Role of simvastatin and RORα activity in the macrophage apoptotic pathway

Neslihan Çoban, Çağrı Güleç, Bilge Özsait Selçuk, Nihan Erginel-Ünaltuna

Department of Genetics, Institute for Experimental Medicine, İstanbul University; İstanbul-*Turkey*

ABSTRACT

Objective: Atherosclerosis is a chronic inflammatory condition and is one of the main causes of death worldwide. Macrophages play important roles in the formation of atherosclerotic plaques. Apoptosis is progressively observed while plaques develop, although the precise mechanisms and outcomes of apoptosis in atherosclerosis development and progression are still contradictory. This study was conducted to explore the effects of simvastatin and retinoic acid receptor-related orphan receptor alpha (RORα) ligands on apoptosis in human acute monocytic leukemia (THP-1) macrophage cells.

Methods: Briefly, the occupancy of RORα in the promoter regions of apoptotic pathway genes was demonstrated in THP-1 cell lines using chromatin immunoprecipitation (ChIP) analysis. In order to modulate RORα activity, THP-1 macrophage cells were treated with specific ligands (CPG52608 and SR1001) and then viability as well as count of THP-1 macrophage cells were analyzed.

Results: We observed that simvastatin and both RORα ligands had a tendency to decrease THP-1 macrophage cell viability in culture. When compared with non-treated controls, simvastatin significantly decreased cell viability (p=0.04) and cell count (p=0.03). However, this negative effect of simvastatin seemed to be partly prevented by RORα ligands. In addition, bioinformatics analysis of ChIP-on-chip data demonstrated that several genes that are involved in the apoptotic pathway were likely RORα target genes. These genes were involved in the regulation of apoptosis through various pathways.

Conclusion: In summary, our study suggest that simvastatin-mediated macrophage apoptosis might be modulated by SR1001 administration. However, involvement of RORα in this modulation through potential apoptotic target genes remains elusive. *(Anatol J Cardiol 2017; 17: 362-6)*

Keywords: RORα ligands, atherosclerosis, simvastatin, apoptosis, THP-1 macrophage, cell viability

Introduction

Macrophages play crucial roles as a primary line of defense against infectious pathogens and function in the phagocytosis of cancer cells, apoptotic cellular debris, and foreign substances. However, macrophages are also involved in the pathogenesis of a number of diseases, including insulin resistance, cancer, and atherosclerosis (1, 2). Atherosclerosis is a chronic inflammatory disease of the arterial wall and one of the leading causes of mortality in the world. Macrophages are key players in atherosclerotic development, progression, and, importantly, stability of the plaque due to their contribution in the formation of the core and degradation of the fibrous cap. Atherosclerosis develops as a failure to resolve the inflammatory response in the arterial wall that was initiated by the accumulation of apolipoprotein B (apoB)-containing lipoproteins within the wall (3). These lipoproteins are taken up by tissue macrophages that become engorged with cholesterol and transform into foam cells and are subsequently activated. The inflammatory response to retained lipoproteins is maladapted because the macrophage foam cells do not leave but are retained in the vessel wall (4). Furthermore, being the part of an essential component of innate immunity, macrophages are capable of differentiating into protean varieties with a range of function (5–7).

Macrophages are a specific group of cells that show plasticity and are capable of differentiating into different functional phenotypes upon exposure to different environmental conditions (8). However, most of these phenotypes were induced in cell-culture conditions supplemented with various growth factors or cytokines (9). Among these, macrophage M1 (classically activated) and M2 (alternatively activated) are the predominantly used terms defining the functioning phenotypes (10). M1 macrophages are considered to be inflammatory and take part in tissue destruction (7). However, M2 macrophages produce anti-inflammatory cytokines and are involved in immune regulation and wound healing (7). With the progression of the atheroscle-

Anatol J Cardiol 2017; 17: 362-6

Çoban et al.
Simvastatin and RORα activity in the macrophages   *363*

rotic lesions, microenviroment of the plaque becomes more inflammatory, and this change causes M1 macrophage phenotype to dominate M2 phenotype (11). Due to their opposing actions, M1 and M2 act differentially in atherosclerotic plaque. For example, M1 macrophages are pro-atherogenic, whereas M2 macrophages might promote plaque stability.

