated with rapamycin (activation) and LY290042 (inhibition). Scale bar = 50 µm. **(B)** Relative gene expression of *ACAN*, *COL2*, *SOX9*, *MMP13*, *ADAMTS4*, and *ADAMTS5* in response to treatment with SFA under autophagy activation or inhibitory conditions was determined by qPCR. All experimental samples were performed in triplicate. The values are presented as mean ± SD. ($P < 0.05$); (n=5).

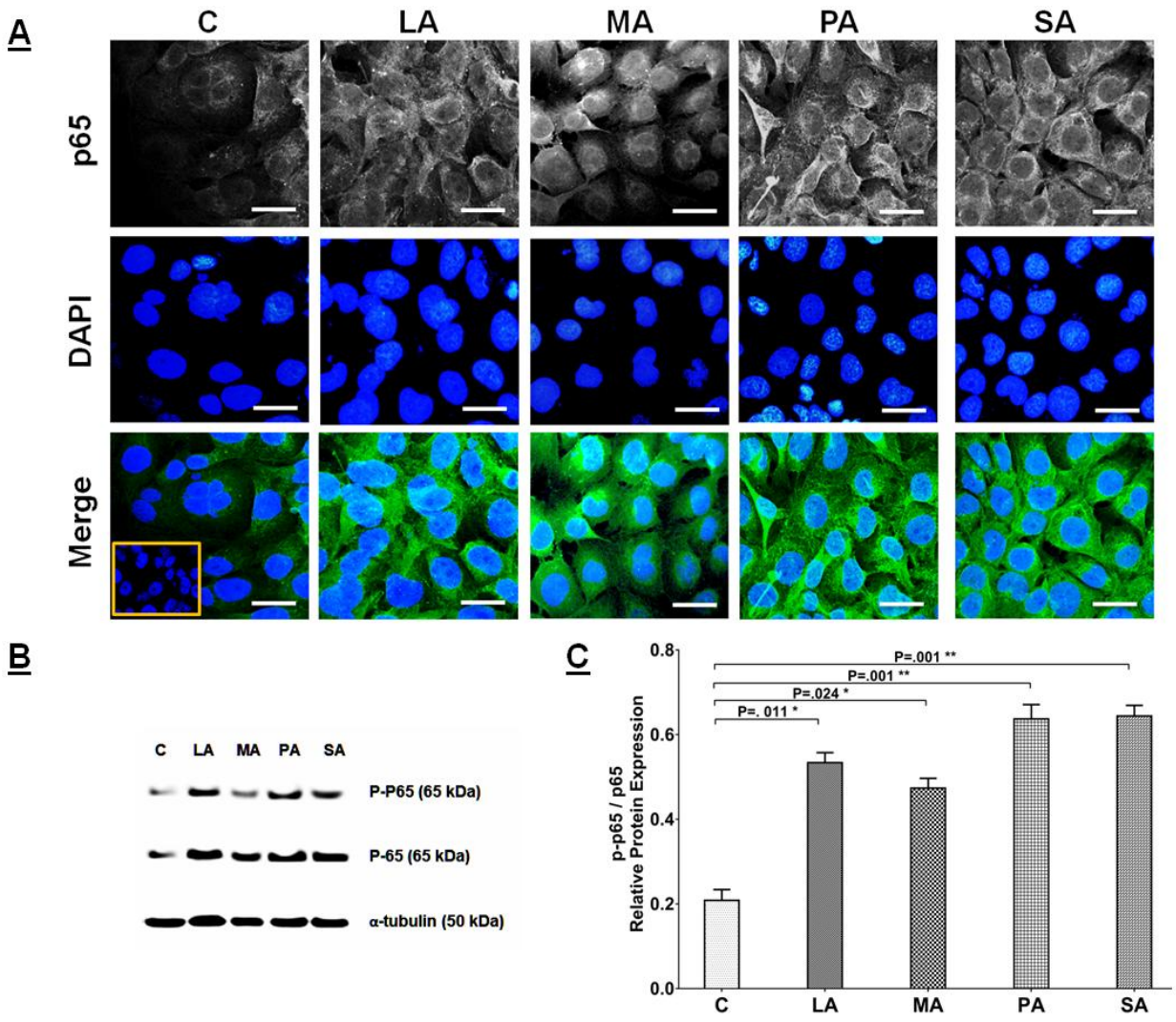

Figure 6: SFA-mediated activation of NF-κβ Pathway. (A) Nuclear translocation of NF-κβ p65 in SFA treated C28/I2 cells