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3-Methyl-1,2-cyclopentanedione Down-Regulates Age-Related NF-κB Signaling Cascade

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Activation of the redox-sensitive transcription factor, nuclear factor-kappa B (NF-κB), plays a central role in inflammation and aging processes by inducing pro-inflammatory genes. The present study was designed to unravel the molecular mechanisms underlying the anti-inflammation effects of 3-methyl-1,2-cyclopentanedione (3-MCP) in coffee extracts. In particular, we investigated the effects of 3-MCP on the modulation of NF-κB signaling pathways and its target genes in the kidneys of aged animal rats: young (6 months old), old (21 months old), and old 3-MCP-fed (4 and 8 mg/kg/day for 10 days). The results strongly show that 3-MCP exerted potent anti-inflammatory effects, significantly reducing (i) the phosphorylation of inhibitor κB (IκB) and other NF-κB upstream events; (ii) the translocation of NF-κB into the nucleus; (iii) the expression of iNOS and COX-2; and (iv) pro-inflammatory genes such as VCAM-1, MCP-1, and IL-6. Furthermore, 3-MCP suppressed reactive oxygen species levels. Taken together, our results clearly demonstrate that 3-MCP modulated the age-related NF-κB signaling cascade and its pro-inflammatory genes. Therefore, 3-MCP is proposed to be an effective anti-inflammatory agent that can be a novel approach for the therapy of inflammation.

KEYWORDS: 3-MCP; coffee extracts; NF-κB; aging; inflammation

INTRODUCTION

Innate inflammation responses are essential for organisms to counteract invading pathogens or other insults. With aging, however, chronic inflammatory responses may cause damage, which results in adverse pathological conditions including cardiovascular disorders, arthritis, and cancers (*1*−*3*). Because the cellular signaling pathways of inflammation almost always lead to activation of the transcription factor, nuclear factor-kappa B (NF-κB, a transcription factor involved in the induction of several genes) has been of great interest for many researchers who investigate anti-inflammatory properties.

NF-κB is a redox-sensitive transcription factor that controls various aspects of the immune and inflammatory response (*4*), and its activation along with those of its target genes is associated with various pathological processes. The regulation of NF-κB centers around its interaction with specific inhibitory proteins, called inhibitor κB (IκB), of which the most important may be IκBα, IκBβ, and IκBγ. Two closely related kinases, IκB kinase α (IKKα) and IKKβ, have been identified as key players in NF-κB modulation (*5*). Upon activation of the NF-κB inducing kinase (NIK), NIK is recruited to the IKK complex via IKKγ, resulting in the phosphorylation of IKKβ associated with the regulatory subunit, IKKγ (i.e., activation of IKK). The activated IKK complexes initiate IκB phosphorylation to trigger the ubiquitination of IκB, leading to the activation of NF-κB and its translocation into the nucleus (*6*).

NF-κB also is shown to activate target genes, inducing transcription of pro-inflammatory cytokines, cell adhesion molecules, intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), cyclooxygenase-2 (COX-2), and inducible NO synthase (iNOS) (*7*). Inflammatory arthritis animal models support the notion that NF-κB activation plays a pathogenic role in vivo (*8*). The modulation of an abnormal up-regulation of the redox-sensitive transcription factor, NF-κB, therefore, is essential in the prevention of chronic inflammation, which in turn could regulate inflammation-related diseases.

Coffee is one of the most widely consumed beverages in the world. Herein, it has been studied extensively and shown to have pharmacologically beneficial effects. It has been reported to possess anti-diabetic effects (9) and anti-hypersensitive (*10*) and anti-oxidant activity, inhibiting lipid peroxidation and exerting a strong protective effect against mutagenicity and cytotoxicity (*11*).

The present study was designed to elucidate the effects of coffee extract, 3-MCP (**Figure 1**), in animal models of chronic inflammation. In particular, we investigate the effects of 3-MCP

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on the age-related NF-κB signaling cascade via the NIK/IKK pathway, NF-κB targeting genes, and ROS scavenging activity in rat kidneys.