Previous studies demonstrated that orphan receptor alpha (RORα) functions as a ligand-dependent transcriptional factor. Melatonin and cholesterol are the two known ligands of RORα and both were shown to be important in atherosclerosis (12, 13). RORα binds to compounds such as cholesterol and its metabolites that are abundant in cells and modulates the transcription of the target genes. However, the function of RORα in monocytic cells, which play critical roles in the development of atherosclerosis, is not clear. In the vascular system, RORα mRNA was detected in human smooth muscle cells (SMCs), endothelial cells (ECs), and mammary arteries (14, 15). Furthermore, RORα expression in atherosclerotic plaque was found to be significantly decreased (14). These findings suggest that RORα might play an important role in modulating inflammatory responses and lipoprotein metabolism thus take part in the switching of macrophage phenotype in vasculature.

In this study, our aim was to investigate the role of specific ROR-response elements (ROREs) in atherosclerosis-associated molecular mechanisms in macrophages. In the scope of this study, we identified ROREs within the promoter regions of the apoptotic pathway genes and subsequently investigated whether these ROREs were occupied by RORα in monocyte cells. In order to complement the study, we then analyzed whether the RORα activity in the macrophages, influencing cell viability and cell count, is affected by the presence of simvastatin, a lipid-lowering drug.

Methods

Reagents and chemicals

Cell culture media and supplements were purchased from Biochrom (Berlin, Germany). A synthetic ROR agonist (CGP 52608), simvastatin, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A synthetic ROR inverse agonist (SR1001) was purchased from Cayman (Hamburg, Germany). We used dimethyl sulfoxide (DMSO, Wako) as a solvent, and all compounds were dissolved in DMSO. Other chemicals and kits used in the experiments were formaldehyde (Merck), glycine (Sigma), and Millipore EZ-ChIP Chromatin Immunoprecipitation Kit (Millipore, Billerica, MA, USA).

Cell culture and treatment

Human monocytic cell line (THP-1) was obtained from ATCC (THP-1, ATCC No: TIB-202, Manassas, VA) and cultured according to the manufacturer's recommendations. Briefly, both cell lines were grown in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom, Ber-

lin, Germany), penicillin/streptomycin (100 U/100 µg/mL), and L-glutamine (2 mmol/L) at 37°C in 5% CO_2 atmosphere. THP-1 cells were treated with RORα-specific ligands (CPG 52608, 5 nM and SR1001, 5 µM). Treatment doses of ligands were determined using toxicity tests performed by serial dilutions of ligands, where dilution ranges were selected based on the previously published data by other authors (16, 17). Next, macrophages were obtained by 200-nM PMA treatment of THP-1 cells. Macrophage cells were treated with RORα-specific ligands (CPG 52608, 5 nM and SR1001, 5 µM), and in order to inhibit cholesterol synthesis, cells were treated with simvastatin (10 µM). Cell viability was analyzed by staining with trypan blue-based method using ViCell instrument (Bekman Coulter, USA).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using Magna ChIP kit (Millipore) according to manufacturer's instructions with modifications. Briefly, THP-1 cells were cultured in 10-cm dishes, and experiments were conducted using 1×107 cells. After administration of THP-1 cells with RORα-specific ligand CPG 52608 (5 nM), ChIP was performed. After 24 hours of incubation, cells were treated with 1% formaldehyde (Merck). Cell lysates were sonicated on ice, 20 times for 10 s and separated by 20 s. Immunoprecipitations were performed by incubation of fragmented chromatin with appropriate antibodies overnight at 4°C. ChIP reactions contained the following antibodies: anti-mouse IgG (Millipore), anti-RNA Pol II (Millipore), and anti-RORα antibody (Santa Cruz Biotechnology). An aliquot of the cell lysates was used to isolate total input DNA. Amplifications of the immunoprecipitated DNA were performed using PCR. GAPDH gene was used as control for ChIP–PCR with primers 5'-TACTAGCGGTTTTACGGGCG-3' (forward) and 5'-TCGAACAGGAGGAGCAGAGAGCGA-3' (reverse).

ChIP–chip assays

In addition, enriched DNA fragments were then amplified using whole genome amplification kit (WGA, Sigma). Identification of DNA fragments was performed using whole-genome tiling array (NimbleGen). Microarray hybridizations were performed by the NimbleGen Service Facility (Iceland, WI) using standard protocols for ChIP–chip arrays, and data analysis was performed using NimbleGen SignalMap Software (signalmap_v1.9).

Bioinformatic analysis of potential RORα target genes

To perform annotation of potential target genes within the ChIP–array data, we used DAVID bioinformatics (http://david.abcc.ncifcrf.gov/). For annotation analysis, genes with FDR value less than 0.05 of array data were selected.