at 24 hours. The negative control is shown (insert). Scale bar = 50 µm. **(B)** Chondrocytes were treated with SFA for 24 hours and p65 and p-p65 expression determined by western blot analysis. **(C)** The optical density of p-p65 was analyzed. All experiments were performed in triplicate. The values are presented as mean ± SD. ($P < 0.05$); (n=5).

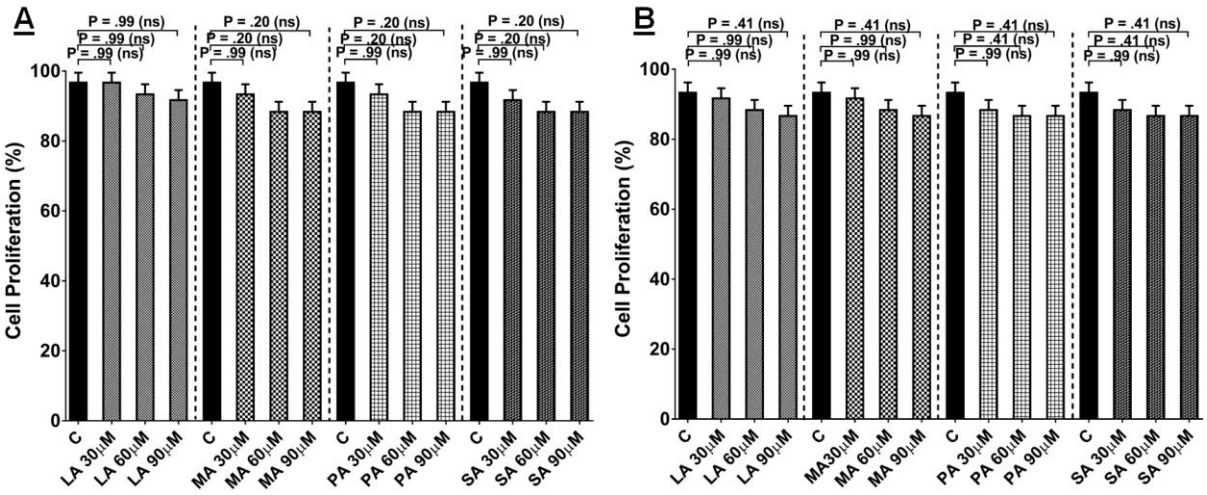
A
B
Cell Proliferation (%)
P = .99 (ns)
P = .20 (ns)
P = .41 (ns)
C
LA 30μM
LA 60μM
LA 90μM
MA 30μM
MA 60μM
MA 90μM
PA 30μM
PA 60μM
PA 90μM
SA 30μM
SA 60μM
SA 90μM