MATERIALS AND METHODS

Materials. Unless otherwise stated, all compounds were obtained from Sigma Chemical Co. (St. Louis, MO). 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes, Inc. (Eugene, OR). Immobilon-P transfer membrane was obtained from Millipore Corp. (Bedford, MA). Antibodies to p65, p50, phospho-NIK, phospho-IKKα/β, phospho-IκBα, IκBα, COX-2, iNOS, VCAM-1, MCP-1, and IL-6 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced chemiluminescence (ECL) Western blot detection reagents were from Amersham Life Sciences Inc. (Arlington Heights, IL).

Animals. Male specific-pathogen free (SPF) Fischer 344 rats (Samtako, Osan, Korea) were used for these studies. Rats were fed a diet of the following composition: 21% soy bean protein, 15% sucrose, 43.65% dextrin, 10% corn oil, 0.15% α-methionine, 0.2% choline chloride, 5% salt mix, 2% vitamin mix, and 3% Solka-Floc. Animals were housed and handled in a controlled environment (24 °C; 50−60% humidified atmosphere) according to the guidelines of the Animal Care Committee of the Pusan National University.

Experimental Conditions. Rats at 6 and 21 months of age were grouped as young (Y) and old (O), respectively. 3-MCP (Sigma) was mixed with powder and fed to the 21-month-old rats at a dose of 4 and 8 mg/kg/day (3-MCP 4 and 3-MCP 8). Each group contained five rats. After 10 days of feeding, the rats were sacrificed by decapitation, and the kidneys were quickly removed and rinsed in ice-cold buffer [100 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 M phenylmethyl-sulfonyfluoride (PMSF), 1 μM pepstatin, 2 μM leupeptin, 80 mg/L trypsin inhibitor, 20 mM β-glycerophosphate, 20 mM NaF, and 2 mM sodium orthovanadate (pH 7.4)]. The tissue was immediately frozen in liquid nitrogen and stored at −80 °C.

Preparation of Kidney Homogenates from Rats. All solutions, tubes, and centrifuges were maintained at 0−4 °C. The preparation of nuclear extract was based on previous methods (*12*). Three hundred milligrams of kidney was homogenized with 2 mL of homogenate buffer A (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.1 mM EDTA, 0.1 mM PMSF, 1 μM pepstatin, and 1 mM P-aminobenzamidine) with a tissue homogenizer for 20 s. Homogenates were kept on ice for 15 min, 125 μL of a 10% Nonidet p40 (NP 40) solution was added and mixed for 15 s, and the mixture was centrifuged for 2 min at 12 000 rpm. The supernatant contained cytosol proteins. The pelleted nuclei were washed once with 400 μL of buffer A plus 25 μL of 10% NP 40, centrifuged, then suspended in 50 μL of buffer C (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% (v/v) glycerol), mixed for 20 min, and centrifuged for 5 min at 12 000 rpm. The supernatant containing nuclear proteins was stored at −80 °C.

Measurement of ROS Generation by DCF-DA Assay. Nonfluorescent 2,7-dihydrofluorescin-diacetate (DCF-DA) was converted into DCFH esterase and subsequently oxidized to highly fluorescent 2,7-dichlorofluorescein (DCF) by reactive species (RS) (*13*, *14*). Briefly, DCF-DA was dissolved in absolute ethanol at a concentration of 12.5 mM and kept at −70 °C in the dark. Phosphate buffer (50 mM) at pH 7.4 was used to dilute the DCF-DA stock to working solution. The 25 μM DCFDA was added to the kidney homogenate for a 250 μM final volume, and then changes in fluorescence intensity were measured for 30 min. The fluorescence was determined using microplate fluorescence GENios (TECAN, Schweiz AG, Mannedorf, Switzerland) at excitation and emission wavelengths of 485 and 535 nm, respectively.

