Free Radical Biology and Medicine 75 (2014) 105–120

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Carotenoid derivatives inhibit nuclear factor kappa B activity in bone and cancer cells by targeting key thiol groups

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ARTICLE INFO

Article history:
Received 8 February 2014
Received in revised form
5 July 2014
Accepted 19 July 2014
Available online 29 July 2014

ABSTRACT

Aberrant activation of the nuclear factor kappa B (NFkB) transcription system contributes to cancer progression, and has a harmful effect on bone health. Several major components of the NFkB pathway such as IkB Kinase (IKK) and the NFkB subunits contain cysteine residues that are critical for their activity. The interaction of electrophiles with these cysteine residues results in NFkB inhibition. Carotenoids, hydrophobic plant pigments, are devoid of electrophilic groups, and we have previously demonstrated that carotenoid derivatives, but not the native compounds activate the Nrf2 transcription system. The aim of the current study was to examine whether carotenoid derivatives inhibit NFkB, and, if so, to determine the molecular mechanism underpinning the inhibitory action. We report in the present study that a mixture of oxidized derivatives, prepared by ethanol extraction from partially oxidized lycopene preparation, inhibited NFkB reporter gene activity. In contrast, the intact carotenoid was inactive. A series of synthetic dialdehyde carotenoid derivatives inhibited reporter activity as well as several stages of the NFkB pathway in both cancer and bone cells. The activity of the carotenoid derivatives depended on the reactivity of the electrophilic groups in reactions such as Michael addition to sulfhydryl groups of proteins. Specifically, carotenoid derivatives directly interacted with two key proteins of the NFkB pathway: the IKKβ and the p65 subunit. Direct interaction with IKKβ was found in an in vitro kinase assay with a recombinant enzyme. The inhibition by carotenoid derivatives of p65 transcriptional activity was observed in a reporter gene assay performed in the presence of excess p65. This inhibition action resulted, at least in part, from direct interaction of the carotenoid derivative with p65 leading to reduced binding of the protein to DNA as evidenced by electrophoretic mobility shift assay (EMSA) experiments. Importantly, we found by using mutation in key cysteine residues of both p65 and IKK that specific thiol groups are essential for NFkB inhibition by carotenoid derivatives. In conclusion, we propose that electrophilic carotenoid derivatives contribute to cancer prevention as well as bone health maintenance via the inhibition of the NFkB transcription system. Pivotal thiol groups of both IKK and p65 play a key role in this process.

Introduction

Aberrant constitutive activation of the nuclear factor kappa B (NFkB) transcription system plays a key role in inflammatory processes and therefore affects chronic diseases such as osteoporosis and cancer [1–6]. It is well documented that NFkB activation increases proinflammatory cytokine production and cancer cell proliferation, decreases apoptosis, and promotes tumor metastasis, all of which lead to cancer progression [1]. A prevalent view is that NFkB is a pivotal link between inflammation and cancer [6]. Indeed, constitutive NFkB activation has been observed in many human cancers. NFkB-dependent transcription is induced by a variety of stimuli including proinflammatory cytokines (e.g., tumor necrosis factor α (TNFα), IL-1β), oxidative stress, and UV radiation. The resultant activation of proinflammatory genes by NFkB thus creates a vicious positive feedback reinforcing NFkB proinflammatory, pro-tumorigenic action. Consequently, inhibition of NFKB is considered a promising therapeutic approach for blocking tumor growth or sensitizing tumor cells to more conventional therapies, such as chemotherapy.