FIGURE 1.tif

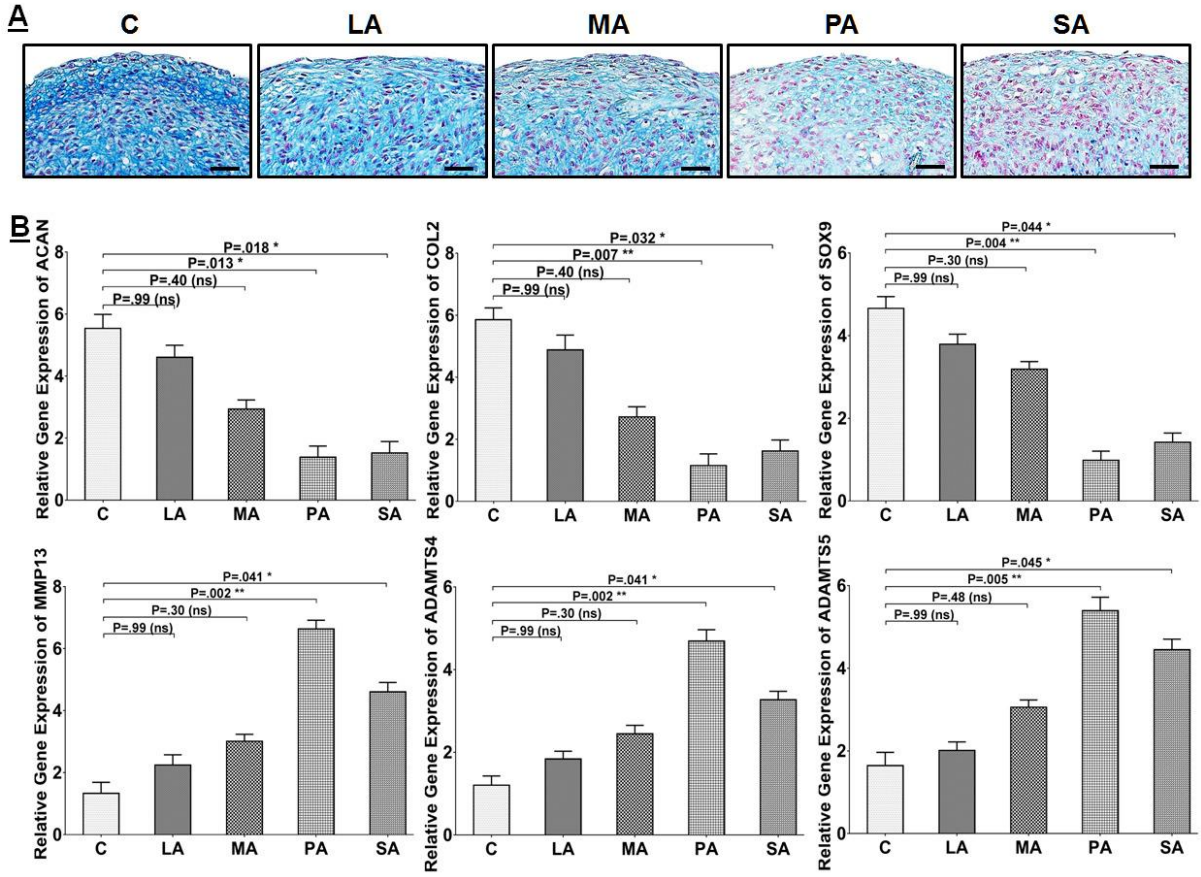
A
C
LA
MA
PA
SA
B
Relative Gene Expression of ACAN
P=.018 *
P=.013 *
P=.40 (ns)
P=.99 (ns)
Relative Gene Expression of COL2
P=.032 *
P=.007 **
P=.40 (ns)
P=.99 (ns)
Relative Gene Expression of SOX9
P=.044 *
P=.004 **
P=.30 (ns)
P=.99 (ns)
Relative Gene Expression of MMP13
P=.041 *
P=.002 **
P=.30 (ns)
P=.99 (ns)
Relative Gene Expression of ADAMTS4
P=.041 *
P=.002 **
P=.30 (ns)
P=.99 (ns)
Relative Gene Expression of ADAMTS5
P=.045 *
P=.005 **
P=.48 (ns)
P=.99 (ns)
C
LA
MA
PA
SA