Western Blot Analysis. The protein concentration was determined by the Lowry method (Sigma) using bovine serum albumin (BSA) as a standard. Homogenized samples were boiled for 5 min with a gel-loading buffer (pH 6.8, composed of 0.125 M Tris-HCl, 4% SDS, 10% 2-mercaptoethanol, and 0.2% bromophenol blue) in a ratio of 1:1. Equal amounts of protein were separated by SDS-PAGE using 6−17% acrylamide gels. The gels were subsequently transferred onto a nitrocellulose membrane (Hybond C, Amersham Corp.). The membrane was immediately placed in a blocking solution (5% non-fat dry milk in TBS−-T buffer containing 10 mM Tris, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) at room temperature for 1 h. The membrane was washed in TBS−-T buffer for 30 min and then incubated with a primary antibody at room temperature for 2 h. After three 10 min washings in TBS−-T buffer, the membrane was incubated with a second antibody at room temperature for 1 h. After four 10 min washings in TBS−-T buffer, antibody labeling was detected using ECL per the manufacturer's instructions and exposed to a radiographic film. Prestained blue protein markers were used for molecular weight determination.

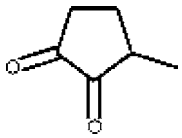

Figure 1. Chemical structure of 3-methyl-1,2-cyclopentanedione.

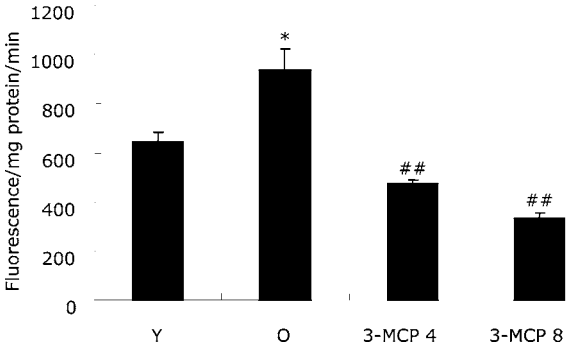

Figure 2. Suppression of ROS level by 3-MCP in aged rats. ROS generation in aged rats was determined using the DCF-DA method in kidney homogenates for control and 3-MCP-fed rats. Each value is the mean ± SE of five rats. Y, 6-month-old rats; O, 21-month-old rats; 3-MCP 4, 21-month-old rats fed 3-MCP 4 mg/kg/day for 10 days; 3-MCP 8, 21-month-old rats fed 3-MCP 8 mg/kg/day for 10 days; and 3-MCP, 3-methyl-1,2-cyclopentanedlone. Statistical significance: $*p < 0.05$ as compared to Y and $\#\#p < 0.01$ as compared to O, respectively.

Statistical Analysis. The results are presented as the mean ± SE of individual experiments, and each measurement was performed in triplicate. The statistical significance was tested using a one-way ANOVA/post-hoc test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Anti-oxidant Effects of 3-MCP on Age-Related Oxidative Status in Aged Rats. To investigate the age-related oxidative status, ROS generations were studied using the DCFH-DA method in kidney homogenate. **Figure 2** depicts an increase of ROS with age, showing a fluorescence intensity increase of ∼30% as compared to young rats (6 months old). In contrast, the ROS generation significantly decreased by 64% in the 3-MCP-fed (8 mg/kg/day) old rats as compared to their age-matched controls. The results indicate that 3-MCP suppressed an increase in oxidative stress during the aging process.

Suppression of Translocation and Activation of NF-κB by 3-MCP in Aged Rats. NF-κB is normally present in the cytoplasm in an inactive state and bound to a member of the IκB inhibitor protein family. Upon stimulation, IκBα is phosphorylated and degraded. Unbound NF-κB then translocates into the nucleus and activates various downstream genes (*15*). Western blot analysis revealed that old rats showed significantly increased NF-κB p65 and p50 levels about 38 and 93%, relative to young rats, and that the 3-MCP-fed old rats exhibited a decrease of p65 and p50 protein levels in nuclear extracts (**Figure 3**).

