

7-Ketocholesterol Induces Cell Apoptosis by Activation of Nuclear Factor kappa B in Mouse Macrophages

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We investigated the molecular mechanisms responsible for the induction of apoptosis in mouse monocytic macrophage cell line J774A.1 stimulated by 7-ketocholesterol (7-KC). Cell apoptosis was detected by Annexin V-propidium iodide (PI) staining. The DNA-binding activity of nuclear factor kappa B (NF- κ B) was assessed by electrophoretic mobility shift assay (EMSA). Results showed that 7-KC-stimulation in J774A.1 cells activated NF- κ B, which is involved in cell apoptosis, in a time- and dose-dependent manners. 7-KC was also found to increase the binding activity of NF- κ B to specific DNA binding sites, a possible mechanism for the induction of the cell apoptosis. Moreover, these effects were partially inhibited by pyrrolidine dithiocarbamate (PDTC), an NF- κ B inhibitor. Taken together, 7-KC may be an important factor in atherosclerosis due to the ability of 7-KC to induce cell apoptosis, which is at least partially mediated through the activation of NF- κ B.

Key words: 7-KC, NF- κ B, apoptosis, atherosclerosis

Atherosclerosis (AS) is a primary cause of coronary artery and cerebrovascular disease, 2 of the most common causes of illness and death worldwide [1]. AS is also a chronic inflammatory disease related to oxidative modification stress [2, 3]. The oxidative modification hypothesis of AS predicts that low-density lipoprotein (LDL) oxidation is an early event in AS and that oxidized LDL (oxLDL) contributes to AS [4]. The oxLDL, along with several lipid-derived bioactive molecules generated in LDL,

including oxysterols, oxidized fatty acids, lysophospholipids and aldehydes, induce the expression of several inflammation genes that involved in the pathogenesis of AS [5].

Among the oxysterols, 7-ketocholesterol (7-KC) is the most abundant in human atherosclerotic plaques; it has strong preoxidation properties. It is involved in cell apoptosis and lipid peroxidation [6]. Some diseases like AS and diabetes elevate 7-KC levels [7]. Pervious studies have indicated that 7-KC could stimulate adhesive molecular expression, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and induce endothelial cell apoptosis [8, 9]. These behaviors of

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7-KC can promote AS formation and development.

The role of the macrophage is of fundamental interest in understanding atherosclerotic lesion development and thrombogenicity. Macrophages are mainly differentiated from blood peripheral monocytes; after that, they are incorporated with modified lipoproteins through the scavenger receptor pathway [10], which transforms the macrophage into lipid-rich foam cells, a hallmark feature of AS. Further, the accumulation of free cholesterol or oxidatively modified cholesterol loading of macrophages, including 7-KC or other oxysterols, induces macrophage apoptosis [11] and also can induce NF- κ B activation [12]. Apoptosis is a well-characterized feature of AS; it increased the risk of lesion rupture caused by the release of matrix metalloproteinases from the macrophages [13]. This finally drives the progression of AS.

As a major transcription factor in inflammatory responses, nuclear factor kappa B (NF- κ B) is involved in the regulation of inflammatory and immune genes, apoptosis, and cell proliferation. Under normal conditions, NF- κ B combines with inhibitor kappa B (I κ B) and is stored in the cytoplasm. Among the members of the I κ B family, I κ B α plays an important function [14]. Upon activation, I κ B undergoes phosphorylation and ubiquitination-dependent degradation by the 26S proteasome, thus leading to nuclear translocation of NF- κ B, the p50-p65 heterodimer [15]. It is well-known that NF- κ B can affect different steps in the atherosclerotic process, such as initiation of AS, foam cell formation, proliferation of smooth muscle cells and fibrous cap formation [16]. NF- κ B inducers and genes regulated by NF- κ B, such as tumor necrosis factor α (TNF- α), ICAM-1, VCAM-1 *etc.*, have been implicated directly and indirectly in AS [16].

It has been reported that oxLDL modulation induces AS by NF- κ B activation. For example, some oxidative stresses enhance NF- κ B activation, and then up-regulate several genes involved in AS [17]. Indeed, oxLDL is involved in NF- κ B activation in the pathogenesis of AS. Numerous studies have reported that 7-KC can inhibit cell proliferation, accelerate formation of reactive oxygen species, induce apoptosis and decrease cell viability [9–12]. However, no research has been reported on the activation of NF- κ B after treatment with 7-KC during cell apoptosis.

