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Elevated COX2 expression and PGE2 production by downregulation of RXRα in senescent macrophages

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ABSTRACT

Increased systemic level of inflammatory cytokines leads to numerous age-related diseases. In senescent macrophages, elevated prostaglandin E2 (PGE2) production contributes to the suppression of T cell function with aging, which increases the susceptibility to infections. However, the regulation of these inflammatory cytokines and PGE2 with aging still remains unclear. We have verified that cyclooxygenase (COX)-2 expression and PGE2 production are higher in LPS-stimulated macrophages from old mice than that from young mice. Downregulation of RXRα, a nuclear receptor that can suppress NF-κB activity, mediates the elevation of COX2 expression and PGE2 production in senescent macrophages. We also have found less induction of ABCA1 and ABCG1 by RXRα agonist in senescent macrophages, which partially accounts for high risk of atherosclerosis in aged population. Systemic treatment with RXRα antagonist HX531 in young mice increases COX2, TNF-α, and IL-6 expression in splenocytes. Our study not only has outlined a mechanism of elevated NF-κB activity and PGE2 production in senescent macrophages, but also provides RXRα as a potential therapeutic target for treating the age-related diseases.

1. Introduction

It is well known that many physiological functions of the body are changed during the aging processes. Dysregulation of immune and inflammatory responses with aging has been well documented in both human and animals. Aging is related to a proinflammatory environment characterized by constitutively elevated levels of NF-κB-driven proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in the absence of overt disease conditions, the called inflamm-aging [1]. Emerging pathological evidences indicate that major chronic age-related diseases such as atherosclerosis, arthritis, dementia, osteoporosis, and cardiovascular diseases, are inflammation-related [2,3]. However, it remains controversial whether inflammatory mediators have a primary causal or consequential relationship with the pathologies of age-related diseases or simply aggravate them. Thus, understanding the molecular mechanisms that regulate the expression of NF-κB-driven proinflammatory cytokines associated with aging is important for understanding the development of age-related diseases. Elevated expression of cyclooxygenase (COX)-2 and its product, particularly prostaglandin E2 (PGE2), plays an important role in the age-associated dysregulation of the immune and inflammatory responses [4]. For example, increased PGE2 production in senescent macrophages contributes to the suppression of T cell immunity with aging, which explains why the elderly are more susceptible to peripheral infections and with increased incidence of disability and mortality rates for individuals 65 years of age and older [5–7]. Several studies have indicated that elevated NF-κB activity leads to COX2 transcription and thus increases PGE2 production with aging [4,8–10]. However, the mechanism for regulation of NF-κB activity and PGE2 production with aging remains unclear.

Retinoid X receptors (RXRs) occupy a central position in the nuclear receptor superfamily because they can form heterodimers with many other nuclear receptor family members, hence are involved in the control of a variety of physiological processes including cell differentiation, immune response, and cellular metabolism [11,12]. For example, activation of LXR/RXR induces expression of

Abbreviations: RXRα, retinoid X receptor alpha; 9cRA, 9-cis-retinoid acid; TLR, toll-like receptor; COX2, cyclooxygenase 2; PGE2, prostaglandin E2; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1.

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genes such as ABCA1, ABCG1, and apoE, which involve in cholesterol efflux. Therefore, LXR/RXR heterodimer are critical regulators of cholesterol metabolism and determinants of atherosclerosis susceptibility [13,14]. There are three RXR isotypes, RXRα (NR2B1), RXRβ (NR2B2), RXRγ (NR2B3). The most abundant RXR in myeloid cells, or at least the most functionally important, is RXRα [15]. Vitamin A derivative retionid 9-cis retinoic acid (9cRA) is the first identified natural ligand of RXR [16]. Endogenous fatty acids such as docosahexaenoic acid, oleic acid, and phytanic acid, are also ligands for RXRs [17]. Several RXR-specific synthetic ligands such as LG100268, AGN194204, and bexarotene, known as rexinods, have also been used in research or clinic [16]. Peroxisome proliferater-activated receptor α (PPARα), a partner of RXRα, has been reported to repress NF-κB signaling and reduce proinflammatory cytokines production in aged mice [18]. In our current study, we have found that RXRα is also downregulated in senescent macrophages, and thus leads to upregulation of COX2 expression and PGE2 production. We also demonstrate that systemic blocking RXRα signaling by synthetic antagonist HX531 increases inflammatory genes expression in splenocytes from young mice and displays similar expression levels of these genes in aged splenocytes.