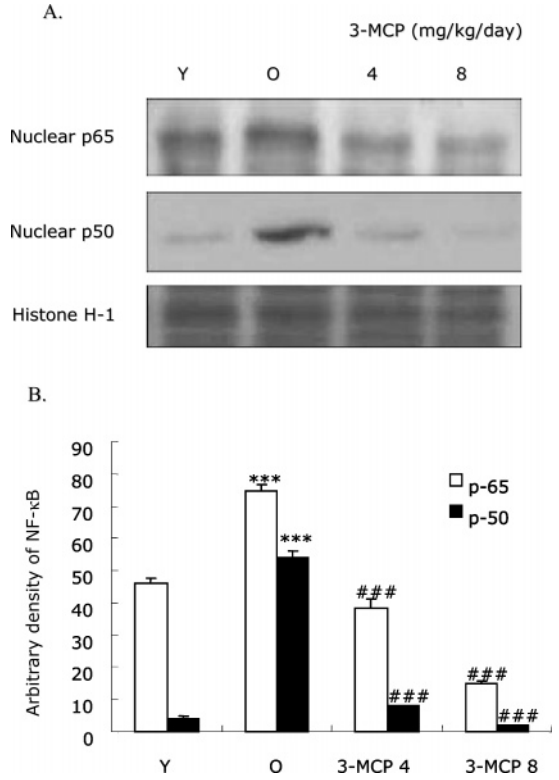

Figure 3. (**A**) Suppression of NF-κB translocation and activation by 3-MCP in aged rats. Western blot analysis was performed to detect NF-κB (p65, p50) and histone H-1 protein levels in nuclear extracts (40 μg of protein) from each group. Western blot analysis was probed with antibodies specific for p65, p50, and histone H-1. Y, 6-month-old rats; O, 21-month-old rats; 3-MCP 4, 21-month-old rats fed 3-MCP 4 mg/kg/day for 10 days; 3-MCP 8, 21-month-old rats fed 3-MCP 8 mg/kg/day for 10 days; and 3-MCP, 3-methyl-1,2-cyclopentanedlone. (**B**) Each bar is the mean ± SE of three experiments. Statistical significance: ***$p < 0.001$ as compared to Y and ###$p < 0.001$ as compared to O, respectively.

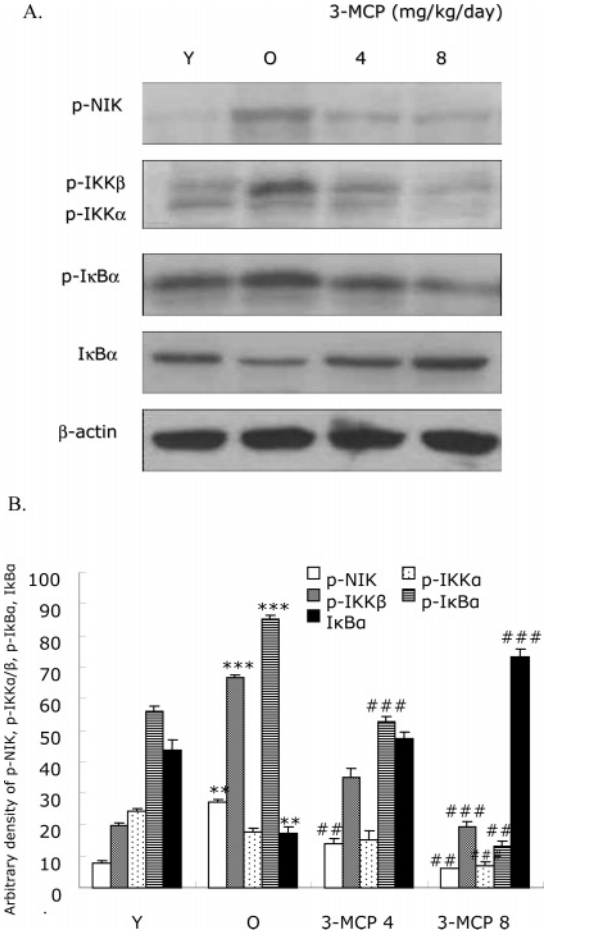

Figure 4. (**A**) Suppression of NF-κB signaling by 3-MCP in aged rats. Western blot analysis was performed to detect phosphor-NIK, phosphor-IKK, phosphor-IκBα, IκBα, and β-actin protein levels in cytosolic extracts (80 μg of protein) from each group. Western blot analysis was probed with antibodies specific for phosphor-NIK, phosphor-IKK, phosphor-IκBα, IκBα, and β-actin. Y, 6-month-old rats; O, 21-month-old rats; 3-MCP 4, 21-month-old rats fed 3-MCP 4 mg/kg/day for 10 days; 3-MCP 8, 21-month-old rats fed 3-MCP 8 mg/kg/day for 10 days; and 3-MCP, 3-methyl-1,2-cyclopentanedlone. (**B**) Each bar is the mean ± SE of three experiments. Statistical significance: **$p < 0.01$ and ***$p < 0.001$ as compared to Y and ##$p < 0.01$ and ###$p < 0.001$ as compared to O, respectively.