Abbreviations: BCO1, 15,15′-β-carotene oxygenase 1; BCO2, β,β-carotene-9,10-oxygenase 2; DMSO, dimethyl sulfoxide; DTT, dithiotreitol; EMSA, electrophoretic mobility shift assay; EpRE/ARE, electrophile/antioxidant response element; IkB, NF-kB inhibitory protein; IKKβ, IkB kinase β; MCP-1, monocyte chemoattractant protein 1; NAC, *N*-acetyl-L-cysteine; NFkB, nuclear factor-kappa B; NQO1, NAD(P)H: quinone oxidoreductase; Nrf2, nuclear factor E2-related factor 2; SEAP, secreted alkaline phosphatase; THF, tetrahydrofuran; TNFα, tumor necrosis factor α; WT, wild type; 10,10′, 10,10′-diapocarotene-10,10′-dial

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http://dx.doi.org/10.1016/j.freeradbiomed.2014.07.024
0891-5849/

Deviant NFkB regulation was found to be involved in impaired bone formation in several inflammatory bone diseases, such as osteoporosis. Bone is a very dynamic organ constantly being remodeled in a process whereby osteoblasts are responsible for bone formation and osteoclasts for its resorption. Studies pertaining to the role of NFkB in the skeleton are focused mainly on the pivotal role of this transcription system in differentiation and activity of osteoclast bone-resorbing cells [5]. However, only recently the inhibitory effect of NFkB on osteoblastic bone formation was recognized and led to the conception that inhibition of the NFkB pathway in bone tissue could contribute not only to reduced bone loss but also to increased bone formation [2].

The NFkB family is composed of five structurally related transcription factors: p65 (RelA), p50, p52, c-Rel, and RelB. These proteins share a Rel homology domain in their N-terminus, important for the formation of homo- or heterodimers. The p65/p50 factor is the most studied heterodimer of the canonical NFkB pathway. The tumor promoting role of the p65/p50 heterodimer is well established [7]; moreover, the p65 subunit was recently shown to be involved in the inhibition of osteoblastic bone formation [2]. Under basal conditions, the NFkB subunits are confined to the cytoplasm by the inhibitory protein IkB. Upon activation of the system, IkB is phosphorylated by the IkB kinase (IKKβ), leading to ubiquitination and proteosomal degradation of the inhibitory IkB and allowing the nuclear translocation of the free NFkB dimers [8].The NFkB dimers then bind to the consensus sequences in the DNA and regulate the expression of proinflammatory proteins.

NFkB activity is tightly regulated by a number of different mechanisms. For example, it is well documented that NFkB is a redox-regulated transcription system [9]. Many natural and synthetic antioxidants inhibit NFkB activity, while oxidants such as H_2O_2 activate transcription [10–16]. Interestingly, several protein components of the NFkB pathway contain redox-sensitive cysteine residues that are surface exposed and thereby may undergo direct modification by various inhibitors. For example, cysteine 179 in the activation loop of IKKβ is responsible for redox regulation of NFkB activity. Similarly, cysteine 38 in the DNA binding domain of the p65 subunit was found to be a target residue for thiol-modifying agents [14,15]. Indeed, many chemopreventive agents, such as the sesquiterpene lactone parthenolide from the medicinal Herb feverfew [11,13], have been found to target these exposed cysteine residues by direct alkylation, thereby suppressing aberrant NFkB activation.

The use of natural NFkB inhibitors from the diet seems to provide a promising approach for prevention of various inflammatory diseases. The polyphenol curcumin, a component of the Indian culinary spice turmeric used in traditional medicine for many years, has been studied intensively in this regard over the past decade [10]. Nonetheless, components with anti-inflammatory properties that can be regularly added to the prevailing Western style diet are desirable. The beneficial effect of a diet rich in fruits and vegetables has long been appreciated [17–20]. Carotenoids, major components of such a diet, have been suggested to contribute to this favorable effect. More specifically, carotenoids such as lycopene, the red pigment of tomato, were found to have a cancer preventive effect [21–23] as well as to contribute to bone health maintenance [24,25]. However, the molecular mechanisms underpinning the beneficial effects of carotenoids are not fully elucidated. It has been widely accepted that carotenoids can function as potent antioxidants, and this is clearly a major mechanism of their action. In addition, carotenoids modulate various signaling pathways such as the estrogenic signal [26], the IGF-I pathway [27–30], and the redox-sensitive transcription systems dubbed the activator protein 1 (AP-1) [27] and the electophile/antioxidant response element (EpRE/ARE) [31–33].