2. Materials and methods

2.1. Mice and reagents

C57BL/6 mice (2- and 24-month old) were purchased from Laboratory Animal Center (China Medical University, Shenyang, China). All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of China Medical University (Shenyang, China). Antibodies against COX2, RXRα and GAPDH were purchased from Cell Signaling Technology (Boston, MA). Anti-α-tubulin antibody, TLR4 ligand LPS, RXR agonist 9cRA, and RXR antagonist HX531 were ordered from Sigma–Aldrich (St. Louis, MO). RXRα specific siRNA and its control siRNA were purchased from Thermo Scientific (Pittsburgh, PA).

2.2. Cell culture and transfection

For elicitation of peritoneal macrophages, 2- or 24-month old mice were injected intraperitoneally (*i.p.*) with 2 ml of 3% thioglycollate (Bacterius LTD, Houston, Texas). 72 h later, peritoneal cells were harvested by lavage and washed twice with completed medium. Approximately 1×10^6 cells per well were cultured in 6-well plates. The non-adherent cells were removed by changing the medium after 1 h culturing in RPMI1640 medium with 10% FBS (37 °C, 5% CO_2, 95% air). The adherent cells are peritoneal macrophages (more than 95% cells are $CD11b^+$ cells). RAW264.7 cells were obtained from the ATCC (Manassas, VA) and maintained at 37 °C (5% CO_2, 95% air) in culture medium consisting of DMEM supplemented with 10% FBS. For p65 and RXR transfection in RAW264.7 cells, 10^4 cells per well were seeded into 96-well plates and incubated overnight. Lipofectamine 2000 (Life Technologies, Grand Island, NY) was used to transfect the plasmids according to the manufacturer's instructions. INTERFERin (PolyPlus transfection) was used to transfect siRNA into RAW264.7 cells according to the manufacturer's standard protocol.

2.3. ELISA

Supernatant PGE2 were measured by using Prostaglandin E2 EIA Kit (Cayman Chemical, MI) according to the manufacturer's instructions.

2.4. RNA quantification

Total RNA was extracted with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Real-time quantitative RT-PCR analysis was performed using the LightCycler (Roche) and SYBR RT-PCR kits (Takara, Beijing, China). The relative expression level of COX2, RXRα, TNF-α, and IL-6 mRNAs was normalized to the internal control β-actin by using $2^{-\Delta\Delta Ct}$ cycle threshold method [19]. The primers used in quantitative PCR are designed by PrimerBank [20], and the primer sequences are available upon request.

2.5. Western blot

Cells were lysed using M-PER protein extraction reagent (Thermo Scientific) supplemented with protease inhibitor mixture (Merck Millipore, Billerica, MA). Protein concentrations of the extracts were measured with a BCA assay (Thermo Scientific) and equalized with the extraction reagent. Equal amounts of the extracts were loaded and subjected to SDS–PAGE, transferred onto nitrocellulose membranes, and then blotted.

2.6. Dual luciferase reporter assay

RAW264.7 cells were cotransfected with IFNb *firefly* luciferase reporter, pRL-TK-*Renilla* luciferase reporter, p65, and/or hRXRα by Lipofectamine2000 according to the manufacturer's instructions. 24 h later, luciferase activities were measured by using the Dual-Luciferase Reporter Assay System (Promega). Data was normalized for transfection efficiency by dividing *firefly* luciferase activity with that of *Renilla* luciferase.