In the present study, we aimed to explore the relationship between 7-KC and NF- κ B activation dur-

ing cell apoptosis. Our findings showed that 7-KC induces NF- κ B activation and enhances cell apoptosis in the mouse monocytic macrophage cell line J774A.1. We believe that this is the first evidence that 7-KC induces apoptosis in J774A.1 cells at least partially through NF- κ B activation.

Materials and Methods

Reagents and Abs. The mouse monocytic macrophage cell line J774A.1 was purchased from the American Tissue and Culture Collection (Manassas, VA, USA). The oxysterol 7-KC (5-cholesten-3 β -ol-7-one) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) The prestained molecular weight marker was from Ferment Corporation. Pyroxylin membrane was purchased from Glean Corporation. Anti-NF- κ B/p65 polyclonal antibody (sc-109), anti-I κ B α polyclonal antibody (sc-371), Lamin A (H-102; sc-20680) and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (sc-25778) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture. J774A.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen Corporation, CA, USA) containing 1.5g/L sodium bicarbonate, and supplemented with 10% heat-inactivated fetal calf serum (Gibco), penicillin 100U/ml and streptomycin 100 μ g/ml (Gibco). The cells were seeded at 2×10^5 cells/ml in culture medium and passaged twice a week. They were incubated at 37°C under 5% CO₂/95% air atmosphere.

In all experiments, initial solutions of 7-KC were prepared at 5mg/ml in ethanol and stored at 4°C prior to use. Ethanol concentrations were <0.5% in culture. At this dose, ethanol did not affect any parameter of proliferation that was examined in the study.

Western blot analysis. J774A.1 cells were challenged for different periods at different concentrations of 7-KC as indicated in the figure. Cells lysates were prepared using nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA). The protein concentration of each sample was determined by BCA protein assay kit (Pierce, Rockford, IL, USA) before storage at -80°C.

Protein samples (20 μ g/lane) were electrophoresed on 12% acrylamide gradient gels using 80 V. After electrophoresis, proteins were transferred to a nitro-

cellulose membrane (Gelman, Biosharp) by standard electroblotting procedures at 100 V for 1h. After being transferred, blots were blocked with 5% w/v bovine serum albumin (BSA) in TBS-T (10mM Tris-HCl, pH7.5, 150mM NaCl, and 0.05% Tween 20) for 1h at room temperature, then washed 3 times for 5min each with 15ml of TBS-T. They were further probed with an antibody to I κ B α (1:1000 dilution) or GAPDH (1:1000 dilution) with gentle agitation overnight at 4°C, then developed with anti-rabbit secondary antibody conjugated to HRP (1:5000 dilution) and subjected to an enhanced chemiluminescence assay. We analyzed the results with Labworks 4.5 software (UVP Company).

Electrophoretic mobility shift assay (EMSA).

To determine NF- κ B activation, we performed EMSA. The specificity of binding was examined by competition with an unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from 7-KC treated cells were analyzed by EMSA. The NF- κ B activation was assayed using an EMSA kit (Viagene Biotech, Ningbo, China) according to the manufacturer's protocol. The consensus NF- κ B oligonucleotides included in the kit were 5'-TTG TTA CAA GGG ACT TTC CGC TGG GGA CTT TCC AGG GAG GCG TGG-3'. The dried gels were visualized and analyzed with Labworks 4.5 software.

Immunofluorescence. Cells were treated with 7-KC for different concentrations or times as indicated in the figure, and fixed in 4% formaldehyde for 15min at room temperature. After fixation, the cells were rinsed 3 times with Phosphate Buffered Saline (PBS). The cells were then pretreated with normal goat serum block working solution for 15min, incubated with primary polyclonal antibodies NF- κ B/p65, 1:50 overnight at 4°C, and rinsed 3 times in PBS for 5min each. After additional washing, bound primary antibodies were detected by incubation with fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-rabbit IgG (sc-1012) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The slides were counterstained for nuclei with PI for 5min. To exclude nonspecific binding of primary antibodies, slides were rinsed 3 times in PBS for 5min. The slides were visualized with a laser scanning confocal microscope (LSCM).