FIGURE 2.tif

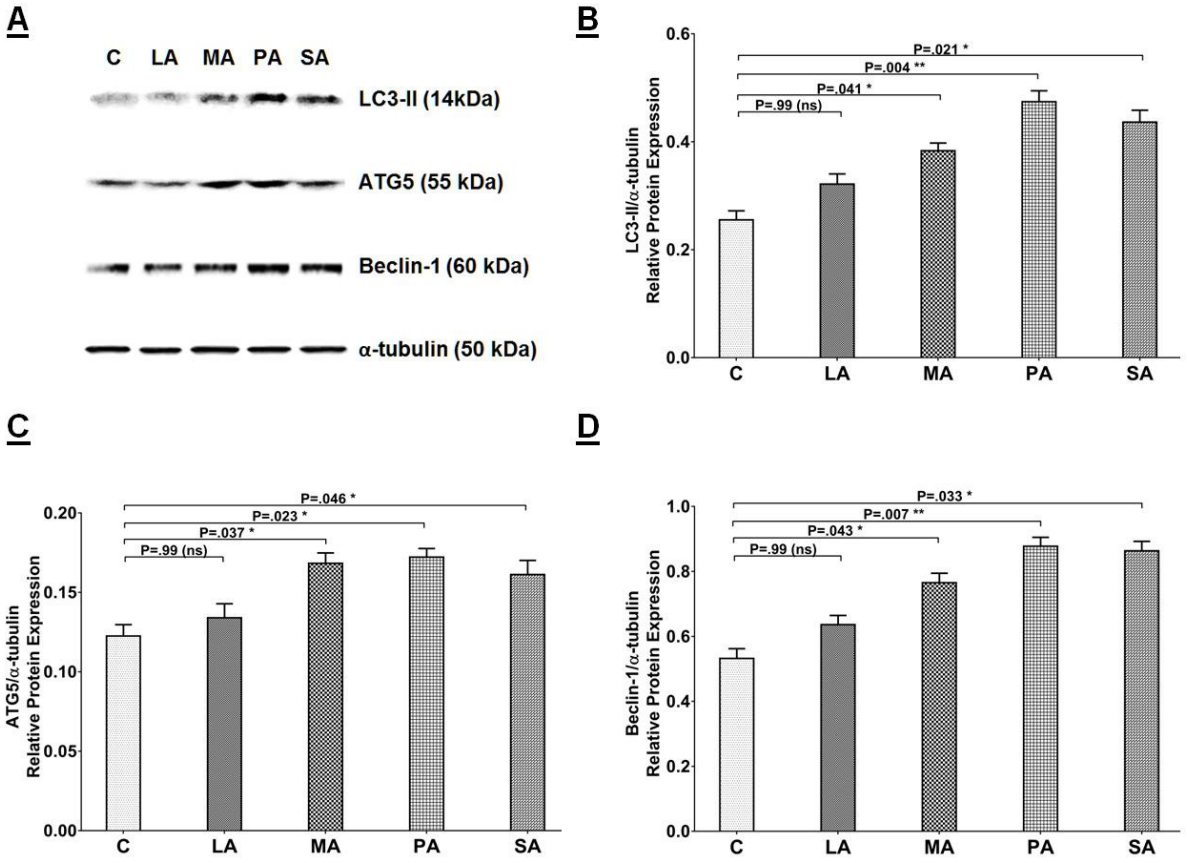
A
C LA MA PA SA
LC3-II (14kDa)
ATG5 (55 kDa)
Beclin-1 (60 kDa)
α-tubulin (50 kDa)
B
LC3-II/α-tubulin
Relative Protein Expression
P=.021 *
P=.004 **
P=.041 *
P=.99 (ns)
C LA MA PA SA
C
ATG5/α-tubulin
Relative Protein Expression
P=.046 *
P=.023 *
P=.037 *
P=.99 (ns)
C LA MA PA SA
D
Beclin-1/α-tubulin
Relative Protein Expression
P=.033 *
P=.007 **
P=.043 *
P=.99 (ns)
C LA MA PA SA