2.7. HX531 treatment in vivo

2-month old mice (Young) or 24-month old mice (Old) were injected *i.p.* with DMSO or HX531 (10 mg/kg) solubilized in corn oil every 24 h for 7 days, and then the spleens from these mice were harvested, subsequently grinded in Trizol, RNA was extracted by standard procedure of the Trizol Reagent (Life Technology) .

3. Results

3.1. Elevated COX2 expression and PGE2 production in macrophages from aged mice

Emerging evidences have demonstrated that upregulation of COX2 and its product PGE2 plays a critical role in the age-associated dysregulation of immune and inflammatory responses [4]. To verify if PGE2 production and COX2 expression are increased in senescent macrophages, we checked the PGE2 production and COX2 expression in resting and LPS-stimulated peritoneal macrophages from young and old mice. Macrophages from old mice produced more PGE2 than that from young mice in both resting and LPS-stimulated conditions (Fig. 1A). Similarly, COX2 mRNA and protein were significantly induced in LPS-stimulated macrophages from old mice than that from young mice (Fig. 1B and C), which is consistent with that COX2 is the key enzyme in catalyzing arachidonic acid into PGE2 in macrophages.

3.2. Downregulation of RXRα and its downstream genes in macrophages from aged mice

PPARα expression level is reduced in aged mice, which contributes to dysregulation of proinflammatory cytokines production with aging [18]. To determine if other nuclear receptors have sim-

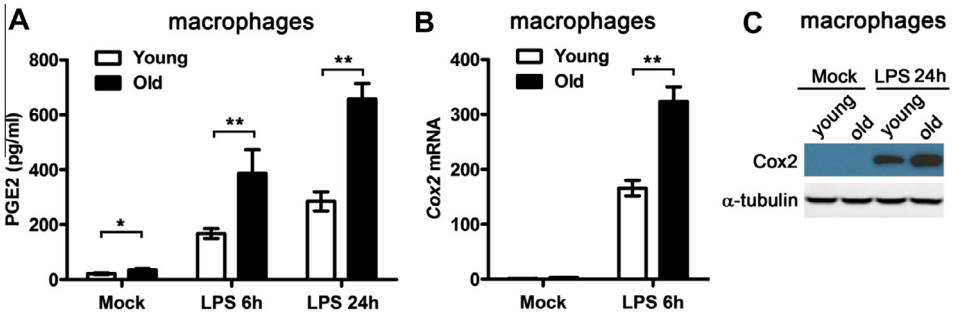

Fig. 1. Elevated COX2 expression and PGE2 production in macrophages from aged mice. (A) Peritoneal macrophages from 2-month old mice (Young) and 24-month old mice (Old) were cultured for 24 h (Mock) or stimulated with 100 ng/ml LPS for indicated time (LPS 6 h or LPS 24 h), PGE2 in the supernatant of these macrophages were detected by ELISA. (B) RNA from Mock-treated cells and LPS 6 h-stimulated macrophages were extracted, and the COX2 mRNA expression was quantified by quantitative PCR (qPCR). (C) Protein from Mock-treated cells and LPS 24 h-stimulated macrophages were extracted, and the COX2 protein expression was determined by Western blot (WB). Data of (A, B) are from three independent experiments (mean ± s.e.m), $^{*}p < 0.05$, $^{**}p < 0.01$ (Student's *t*-test). Data of (C) are from one experiment representative of three independent experiments with similar results.

ilar expression patterns and functions with aging, we checked the expression level of numerous nuclear receptors. Surprisingly, the expression of RXRα, a partner of PPARα, was significantly downregulated in old macrophages, both in mRNA level and protein level (Fig. 2A and B). RXRα can form heterodimer with LXR to regulate cholesterol efflux by inducing its downstream genes including ABCA1 and ABCG1 [13,14]. 9cRA treatment increased ABCA1 and ABCG1 expression in macrophages from both young and old mice (Fig. 2C and D). However, ABCA1 and ABCG1 mRNA were significantly downregulated in both DMSO-treated and 9cRA-treated cells from old mice than that from young mice (Fig. 2C and D). No difference of LXRα and LXRβ expression was found between young and senescent macrophages (Fig. 2E), which excluded that the downregulation of ABCA1 and ABCG1 in senescent macrophages is due to the decreased LXR expression. These results suggest that the downregulation of RXRα in senescent macrophages and partially accounts for higher risk of atherosclerosis in aged population.