DNA fragmentation assay. DNA fragmentation analysis was carried out as previously described

[18]. In brief, cells were pelleted and analyzed in DNA isolation buffer containing 50mM Tris-HCl, pH8.0, 2mmol/L ethylenediaminetetraacetic acid (EDTA), 10mmol/L NaCl, 1% sodium dodecyl sulfate (SDS) and 500 μ g/mL proteinase K at 50°C for 6h, and centrifuged in order to separate intact from fragmented DNA. The supernatant containing the fragmented DNA was digested with Rnase A (100 μ g/mL) and proteinase K (0.2mg/mL) for 2h at 37°C. After precipitation with 75% ethanol, the DNA was collected and resuspended in 20 μ L TE buffer (Tris-HCl 10mmol/L, pH7.4, acetic acid 1mmol/L, pH8.0) and separated by 1% agarose gel electrophoresis.

Cell growth assay. The growth rate of cells was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl (MTT) assay. Briefly, exponentially growing J774A.1 cells were seeded into a 96-well plate at 1×10^4 cells per well in triplicate. After incubation for 12h, cells were treated with 15 μ g/mL 7-KC for 6, 12, 24, 48h at increasing concentrations as indicated in the figure for 24h. At the end of treatment, 20 μ L MTT (5mg/mL) was added to each well and incubated for an additional 4h. The formazan grains formed by viable cells were solubilized with dimethyl sulfoxide (DMSO) and the color intensity was measured at 570nm with an enzyme-linked immuno-absorbent assay reader (Rayto, Shenzhen, China). J774A.1 cells were pretreated with an NF- κ B inhibitor, PDTC, for 20min prior to the treatment with 7-KC.

Flow cytometric (FCM) analysis. J774A.1 cells were plated at 1×10^6 per well in a 6-well plate, then treated with 7-KC as described in the figures. Cells were adjusted at 1×10^6 cells/ml with combining buffer. One hundred μ L of cell suspended solution was added to a new tube, and incubated with 5 μ L Annexin V and 10 μ L 20 μ g/ml propidium iodide (PI) solution at room temperature in the dark for 15min. J774A.1 cells were stimulated with 15 μ g/mL of 7-KC for 24h with or without pretreatment by PDTC for 20min. FCM was performed using a FACS-Caliber (BD, USA) and data were analyzed using the Cell Quest software program. Cell debris was excluded by appropriate 2-dimensional gating methods.

Statistical analysis. The experimental data were analyzed using Student's t-test. Significance was established at values of $p < 0.05$ and $p < 0.01$.

Results

The activation of NF- κ B by 7-KC. To determine the NF- κ B activation by 7-KC, cells were treated with 15 μ g/mL 7-KC for 6h and 12h. The expression of NF- κ B-p65 protein located in the nucleus has a tendency to increase when treated with 7-KC, while in the cytosol we found a reverse phenomenon (Fig. 1A, 1B). Furthermore, the stimulation of J774A.1 cells with 7-KC decreased I κ B α protein levels in a time- (Fig. 1C) and dose- (Fig. 1D) dependent manner in the cytosol, indicating that I κ B α was degraded during NF- κ B activation. After demonstrating that 7-KC induced NF- κ B protein expression as measured by western blots, we tried to further examine the effect of 7-KC on the transcriptional activity of NF- κ B by EMSA. Nuclear extracts of J774A.1 cells were incubated with end-labeled double-stranded oligonucleotides possessing the NF- κ B consensus site. The nuclear extracts from the J774A.1 cells stimulated by 7-KC for 12h yielded a large band in comparison to the unstimulated cells (Fig. 2A, 2B).

Modulation of oxLDL in other systems is linked to the transcription factor NF- κ B, whose activation requires translocation to the cell nucleus [16]. To detect nuclear translocation of NF- κ B, here, we performed immunofluorescence staining. We exposed

J774A.1 cells to 7-KC for 12h and visualized the localization of NF- κ B with LSCM after probing with anti- NF- κ B/p65 antibody (green). In the control without 7-KC treatment, NF- κ B was expressed only slightly (shown by arrows) in the nucleus, and was mainly dispersed in the cytosol (Fig. 2C). When exposure to 15 μ g/mL of 7-KC induced an obvious translocation of NF- κ B/p65, the nucleus was stained green as shown by the arrow. Conversely, the green fluorescence became weak in the cytosol (Fig. 2C). In summary, 7-KC was able to up-regulate NF- κ B activation and induce its translocation.