FIGURE 3.tif

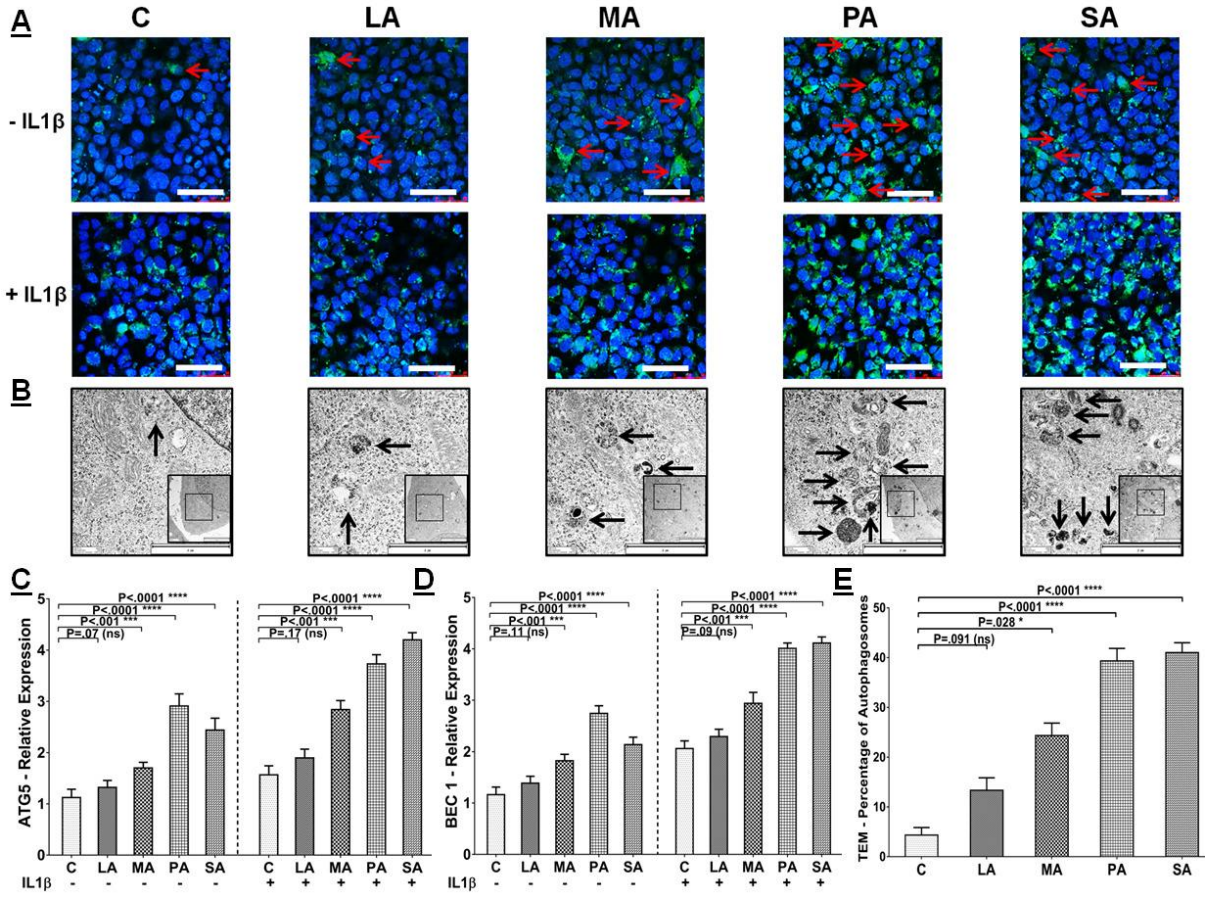
A
C
LA
MA
PA
SA
- IL1β
+ IL1β
B
C
ATG5 - Relative Expression
P<.0001 ****
P<.0001 ****
P<.001 ***
P=.07 (ns)
P=.17 (ns)
IL1β
D
BEC 1 - Relative Expression
P=.11 (ns)
P=.09 (ns)
E
TEM - Percentage of Autophagosomes
P=.028 *
P=.091 (ns)