3.3. RXRα suppresses COX2 expression and PGE2 production by inhibiting NF-κB activity

To verify if downregulation of RXRα leads to elevated PGE2 production and COX2 expression in aged macrophages, we first tested

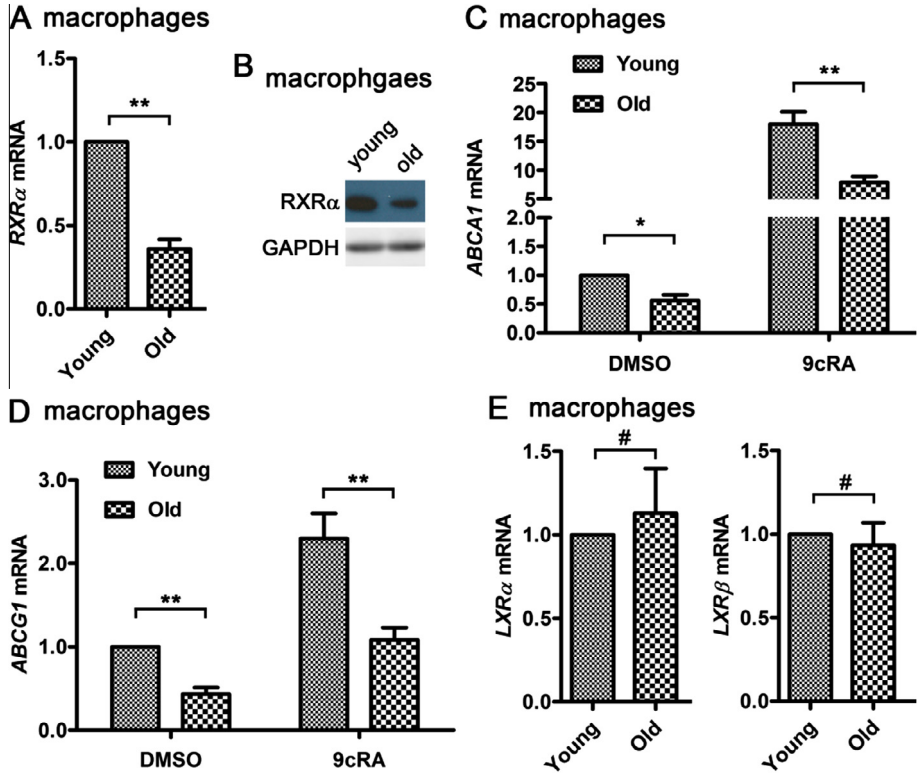

Fig. 2. Downregulation of RXRα and its downstream genes in macrophages from aged mice. (A and B) RNA and protein were extracted from peritoneal macrophages which were from 2-month old mice (Young) or 24-month old mice (Old), RXRα mRNA level was detected by qPCR (A) and RXRα protein level was detected by WB, GAPDH was taken as a loading control (B). (C and D) Peritoneal macrophages from 2-month old mice (Young) or 24-month old mice (Old) were treated with DMSO or 9cRA (100 nM) for 16 h, ABCA1 mRNA (C) and ABCG1 mRNA (D) were detected by qPCR. (E) RNA was extracted from peritoneal macrophages as described in (A), LXRαα and LXRβ mRNA level were detected by qPCR. Data of (A, C, D, and E) are from three independent experiments (mean ± s.e.m), $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{\#}p > 0.1$, no significance (Student's *t*-test). Data of (B) are from one experiment representative of three independent experiments with similar results.