Treatment with 7-KC led to apoptosis of J774A.1 cells. There have been numerous reports about the reduction of cell proliferation by 7-KC [10–12]. Here, the effect of 7-KC cell growth was studied in J774A.1 cells using an MTT assay. Compared to the untreated control group, the cell growth rates when stimulated by 10, 15, 20 μ g/mL of 7-KC for 24h were 70.1 \pm 2.8% ($p < 0.05$), 65.8 \pm 2.0% ($p < 0.05$), and 27.0 \pm 0.6% ($p < 0.01$), respectively (Fig. 3A). About 73% of cell viability was suppressed by treatment with 20 μ g/mL of 7-KC, indicating a crucial role for 7-KC in cell proliferation. Moreover, we determined the blockade of cell proliferation after treatment by 7-KC for 6, 12, 24, 48h (data not shown). The findings clearly showed that 7-KC can induce the reduced the viability of J774A.1 cells.

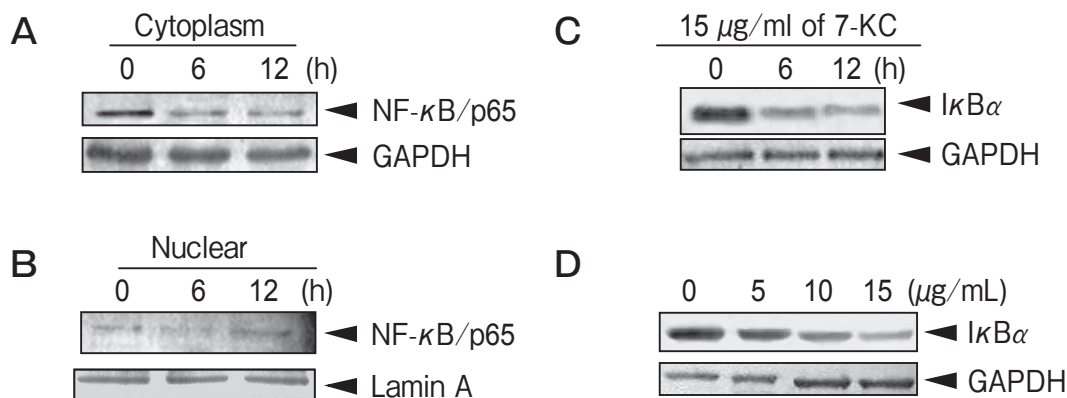


Fig. 1 Effects of 7-KC on the expression of NF- κ B and I κ B α . **A**, the expression of NF- κ B after treatment with 15 μ g/ml of 7-KC in cytoplasm was detected by western blot; **B**, the expression of NF- κ B after treatment with 15 μ g/ml of 7-KC in the nucleus. Protein samples from cytoplasm and nucleus were extracted with nuclear and cytoplasmic extraction reagents. Lamin A and GAPDH were the loading controls for the nuclei and cytosol, respectively; **C**, 7-KC induced I κ B α degradation in a time-dependent manner. J774A.1 cells were incubated with 15 μ g/mL of 7-KC for 0, 6 and 12h; **D**, 7-KC induced I κ B α degradation in a dose-dependent manner. J774A.1 cells were incubated with 0, 5, 10, 15 μ g/ml. Western blot analysis was performed using anti-I κ B α antibody. GAPDH was the loading control.