Statistical analyses

Statistical comparison of the viability and count of the cells cultured under different conditions was performed using Student's t-test in GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, USA). A p value of <0.05 was considered significant.

364 Çoban et al.
Simvastatin and RORα activity in the macrophages

Anatol J Cardiol 2017; 17: 362-6

Results

Simvastatin and RORα ligands had a tendency to decrease cell viability and cell count

We observed that simvastatin and both RORα ligands had a tendency to decrease THP-1 macrophage cell viability in culture (Fig. 1). Compared with non-treated controls, simvastatin significantly decreased cell viability (p=0.04) and cell count (p=0.03). However, this negative effect of simvastatin seemed to be partly prevented by RORα ligands. Furthermore, when simvastatin and SR1001 was used together, cell viability was increased by 5% and cell count increased by 18% (p=0.04 and p=0.05, respectively). Additionally, viability and count of simvastatin-treated cells were significantly lower than those of SR1001-treated cells (p=0.001 and p=0.015, respectively). Interestingly, however, the count of the cells treated with SR1001 and simvastatin together was lower (p=0.036) than that of the cells treated with SR1001 alone, while higher (p=0.059) than that of the cells treated with simvastatin alone (Fig. 1). These findings suggest that SR1001 might prevent the effect of simvastatin by inducing cellular proliferation or by alleviating the negative effect on normal cell division program via undetermined molecular mechanism.

Potential RORα target genes involved in apoptotic pathways

Because CPG 52608-treated and SR1001-treated macrophages showed similar results regarding cell viability and cell count in culture, we concluded that they possibly show their action through common cellular pathways. In order to offer a model to explain the effect of SR1001 on macrophage viability and cell count, we analyzed our preliminary RORα ChIP-array data (manuscript in preparation). Bioinformatics analysis (http://david.abcc.ncifcrf.gov/) of this data demonstrated that several genes that are involved in the apoptotic pathway were likely RORα target genes (Fig. 2). As shown in Figure 2, these genes were involved in the regulation of apoptosis through various pathways. However, cytochrome c, Bcl2, and caspase pathways seemed to be predominantly represented among the target genes. Furthermore, other genes were linked to the TP53-, hypoxia-, endoplasmic reticulum stress-, TGF-beta-, and DNA fragmentation-mediated apoptotic pathways. While some of these genes were known to prevent apoptosis, most of them were the activators of apoptosis. In addition to apoptosis-related genes, we have found several target genes, such as JMJD6 and RBP-1, involved in macrophage differentiation and polarization, respectively (Fig. 3).

Discussion

In our experiments, we found that SR1001 RORα ligand and simvastatin decreased macrophage cell viability. On the other hand, when SR1001 and simvastatin were used together, SR1001 seemed to prevent the negative effect of simvastatin on cell via-
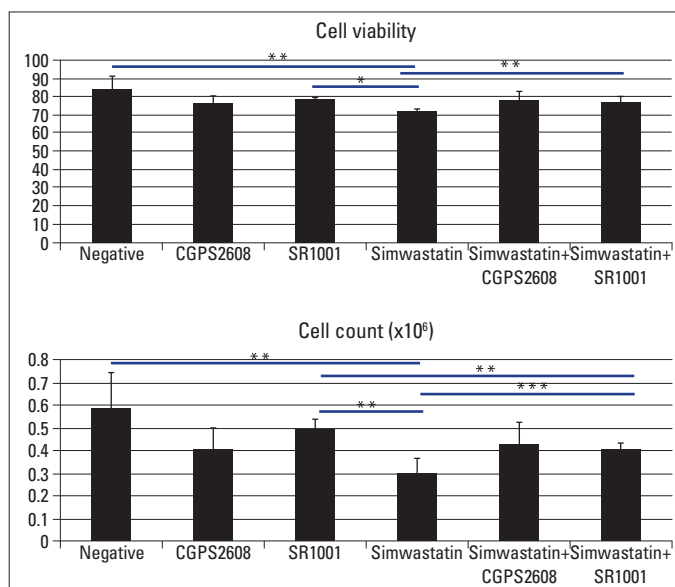