the regulation effect of RXRα on NF-κB activity in macrophages. Ligand activation of RXRα by 9cRA, a natural ligand of RXRα, significantly inhibited the NF-κB reporter activity in RAW264.7 cells (Fig. 3A). Transfection of p65 increased NF-κB activity dramatically (Fig. 3A). However, 9cRA treatment attenuated the induction of p65-drived NF-κB activity, both in wild type or RXRα-overexpressing RAW264.7 cells (Fig. 3A). In addition, overexpression of RXRα alone suppressed the induction of NF-κB activity (Fig. 3A). Subsequently, we found that LPS stimulation induced PGE2 production significantly in RAW264.7 cells, and 9cRA pretreatment inhibited the induction of PGE2 in LPS-stimulated cells (Fig. 3B). Consistently, 9cRA pretreatment suppressed the induction of COX2 significantly in LPS-stimulated RAW264.7 cells (Fig. 3C). Further, RXRα knockdown by specific siRNA significantly facilitated PGE2 production in both unstimulated and LPS-stimulated RAW264.7 cells (Fig. 3D). Similarly, RXRα knockdown increased Cox2 expression in LPS-stimulated RAW264.7 cells (Fig. 3E). Although more PGE2 production was found in macrophages from old mice, however, there was no significant difference of PGE2 production in 9cRA-pretreated young macrophages and old macrophages (Fig. 3F). Meanwhile, we found that less PGE2 production in 9cRA-pretreated macrophages from young mice or old mice comparing to DMSO-pretreated cells (Fig. 3F). To further determine the mechanism of RXRα inhibiting PGE2 production, we compared the expression of calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$), COX1, and microsomal PGE2 synthase (mPGES), three enzymes which are critical for PGE2 production, in young and senescent macrophages [21–23]. $cPLA_2$ expression level is similar