Suppression of NIK/IKK and IκBα Phosphorylation by 3-MCP. To determine whether 3-MCP modulates NF-κB activation through the NIK/IKK pathway, the activation of NIK and IKK and the IκBα phosphorylation were examined in their phosphorylated forms by Western blot analysis. It has been shown that NF-κB activators induce the phosphorylation and degradation of IκB by the activation of the IKK complexes, IKKα and IKKβ (*16*). NIK either directly or indirectly activates the IKKα/IKKβ complex, leading to IκB phosphorylation and degradation and thus NF-κB activation. We used Western blot analysis to probe with antibodies specific for phospho-NIK, phospho-IKKα/β, and phospho-IκBα in kidney homogenate cytosolic extracts.

Our data show that NF-κB signaling significantly increased with age due to the age-related activation of IκBα, IKKα/β, and NIK. In contrast, 3-MCP-fed old rats exhibited decreased phospho-NIK, phospho-IKKα/β, and phospho-IκBα protein levels (**Figure 4**). These findings demonstrate that 3-MCP suppressed NF-κB activation through the NIK/IKK/IκB pathway.

Inhibition of NF-κB-Related Genes by 3-MCP in Aged Rats. To further verify the regulatory role of 3-MCP in age-associated NF-κB activation, the effects of 3-MCP on several NF-κB targeting genes were examined. COX-2 and iNOS are known to have an NF-κB binding site in their promoter region and be controlled by NF-κB regulation. NF-κB activation also increases the expression of the adhesion molecules, VCAM-1 and chemokines and cytokines, MCP-1, and interleukin-6 (IL-6).

Western blot analysis was carried out using antibodies specific for COX-2, iNOS, VCAM-1, MCP-1, and IL-6 to detect protein levels in rat kidney homogenate cytosolic extracts. As shown in the data (**Figures 5A** and **6A**), the expression of these pro-inflammatory genes increased remarkably with age. However, the 3-MCP-fed old rats showed reduced elevations of these proteins. These results suggest that 3-MCP inhibited the expression of pro-inflammatory genes by suppressing NF-κB activation.

DISCUSSION

Of the important transcription factors, NF-κB is shown to play a pivotal role in the regulation of inflammation processes during aging. Activation of NF-κB induces the transcription of a large array of genes that are implicated in inflammation, including adhesion molecules and cytokines and chemokines