Figure 1. THP-1 macrophage cell viability and cell count values after treatment with RORα ligands (CGP52608 and SR1001) and cholesterol synthesis inhibitor simvastatin. *$P<0.005$, **$P<0.05$, ***$P=0.05$
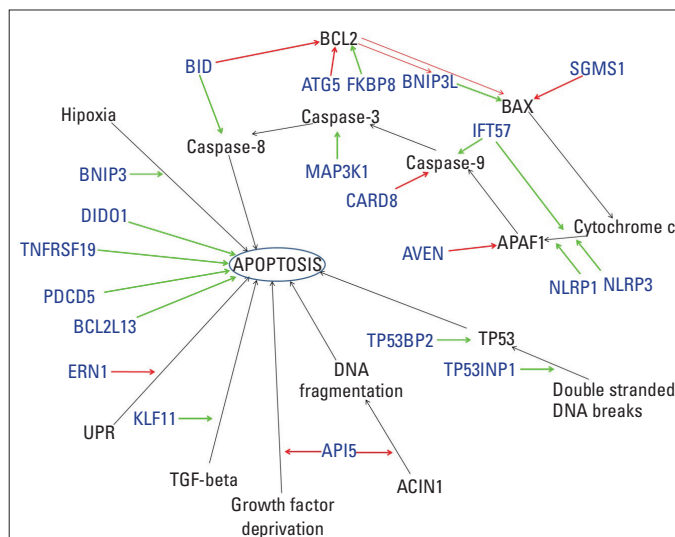

Figure 2. Potential RORα target genes (blue), which may have positive (green) or negative (red) effect on apoptotic pathway
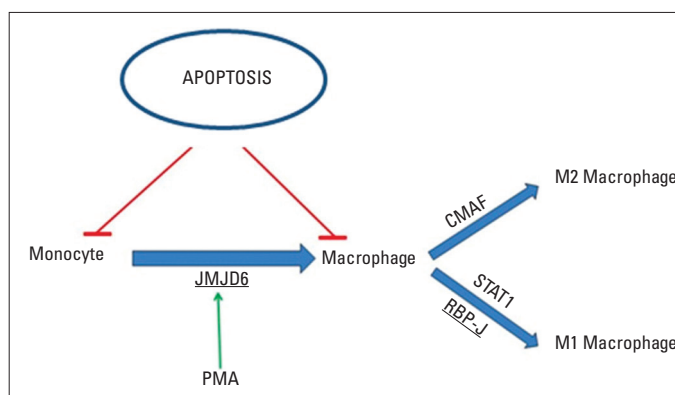

Figure 3. Apoptosis-related genes with respect to macrophage viability or differentiation. Underlined genes are potential RORα target genes

Anatol J Cardiol 2017; 17: 362-6

Çoban et al.
Simvastatin and RORα activity in the macrophages 365

bility and cell count in culture. We concluded that this protective effect might arise either by the induction of cellular proliferation or by alleviation of the negative effect on normal cellular division program. Furthermore, this protective action of SR1001 suggests that the genes involved in cell division and survival processes might be targeted by RORα in the macrophages.

Macrophages are one of the most crucial cell types that are involved in different steps of atherosclerosis, from lipid deposition to plaque rupture, depending on the phenotype of the cell. On the other hand, the plaque rupture has a unique importance because it is largely responsible for the clinical manifestation of the atherosclerosis (18). Although the metalloproteases secreted by macrophages are known to play important roles in atherosclerotic plaque stability and plaque rupture (19–21), apoptotic macrophage accumulation was also shown to be involved in this process (22, 23). Many studies demonstrated that macrophage apoptosis can also be modulated by statins both in vitro and in vivo. However, these studies suggest the involvement of different apoptotic pathways for different statins (24–26). While Liang et al. (25) showed the involvement of Rac1/Cdc42/JNK pathway in lovastatin-mediated apoptosis in macrophages, Qin et al. (26) showed that simvastatin suppressed apoptosis in vivo through the expression of p53, Bcl-2, and Bcl-xL in mice. In this study, we showed that simvastatin-mediated in vitro apoptosis of macrophages could be modulated by SR1001. because SR1001 is known to bind to RORα as a ligand, we attributed these results to the involvement of RORα. Furthermore, we believe that the presence of the apoptotic pathway genes in our preliminary ChIP-array data as potential RORα target genes supports this inference. In accordance with the findings of Qin et al. (26), our data also suggest the involvement of p53 and Bcl-2 pathways in macrophage apoptosis. On the contrary, our results suggested that simvastatin is a possible inducer of apoptosis in macrophages. This contradiction could be the result of differences between species-specific macrophages or between in vitro and in vivo phenotypic behaviors of the macrophages. However, involvement of RORα and its relationship with apoptotic pathways needs to be confirmed with further experiments.