FIGURE 4.tif

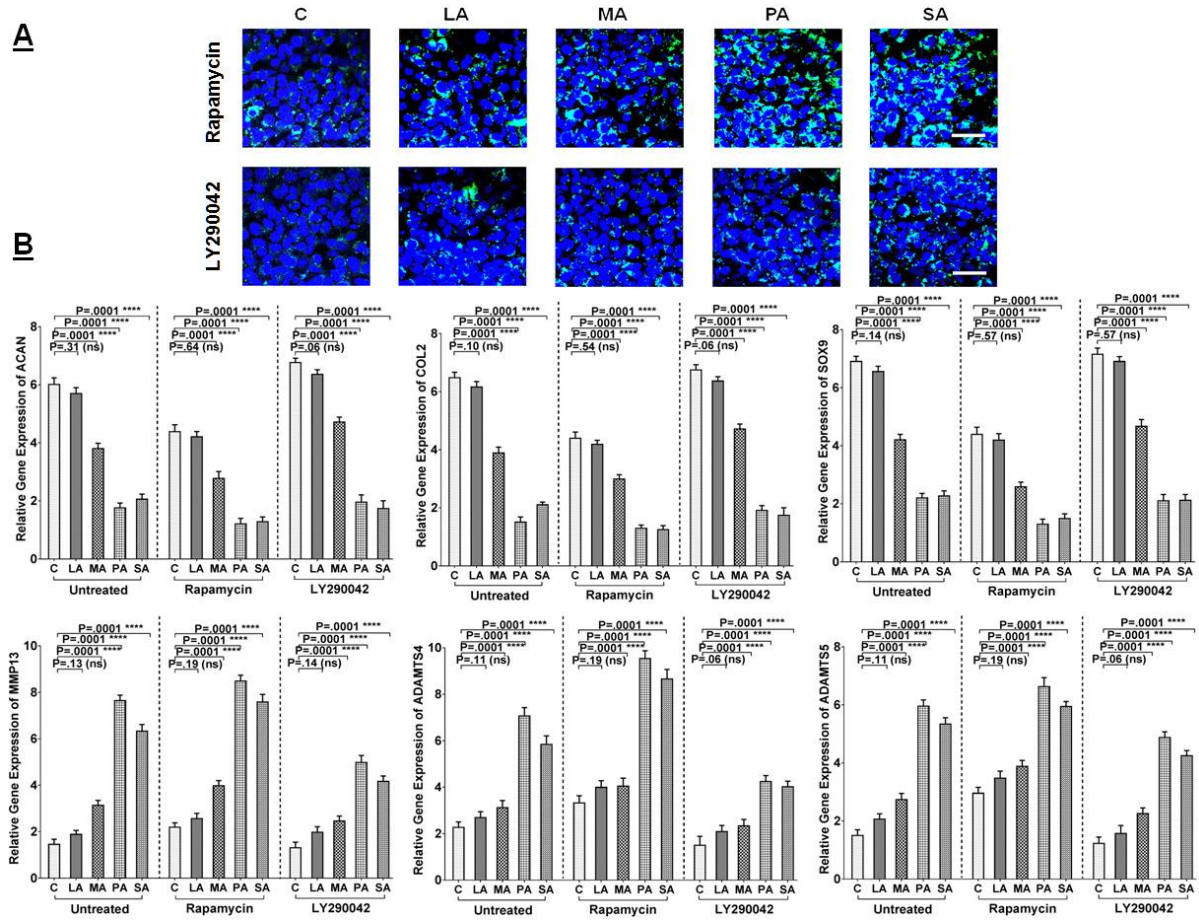
A
B
C
LA
MA
PA
SA
Rapamycin
LY290042
Relative Gene Expression of ACAN
Relative Gene Expression of COL2
Relative Gene Expression of SOX9
Relative Gene Expression of MMP13
Relative Gene Expression of ADAMTS4
Relative Gene Expression of ADAMTS5
Untreated
Rapamycin
LY290042