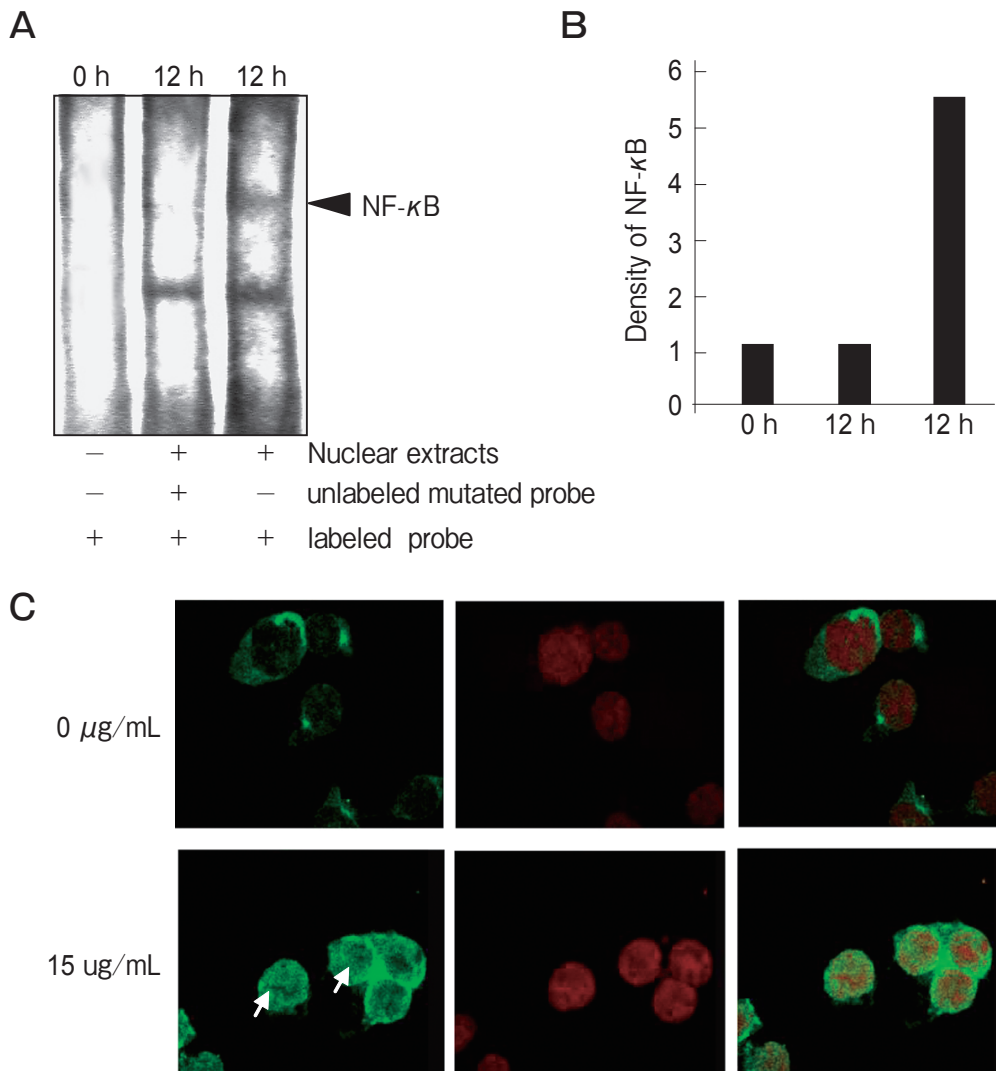


Fig. 2 7-KC induces NF-κB activation. **A**, The activation of NF-κB was examined by EMSA. NF-κB activation was measured by EMSA using a biotin-labeled oligonucleotide encompassing the NF-κB consensus motif. J774A.1 cells were stimulated with 15 μg/mL of 7-KC at different time intervals (0 and 12h). The arrow indicates the specific NF-κB band; **B**, A densitometric analysis is shown; **C**, 7-KC induced nuclear translocation of NF-κB in a dose-dependent manner. Cells were subjected to immunofluorescence analysis as described under "Materials and Methods". Exposure to 15 μg/ml of 7-KC increased the nuclear translocation of NF-κB, shown by the arrow. The results were observed under a laser scanning confocal microscope.

To clarify whether the reduction of cell viabilities by treatment with 7-KC is involved in the cell apoptosis induced by 7-KC or not, we carried out several experiments to detect cell apoptosis and associated events. One of the most important hallmarks of apoptosis is DNA fragmentation. In cell apoptosis, the endonucleases cleave the DNA, leading to the characteristic "ladder" of DNA fragmentation. We analyzed

the time-dependent DNA status of J774A.1 cells treated with 15 μg/mL of 7-KC. After 12h of 15 μg/mL 7-KC treatment, J744A.1 cells began to generate DNA fragments, while the control was not changed. After 24h of 15 μg/mL 7-KC treatment, the fragmentations were more obvious (Fig. 3B).

NF-κB activation partially contributed to the apoptosis of J774A.1 cells in 7-KC treatment.