Macrophage apoptosis is known to have dual role in atherogenesis (27). While playing protective role at the early stages of atherosclerosis, it promotes the plaque instability in advanced lesions. Hence, in vivo significance of our results is supposed to depend on the stage of the lesion where macrophages are involved. Discordance of our data may also be the result of the culture conditions applied in this study. Therefore, further in vitro studies, which may apply various conditions like the presence of oxLDL or hypoxia to the cultured macrophages, will more realistically represent inherent apoptotic behaviors of the macrophages during atherogenesis.

The results of our chip-array data analysis showed that several putative RORα target genes were involved in apoptosis-related pathways. In previous studies, it was shown that RORα could enhance DNA damage-induced apoptosis through p53 and also increase Dox-induced apoptosis in colon cancer cells (28). Additionally, the potential use of RORα ligand melatonin in the prevention of breast cancer is under investigation (29). However, to the best of our knowledge, relationship of RORα with apoptosis has not yet been reported in atherosclerotic lesions. Furthermore, some of the apoptotic pathway genes we detected among the potential RORα target genes were shown to be regulated by melatonin. While Bcl2 pathway-related gene Atg5 was shown to be responsible for neuroprotective role of melatonin (30), cytochrome c pathway-related gene Nlrp3 was found to be involved in anti-inflammatory role of melatonin (31). Though the latter study focused on inflammation and not apoptosis, it revealed the involvement of RORα and melatonin in Nlrp3-NFkappaB pathway. Because the melatonin is known as RORα ligand, its anti-apoptotic and anti-inflammatory actions might arise through RORα target genes. Therefore, identification of the genes and the pathways shared by melatonin and other RORα ligands seems to be helpful.

Study limitations

In this study, we used SR1001 and CPG 52608 as synthetic modulators. Among these, SR1001 has a potential therapeutic usage due to its anti-inflammatory effect. Though SR1001 is accepted to bind to RORα as a synthetic ligand, the possibility of other potential nuclear receptors binding SR1001 was not excluded in our study. In addition to this limitation, apoptotic pathway activity was not measured using specific markers. Further studies overcoming these limitations would be able to elucidate the role of SR1001 in macrophage apoptosis.

Conclusion

In conclusion, our study results suggest that simvastatin-mediated macrophage apoptosis might be modulated by SR1001 administration. However, involvement of RORα in this modulation through potential apoptotic target genes remains elusive. Further research is sorely warranted in this direction.

Authorship contributions: Concept – N.Ç.; Design – N.Ç., Ç.G.; Supervision – N.E.Ü.; Fundings – N.E.Ü., N.Ç., Ç.G., B.Ö.S.; Materials – N.E.Ü., N.Ç., Ç.G.; Data collection &/or processing – N.Ç., Ç.G., B.Ö.S.; Analysis &/or interpretation – N.Ç., Ç.G., B.Ö.S.; Literature search – N.Ç., Ç.G., B.Ö.S.; Writing – N.Ç.; Critical review – N.E.Ü., Ç.G., B.Ö.S.