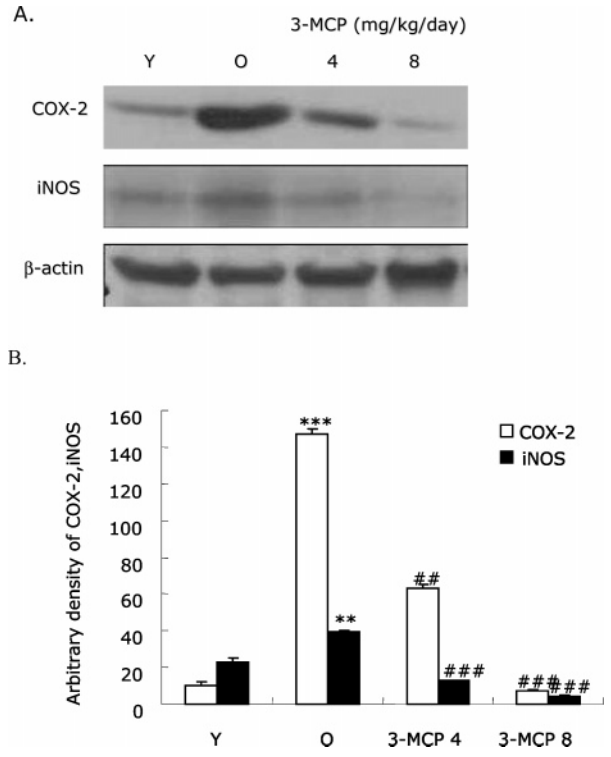

Figure 5. (**A**) Inhibition of NF-κB induced COX-2 and iNOS by 3-MCP in aged rats. Western blot analysis was performed to detect COX-2 and iNOS protein levels in cytosolic extracts (80 μg of protein). Western blot analysis was probed with antibodies specific for COX-2 and iNOS. Y, 6-month-old rats; O, 21-month-old rats; 3-MCP 4, 21-month-old rats fed 3-MCP 4 mg/kg/day for 10 days; 3-MCP 8, 21-month-old rats fed 3-MCP 8 mg/kg/day for 10 days; and 3-MCP, 3-methyl-1,2-cyclopentanedlone. (**B**) Each bar is the mean ± SE of three experiments. Statistical significance: $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to Y and $\#\#p < 0.01$ and $\#\#\#p < 0.001$ as compared to O, respectively.

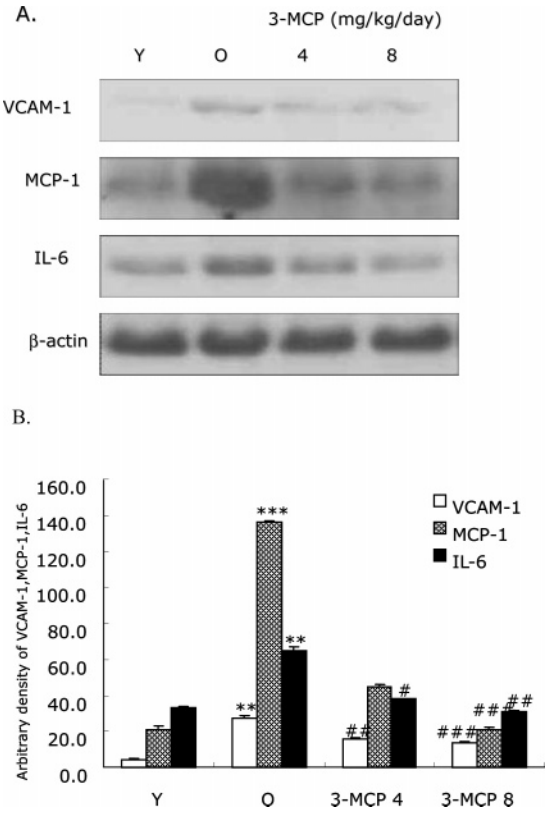

Figure 6. (**A**) Inhibition of NF-κB targeting genes by 3-MCP in aged rats. Western blot analysis was performed to detect VCAM-1, MCP-1, IL-6, and β-actin protein levels in cytosolic extracts (80 μg of protein) from each group. Western blot analysis was probed with antibodies specific for VCAM-1, MCP-1, IL-6, and β-actin. Y, 6-month-old rats; O, 21-month-old rats; 3-MCP 4, 21-month-old rats fed 3-MCP 4 mg/kg/day for 10 days; 3-MCP 8, 21-month-old rats fed 3-MCP 8 mg/kg/day for 10 days; and 3-MCP, 3-methyl-1,2-cyclopentanedlone. (**B**) Each bar is the mean ± SE of three experiments. Statistical significance: $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to Y and $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$ as compared to O, respectively.

(*17*). In the present study, the aging process was found to cause the up-regulation of inflammatory and stress response genes in rat kidneys.

Numerous studies demonstrate that increased levels of ROS activate NF-κB in endothelial cells and many other cell types, leading to the up-regulation of pro-inflammation genes. Moreover, there are studies suggesting that the NF-κB binding activity increases during aging (*18*, *19*). Thus, it is logical to hypothesize that age-related oxidative stress may contribute to inflammation in aging by activating NF-κB. In this respect, many anti-oxidative agents that can detoxify ROS have been purported to suppress the activation of NF-κB (*20*). In this study, we showed that 3-MCP suppressed the ROS level and that NF-κB translocation and activation consequently reduced the age-related up-regulation of pro-inflammatory genes.