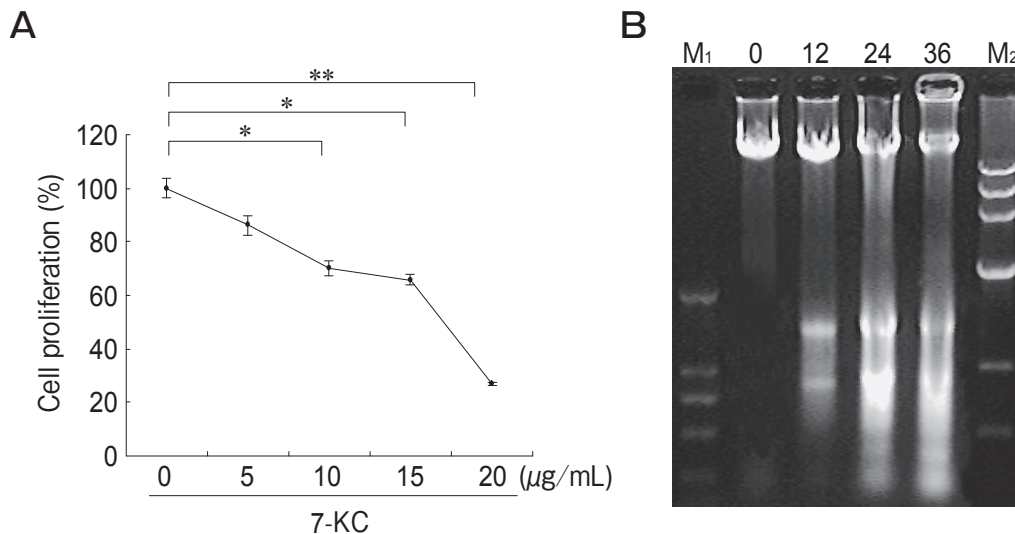


Fig. 3 Effects of 7-KC on the apoptosis of J774A.1 cells. **A**, Effects of 7-KC on the J774A.1 cell viability. The cells were treated with 7-KC at the indicated concentrations for 24h, and the cell viability was determined by an MTT assay as described in "Materials and Methods". The cell viability was expressed as the mean \pm standard deviation (S.D.) of viable cells compared with untreated cells (taken 100% viable) from 3 replicate assays (* $p < 0.05$, ** $p < 0.01$); **B**, Induction of apoptosis by 7-KC in J774A.1 cells. Cells were treated with 15 $\mu\text{g/mL}$ of 7-KC for 0, 12, 24, 36h, and the level of DNA fragmentation was analyzed by electrophoresis in a 1% agarose gel (M, marker).

We found that cell proliferation was partly restored when treated with an NF- κ B inhibitor, pyrrolidine dithiocarbamate (PDTC), for 20min. As shown in Fig. 4A, the rate of cell growth increased from 27.0% to 40.3% after treatment with 100 μM PDTC in J774A.1 cells, suggested that during 7-KC-induced apoptosis, NF- κ B was also activated.

Moreover, we used PI/Annexin V double staining to detect apoptotic and necrotic cells. Apoptotic cells (PI $^-$ /annexin V $^+$ cell and PI $^+$ /annexin V $^+$ cell) were markedly present in the cell treated with 15 $\mu\text{g/mL}$ of 7-KC, but not in the control population. The percentage of early apoptotic cells (PI $^-$ /annexin V $^+$ cells) was 39.30% in cultures treated with 7-KC for 24h compared with only 1.93% in the control (Fig. 4B); the percentage of late apoptotic cells (PI $^+$ /annexin V $^+$ cells) was 2.28% compared with 2.49% in the control. The number of upper left quadrant in Fig. 4B indicates the percentages of cells that take up PI but do not bind to annexin V. These cells would most likely be necrotic. There were 1.76% of PI $^-$ /annexin V $^-$ cells in the 24h 7-KC treatment compared to 3.19% in the control. The dramatic increase of PI $^-$ /annexin V $^+$ cells after 7-KC treatment, which reflects phosphatidylserine externalization early in the apoptotic process, suggested that 7-KC led to reduced prolifera-

tion followed by cell apoptosis. The early apoptotic cells (PI $^-$ /annexin V $^+$ cells) were decreased to 26.86% in the presence of NF- κ B inhibitor, PDTC, which significantly inhibited the cell death (Fig. 4B). Collectively, it is conceivable that 7-KC induced NF- κ B activation during the process of cell apoptosis.