FIGURE 5.tif

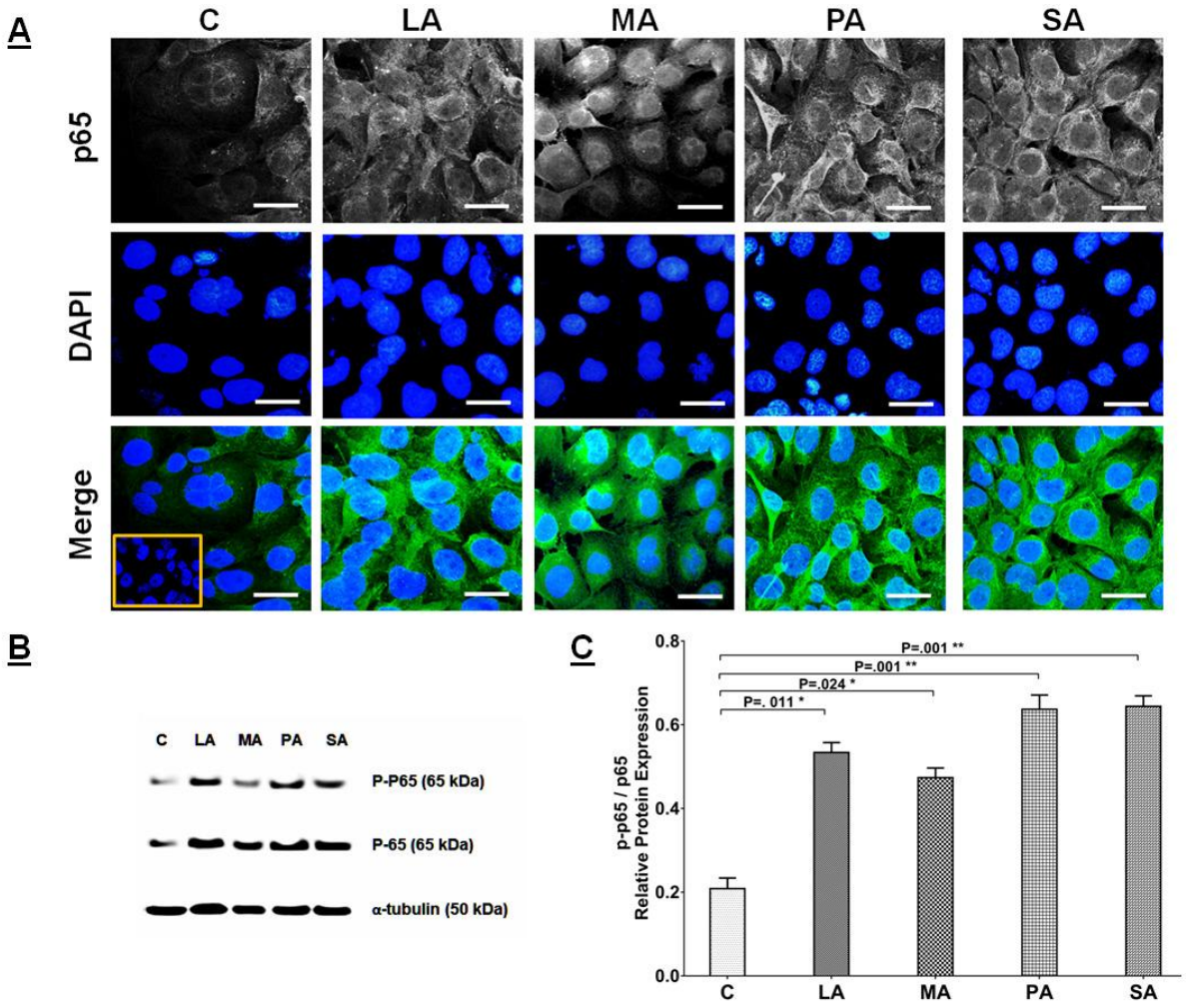
A
C
LA
MA
PA
SA
p65
DAPI
Merge
B
C
LA
MA
PA
SA
P-P65 (65 kDa)
P-65 (65 kDa)
α-tubulin (50 kDa)
C
P=.001 **
P=.001 **
P=.024 *
P=. 011 *
p-p65 / p65
Relative Protein Expression
0.8
0.6
0.4
0.2
0.0
C
LA
MA
PA
SA

FIGURE 6.tif