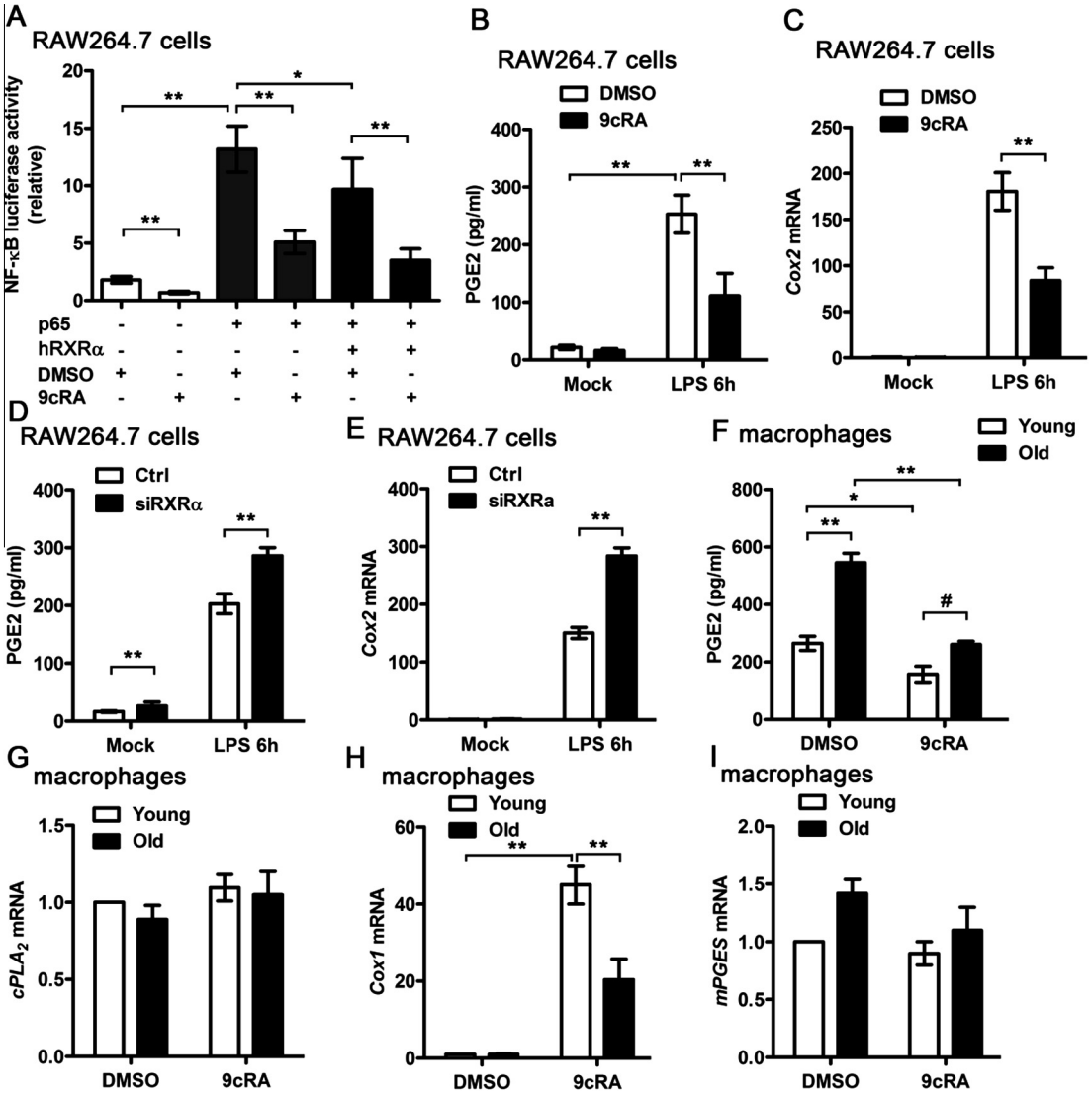

Fig. 3. Overexpression or ligand activation of RXR suppresses COX2 expression and PGE2 production by inhibiting NF-κB activity. (A) NF-κB-*firefly* luciferase reporter vector, pTK-*Renilla* luciferase reporter vector and indicated vectors (p65 and/or RXR) were cotransfected into RAW264.7 cells for 12 h, and then the cells were treated with DMSO or 9cRA (100 nM) for another 12 h. NF-κB *firefly* luciferase activity of these cell lysate were detected and normalized by *Renilla* luciferase activity. Data are shown as mean mean ± s.d ($n = 6$) from one experiment representative of three independent experiments with similar results. (B and C) RAW264.7 cells were treated with DMSO or 9cRA (100 nM) for 6 h, and then the cells were stimulated by LPS (100 ng/ml) for another 6 h, PGE2 in the supernatant was detected by ELISA (B) and COX2 mRNA expression in these cells was quantified by qPCR (C). (D and E) RAW264.7 cells were transfected with 5 nM control siRNA (Ctrl) or RXRα specific siRNA (siRXRα) for 24 h, and then the cells were stimulated by LPS (100 ng/ml) for another 6 h, PGE2 in the supernatant was detected by ELISA (D) and COX2 mRNA expression in these cells was quantified by qPCR (E). (F) Peritoneal macrophages from 2-month old mice (Young) or 24-month old mice (Old) were treated with DMSO or 9cRA (100 nM) for 6 h and stimulated by LPS (100 ng/ml) for another 24 h, PGE2 in the supernatant was detected by ELISA. (G–I) Peritoneal macrophages from 2-month old mice (Young) or 24-month old mice (Old) were treated with DMSO or 9cRA (100 nM) for 6 h, the $cPLA_2$ mRNA (G), Cox1 mRNA (H), and mPGES mRNA (I) were detected by qPCR. Data of (B–I) are from three independent experiments (mean ± s.e.m), $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{\#}p > 0.1$, no significance (Student's *t*-test).

between young and senescent macrophages, no matter the cells were treated with DMSO or 9cRA (Fig. 3G). Interestingly, COX1 expression was dramatically induced by 9cRA treatment in young and senescent macrophages. However, the induction of COX1 was attenuated in senescent macrophages (Fig. 3H). There was no significant difference of mPGES expression between young and senescent macrophages (Fig. 3I). Taken together, these data suggested that RXRα suppresses PGE2 production through inhibiting NF-κB activity and COX2 expression, not via regulating $cPLA_2$, COX1, and mPGES.