The regulation of NF-κB centers on the phosphorylation of the inhibitor of NF-κB, namely, IκB. Two related kinases, IκB kinase (IKKα) and IKKβ, have been identified as key players in NF-κB modulation (*21*). In response to various stimuli, upstream kinases are activated, and IKKβ is phosphorylated accordingly. The activated IKK complexes phosphorylate IκB subunits of NF-κB/IκB to trigger the degradation of IκB, leading to the activation of NF-κB. In our study, we note that 3-MCP suppressed the active form of NIK, IKKα, IKKβ, and IκBα. Thus, the inhibitory effect of 3-MCP on the upstream events of NF-κB appeared to cause NF-κB translocation to the nucleus.

The NF-κB (p65, p50) heterodimer activates the transcription of inflammatory enzymes including COX-2 and iNOS through κB sites in their promoter region. Nitric oxide (NO) derived from iNOS and prostaglandin E2 (PGE2) synthesized by COX-2 play a pivotal role in the pathogenesis of acute and chronic inflammation. Indeed, increased levels of COX-2 and iNOS expression have been reported in various vascular tissues of aged rats (*22*, *23*) and in senescent endothelial cells in cultures as well (*24*). Importantly, a previous study exhibited that overexpression of COX-2 and iNOS was linked to endothelial dysfunction (*25*). In the current study, our results also demonstrated that 3-MCP inhibited COX-2 and iNOS expression as well as pro-inflammatory genes.

Chung (*26*) reported that COX-2 converts arachidonic acid to prostanoids (PGs) during the time that reactive oxygen species (ROS) are generated. ROS production in the PG synthesis pathway can contribute significantly to the overall ROS pool under normal and pathogenic conditions. ROS inhibits the function of mitochondrial respiratory chain enzymes, oxidizes several mitochondrial respiratory chain enzymes and various proteins, and possibly triggers DNA strand breakage (*27*). It is of great interest that a redox imbalance due to the net effect of oxidative stress and a counteracting, anti-oxidative force is responsible for the characteristic changes of the aging process (*28*).

During the aging process, intracellular anti-oxidative defense mechanisms in aerobic organisms do not protect completely

against ROS-mediated damage, thereby enabling ROS to potentially damage proteins, lipids, and most importantly, DNA. A recent report from our laboratory on the modulation of redox-sensitive NF-κB during aging highlighted the essential nature of the redox status in the regulation of inflammatory genes and other transcription factors (*29*). Thus, the inhibition of ROS generation by 3-MCP may affect the suppression of the NF-κB cascade, leading to inflammation.

It has been reported that the transcription factor PPAR plays a role in controlling inflammation (*30*), indicating that negative cross-talk between NF-κB and PPAR may interfere with the trans-activation capacity of NF-κB, and underlies the anti-inflammatory effects of PPAR (*31*). Moreover, activators for PPARγ inhibit age-related NF-κB activation (*32*) in that activation of PPAR decreases soluble IL-6 levels by repressing p65 nuclear translocation. Interestingly, 3-MCP could be a PPAR agonist by up-regulating PPARα and -γ activity in endothelial cells (unpublished work).

Although rats fed soy protein look healthy without showing any sign of demise throughout the experiments, we need to carry out a histological study indicating the effect of 3-MCP on age-related structural changes in kidneys.

In conclusion, our study shows that 3-MCP treatments significantly reduced pro-inflammatory gene expression, including COX-2 and iNOS via a NF-κB signaling cascade in rat kidneys in vivo. Thus, the suppression of pro-inflammatory gene expression through modulation of the NF-κB signaling pathway by 3-MCP provides the molecular basis for its anti-inflammatory effects.

ABBREVIATIONS USED

3-MCP, 3-methyl-1,2-cyclopentanedione; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; VCAM-1, vascular cell adhesion molecule-1; MCP-1, chemokines monocyte chemoattractant protein-1; IL-6, interleukin-6.

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