366 Çoban et al.
Simvastatin and RORα activity in the macrophages

Anatol J Cardiol 2017; 17: 362-6

References

1. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008; 454: 436-44. [CrossRef]
2. Zhang K, Kaufman RJ. From endoplasmic-reticulum stress to the inflammatory response. Nature 2008; 454 :455-62. [CrossRef]
3. Santos-Gallego CG, Picatoste B, Badimón JJ. Pathophysiology of acute coronary syndrome. Curr Atheroscler Rep 2014; 16: 401.
4. Tabas I, Williams KJ, Boren J. Subendothelial lipoprotein retention as the initiating process in atherosclerosis: update and therapeutic implications. Circulation 2007; 116: 1832-44. [CrossRef]
5. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol 2010; 11: 889-96. [CrossRef]
6. Mantovani A, Sozzani S, Locati M, Allavena P, Sica A. Macrophage polarization: tumor associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 2002; 23: 549-55. [CrossRef]
7. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol 2008; 8: 958-69. [CrossRef]
8. Stout RD, Suttles J. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. J Leukoc Biol 2004; 76: 509-13. [CrossRef]
9. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity 2010; 32: 593-604. [CrossRef]
10. Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci 2008; 13: 453-61. [CrossRef]
11. Medbury HJ, Williams H, Fletcher JP. Clinical significance of macrophage phenotypes in cardiovascular disease. Clin Transl Med 2014; 21: 3-63. [CrossRef]
12. Tengattini S, Reiter RJ, Tan DX, Terron MP, Rodella LF, Rezzani R. Cardiovascular diseases: protective effects of melatonin. J Pineal Res 2008; 44: 16-25.
13. Boukhtouche F, Mariani J, Tedgui A. The "CholesteROR" Protective Pathway in the Vascular System. Arterioscler Thromb Vasc Biol 2004; 24: 637-43. [CrossRef]
14. Besnard S, Heymes C, Merval R, Rodriguez M, Galizzi JP, Boutin JA, et al. Expression and regulation of the nuclear receptor RORalpha in human vascular cells. FEBS Lett 2002; 511: 36-40. [CrossRef]
15. Delerive P, Monte D, Dubois G, Trottein F, Fruchart-Najib J, Mariani J, et al. The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. EMBO Rep 2001; 2: 42-8. [CrossRef]
16. Missbach M, Jagher B, Sigg I, Nayeri S, Carlberg C, Wiesenberg I. Thiazolidine diones, specific ligands of the nuclear receptor retinoid Z receptor/retinoid acid receptor-related orphan receptor alpha with potent antiarthritic activity. J Biol Chem 1996; 271: 13515-22.
17. Solt LA, Kumar N, Nuhant P, Wang Y, Lauer JL, Liu J, et al. Suppression of TH17 differentiation and autoimmunity by a synthetic ROR ligand. Nature 2011; 472: 491-4. [CrossRef]
18. Virmani R, Burke AP, Farb A, Kolodgie FD. Pathology of the vulnerable plaque. J Am Coll Cardiol 2006; 47: 13-8. [CrossRef]
19. Newby AC. Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. Physiol Rev 2005; 85: 1-31. [CrossRef]
20. Dollery CM, Libby P. Atherosclerosis and proteinase activation. Cardiovasc Res 2006; 69: 625-35. [CrossRef]
21. Libby P. Collagenases and cracks in the plaque. J Clin Invest 2013; 123: 3201-3. [CrossRef]
22. Seimon T, Tabas I. Mechanisms and consequences of macrophage apoptosis in atherosclerosis. J Lipid Res 2009; 50: 382-7. [CrossRef]
23. Hutter R, Valdiviezo C, Sauter BV, Savontaus M, Chereshnev I, Carrick FE, et al. Caspase-3 and tissue factor expression in lipid-rich plaque macrophages: evidence for apoptosis as link between inflammation and atherothrombosis. Circulation 2004 109: 2001-8. [CrossRef]
24. Wang ZH, Liu XL, Zhong M, Zhang LP, Shang YY, Hu XY, et al. Pleiotropic effects of atorvastatin on monocytes in atherosclerotic patients. J Clin Pharmacol 2010; 50: 311-9. [CrossRef]
25. Liang SL, Liu H, Zhou A. Lovastatin-induced apoptosis in macrophages through the Rac1/Cdc42/JNK pathway. J Immunol 2006; 177: 651-6. [CrossRef]
26. Qin W, Lu Y, Zhan C, Shen T, Dou L, Man Y, et al. Simvastatin suppresses apoptosis in vulnerable atherosclerotic plaques through regulating the expression of p(53), Bcl-2 and Bcl-xL. Cardiovasc Drugs Ther 2012; 26: 23-30. [CrossRef]
27. Santos-Gallego CG. MafB and the role of macrophage apoptosis in atherosclerosis: A time to kill, a time to heal. Atherosclerosis 2016; 252: 194-6. [CrossRef]
28. Kim H, Lee JM, Lee G, Bhin J, Oh SK, Kim K, et al. DNA damage-induced RORalpha is crucial for p53 stabilization and increased apoptosis. Mol Cell 2011; 44: 797-810. [CrossRef]
29. Du J, Xu R. RORa a potential tumor suppressor and therapeutic target of breast cancer. Int J Mol Sci 2012; 13: 15755-66. [CrossRef]
30. Jeong JK, Moon MH, Lee YJ, Seol JW, Park SY. Melatonin-induced autophagy protects against human prion protein-mediated neurotoxicity. J Pineal Res 2012; 53: 138-46. [CrossRef]
31. García JA, Volt H, Venegas C, Doerrier C, Escames G, López LC, et al. Disruption of the NF-κB/NLRP3 connection by melatonin requires retinoid-related orphan receptor-α and blocks the septic response in mice. FASEB J 2015; 29: 3863-75. [CrossRef]