In recent years, the anti-inflammatory effect of carotenoids was demonstrated in several in vitro and in vivo experimental systems [34–40]. Pretreatment with lycopene attenuated the proinflammatory phenotype as shown by the inhibition of NFkB activity, particularly in combination with other phytonutrients [34,39,40]. Several studies indicate that carotenoids inhibit NFkB in experimental cancer models [35,36]. For example, dietary supplementation of lycopene or a tomato extract inhibited nonalcoholic steatohepatitis-promoted hepatocarcinogenesis in rats.This anticancer effect was accompanied by a reduction in the p65 nuclear level and in mRNA levels of proinflammatory cytokines [37]. Altogether, although there is evidence for carotenoid inhibition of NFkB in cancerous as well as in non cancer models, the molecular mechanism of this effect was not thoroughly investigated.

In the current work we focused on the inhibition of the NFkB transcription system in order to clarify mechanisms contributing to the anti-inflammatory, anticancer activity of carotenoids. A key question that we addressed was how the carotenoids and their derivatives affect protein components of the NFkB system. Many modulators of both the NFkB and the EpRE/ARE transcription systems are electrophiles that contain α,β-unsaturated carbonyl groups, which are prone to react with nucleophiles, in a Michael-type addition, especially with cysteine sulfhydryl groups. However, hydrophobic carotenoids such as lycopene do not possess electrophilic groups and therefore it is improbable that they directly modify cysteine thiols. Interestingly, in vitro and in vivo oxidation of carotenoids leads to the formation of mono- and dialdehyde derivatives called apocarotenals and diapocarotene-dials [41,42]. Two homologous genes exist that encode enzymes which oxidize and cleave carotenoids [42]: β,β-carotene-15,15′ oxygenase 1 (BCO1) catalyzes the central cleavage leading to retinal production from provitamin A carotenoids, whereas the carotenoid-oxygenase, β-carotene 9′-10′-oxygenase 2 (BCO2), catalyzes the asymmetric cleavage of carotenoids to apocarotenoids. The expression of these carotenoid-cleaving enzymes in a large number of cells of disparate lineages suggests that the carotenoid derivatives have an important physiological role.

We have recently shown that electrophilic apocarotenoid derivatives activate the EpRE/ARE transcription system, while the intact carotenoids are ineffective in this respect [43]. The reactivity of the carbon–carbon double bond adjacent to the terminal aldehyde group was found to play a determining role in EpRE/ARE activation. This reactivity reflects the tendency of the conjugated double bond to perform Michael addition to nucleophiles such as thiol groups of proteins. Therefore, EpRE/ARE activation by carotenoid derivatives could be mediated by direct modification of redox-sensitive thiols in Kelch-like ECH-associated protein 1 (keap1), an inhibitory cytosolic protein that prevents translocation of the key transcription factor Nrf2 to the cell nucleus and the induction of target genes. Due to the involvement of redox-sensitive thiols in the regulation of both transcriptional systems, we hypothesized that carotenoid derivatives are the active mediators not only in EpRE/ARE activation but also in the inhibition of NFkB, and searched for putative molecular mechanisms underpinning this inhibitory action. The NFkB transcription system was therefore selected to pursue the possibility that carotenoid derivatives directly interact with critical, redox sensor cysteine residues. In the current work we used T47D mammary cancer cells and bone osteoblast-like cells—human HOS and mouse MC3T3-E1 cells. We provide evidence that carotenoid derivatives exert their inhibitory effect by acting on two distinct steps of the NFkB pathways: IKKβ kinase activity and p65 binding to DNA.

Materials and methods

Materials

Crystalline lycopene preparations, purified from tomato extract (> 97%), were a gift of LycoRed Natural Products Industries (Beer