Discussion

While the molecular basis of AS remains unclear, cell apoptosis induced by 7-KC appears to play an important role in the development of AS [19, 20]. In the present study, we first demonstrated that 7-KC is capable of inducing apoptosis of mouse macrophages by activating the NF- κ B pathway, which may directly or indirectly contribute to disease pathogenesis and tissue damage.

The present study was aimed at exploring the mechanism underlying the effect of 7-KC during apoptosis in J774A.1 cells and determining whether this effect is mediated by the activity of its major transcription factor, NF- κ B. NF- κ B is known as a widespread rapid-response transcription factor that is normally expressed in the cytoplasm of a variety of cells. Previous researches have mostly focused on the injury of blood vessels by oxLDL and the inflamma-

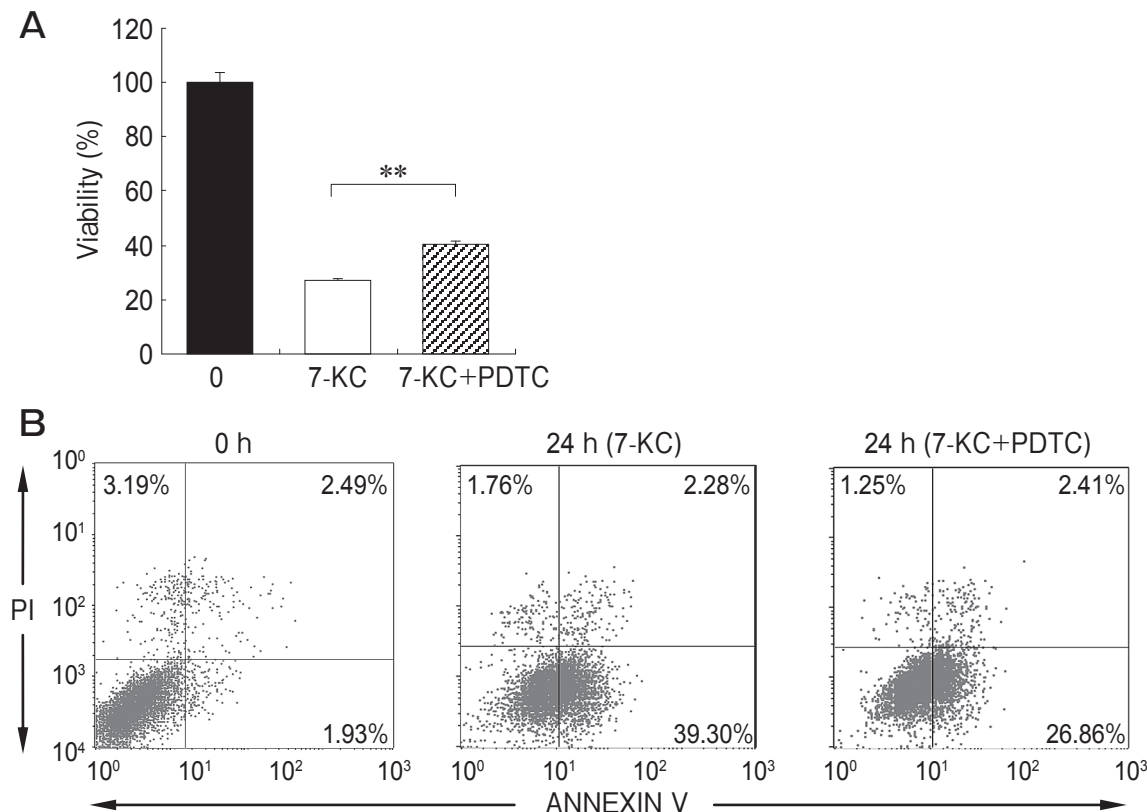


Fig. 4 Cell apoptosis induced by 7-KC was associated with NF- κ B activation. **A**, Reduced cell proliferation was recovered by PDTC pretreatment. Cells were treated with 20 μ g/mL of 7-KC for 12h with or without PDTC (100 μ M) pretreatment (** $p < 0.01$); **B**, Cell apoptosis induced by 7-KC was detected by Annexin-V-PI staining. The untreated cells were the control. Double-negative staining represents living cells, positive staining for Annexin-V-FITC, and negative staining for PI represent the early apoptotic stage, and double-positive staining represents the late apoptotic stage. The fluorescence intensity was quantified for 10,000 individual cells. The experiment shown is representative of 3 others performed.