3.4. Treatment with RXR antagonist HX531 upregulates NF-κB-dependent proinflammatory genes expression

Upregulation of NF-κB-dependent proinflammatory cytokines was found in both serum and splenocytes from aged mice [18,20]. Our data have indicated the downregulation of RXRα, which leads to elevated PGE2 production and COX2 expression in senescent macrophages. To determine whether RXRα modulates proinflammatory cytokines *in vivo*, mice were injected with HX531, a synthetic RXRα antagonist, to mimic the defect of RXRα signaling in aged mice. After injection with HX531 for one week, COX2 mRNA expression was significantly upregualted (Fig. 4A). Despite significantly induction of COX2 expression in splenocytes from old mice than young mice in DMSO-treated group, however, there was no difference in COX2 expression between splenocytes from young and old mice in 9cRA-treated group (Fig. 4A). Similarly, TNFα and IL-6 were upregulated after HX531 treatment and no difference of their expression between splenocytes from young and old mice in 9cRA-treated group (Fig. 4B and C). Together, these data indicates that treatment with RXR antagonist HX531 upregulates NF-κB-dependent proinflammatory genes expression, which is consistent with that downregulation of RXRα contributes to elevation of COX2 and PGE2 in senescent macrophages.

4. Discussion

Age-related upregulation of TNF-α, IL-6, IL-1β and PGE2 contributes to major chronic aging-related diseases [3]. Abnormal activation of NF-κB in aged cells leads to the dysregulated expression of these pleiotropic cytokines [24]. It has been reported that activation of PPARα can restore the cellular redox balance, eliminate the constitutive activation of NF-κB, and inhibit the spontaneous inflammatory cytokine production [18]. Also, PPARγ activation by its agonists may interrupt the NF-κB and AP-1 pathways, downregulate NF-κB- and AP-1-dependent genes, including COX2, inducible NO synthase (iNOS) and inflammatory cytokines [25]. The expression level and function of both PPARα and PPARγ are suppressed with age [18,26]. Our current study has demonstrated that another nuclear receptor, RXRα, has the similar expression pattern and function with PPARs during the aging processes. We have found that RXRα transcript and protein were downregulated in senescent macrophages, and ligand activation of RXRα decrease NF-κB activation, COX2 expression, and PGE2 production. In addition, systemic treatment with RXRα antagonist HX531 increases COX2, IL-6, and IL-1β expression in splenocytes from young mice and displays similar expression levels of these inflammatory genes in old cells. Thus, our study has discovered a novel regulator modulates age-related immune responses.

Understanding the underling mechanisms of the age-associated upregulation of inflammatory genes would help in determining potential targets for treating the age-related diseases. Besides the roles of PPARs in regulation of NF-κB with aging, several other studies have described their own mechanisms. One hypothesis has been raised: despite a poor inflammatory response via toll-like