tion caused by activated NF- κ B [16]. Norata *et al.* [21] indicated that triglyceride-rich lipoproteins contained in the oxLDL could enhance the transcriptional activity of NF- κ B. Also, 13-HPODE (13-hydroperoxy-octadecadienoic acid) could induce NF- κ B activation and subsequent overexpression of VCAM-1 in porcine vascular smooth muscle cells [22]. But the exact composition of oxLDL that can induce AS and activate NF- κ B is unknown at present. Here, we first showed that 7-KC induced an increase of NF- κ B-p65 protein expression in the nucleus (Fig. 1A) and nuclear translocation (Fig. 2C). Furthermore, the effect of 7-KC on NF- κ B activation was shown by EMSA (Fig. 2A). These results clearly indicated that 7-KC could induce NF- κ B activation in J774A.1 cells. On the other hand, it has been reported that the activation of NF- κ B induced by

oxLDL was regulated by the degradation of I κ B α mediated by protein kinase C [23]. The stimulation of J774A.1 cells with 7-KC decreased I κ B α protein levels in the cytosol (Fig. 1C, 1D). We interpreted that the decrease of I κ B α levels was also involved in the 7-KC-induced NF- κ B activation.

The development of atherosclerotic lesions is mainly due to macrophage death. It has been reported that 7-KC plays an important role in the progression of atherosclerotic lesions by inducing cell death in the vascular wall [19–24]. Apoptosis is a major form of cell death, induced initially by 7-KC. A previous study was mainly concerned about the toxicity of 7-KC and pro-oxidation inducing cell apoptosis and emphasized the regulation of macrophage apoptosis by 7-KC toxicity, as a factor that anticipated the development and formation of AS [25]. Fiorella Biasi *et al.* [26]

found that 7-KC could induce mouse macrophage apoptosis associated with the release of cytochrome C and activated caspase-3. Moreover, 7-KC could induce macrophage apoptosis by activation of caspase 3, 7, 8 and 12 [19, 20]. In this study, we showed for the first time that 7-KC also induces cell apoptosis by NF- κ B activation (Fig. 4). As described above, there are various other pathways associated with cell apoptosis by 7-KC, such as its own toxicity, the release of cytochrome C and activation of caspase-3, 7, 8 and 12. Therefore, treatment with the NF- κ B inhibitor, PDTC, could not completely inhibit the cell apoptosis induced by 7-KC.

Although it has been reported that NF- κ B has a role of inhibiting apoptosis [27], numerous studies have also shown that NF- κ B activation participates in cell apoptosis [28–30]. It has been reported that long-term exposure to PDTC for 3h and 6h induces the intracellular antioxidant GSH and anti-oxidative enzymes via nuclear translocation of Nrf2 [31], and suppresses cell apoptosis via the Nrf2/GSH pathway. To effectively inhibit the function of PDTC suppressing cell apoptosis via the Nrf2/GSH pathway, here, J774A.1 cells were pretreated with PDTC for 20min prior to the treatment with 7-KC. It is important to note that 7-KC-induced NF- κ B activation is related to a cell survival pathway, as evidenced by the restoration of J774A.1 cell proliferation from 27.0% to 40.3% with treatment by PDTC for 20min (Fig. 4A). This demonstrated that 7-KC-induced J774A.1 cell death was partially associated with the activation of NF- κ B. Moreover, NF- κ B began to be activated at 6h (Fig. 1, 2), while cell apoptosis was detected from 24h after the treatment by 7-KC (Fig. 3B, 4B). It is noteworthy that 7-KC-induced NF- κ B activation preceded the cell apoptosis, suggesting that NF- κ B activation contributes to the cell apoptosis induced by 7-KC. Further studies are in progress to clarify the molecular mechanism of cell apoptosis involved in the 7-KC/NF- κ B pathway.

Overall, the most important finding of this study is that 7-KC plays an important role during macrophage apoptosis, following the activation of NF- κ B. Our study provides new insights into the biological functions of 7-KC during the AS process and provides a hint for the future development of new therapeutic strategies to prevent tissue damage by AS.

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