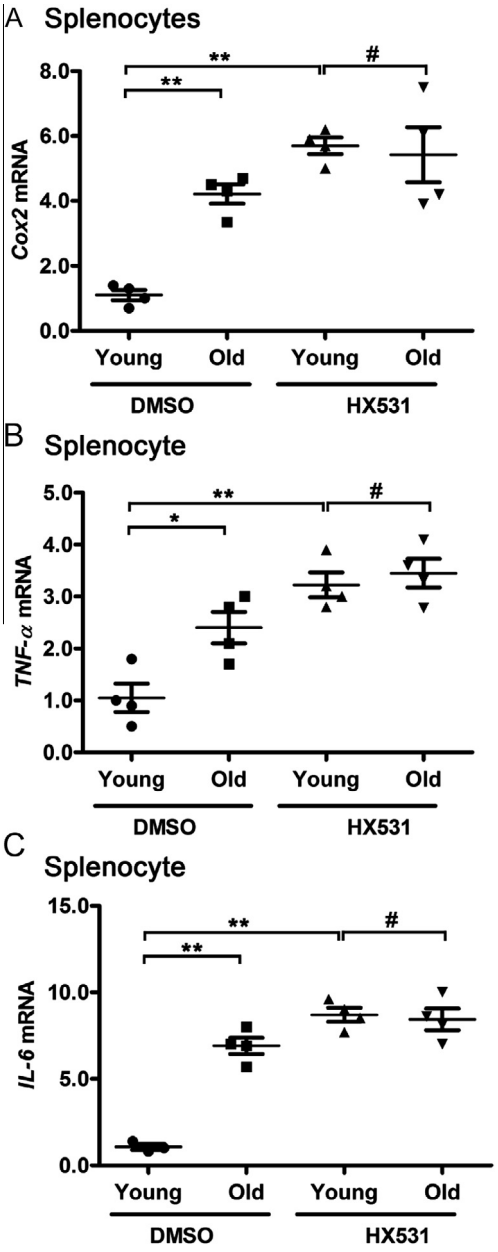

Fig. 4. Treatment with RXR antagonist upregulates NF-κB-dependent proinflammatory genes expression. 2-month old mice (Young) or 24-month old mice (Old) were injected (*i.p*) with DMSO or 10 mg/kg HX531 ($n = 4$) solubilized in corn oil every 24 h for 7 days, subsequently the spleens from these mice were harvested, and then the RNA from these spleens was extracted. COX2 mRNA (A), TNF-α mRNA (B), and IL-6 mRNA (C) in these splenocytes were detected by qPCR. Data are shown as mean mean ± s.d ($n = 4$) from one experiment representative of three independent experiments with similar results, $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{\#}p > 0.1$, no significance (Student's *t*-test).

receptor (TLR) activation, the ineffective clearance of pathogens by macrophages increases the duration of their activation and contributes perpetuation of inflammatory response with aging [27]. In addition, miRNAs have also been implicated in the control of aged and cellular senescence [28–30]. miR-146a and miR-146b increased in senescent human fibroblasts and inhibited excessive secretion of the inflammatory cytokines IL-6 and IL-8, thereby limited age-related inflammation [29]. Dysregulated expression of miR-146a contributes to age-related dysfunction of macrophages [31]. Nuclear receptors (NR) represent a large family of transcriptional factors involved in broad spectrum of biological events such as immune regulation and metabolism [32]. It is not surprising that

dysregulation of NRs will lead to age-related diseases. However, the roles of other NRs members besides PPARs and RXRα with aging still need to be further investigated.

Increased PGE2 production has been implicated in the pathogenesis of several age-related diseases such as arthritis, atherosclerosis, and cancer [4]. It is well known that elevated production of PGE2 results in increased replication of the majority of virus, such as CMV, VSV, BLV, HTLV-I, HTLV-III, HSV-1, and HHV-6 [33]. Considering that ligand activation of RXRα could suppress PGE2 production, RXRα agonist such as bexarotene, known as rexinoids, has the potential to treat the above aged-related diseases and infections. Given that basal expression or induction of RXRα-dependent genes such as ABCA1 and ABCG1 are downregulated in senescent macrophages, rexinoids treatment will induce ABCA1 and ABCG1 and thus may have extra effect on treating atherosclerosis. Recently, it has been reported that abnormal polarization in older macrophages is caused by programmatic changes that lead to reduced expression of ABCA1, which demonstrates that impaired cholesterol efflux in senescent macrophage promotes age-related diseases such as macular degeneration [34]. Given that ABCA1 is an LXR/RXR heterodimer-dependent gene, downregulation of RXRα not only alleviates the suppression on NF-κB activity and subsequent PGE2 production, but also causes atherosclerosis and macular degeneration by reducing ABCA1 expression. In summary, our study not only has outlined a mechanism of elevated NF-κB activity and inflammatory gene expression in senescent macrophages, but also has provided RXRα as a potential therapeutic target for treating the age-related diseases.

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