The bone anabolic carotenoids *p*-hydroxycinnamic acid and ß-cryptoxanthin antagonize NF-κB activation in MC3T3 preosteoblasts

MASAYOSHI YAMAGUCHI and M. NEALE WEITZMANN

Division of Endocrinology, Metabolism and Lipids, Department of Medicine, Emory University, Atlanta, GA 30322-0001, USA

Received December 11, 2008; Accepted February 17, 2009

DOI: 10.3892/mmr_00000150

Abstract. The carotene *p*-hydroxycinnamic acid and the xanthophyll ß-cryptoxanthin are members of the carotenoid family of plant-derived pigments, which are endowed with anti-osteoporotic properties *in vivo*. *p*-Hydroxycinnamic acid and ß-cryptoxanthin have been demonstrated to stimulate osteoblastic bone formation while simultaneously repressing osteoclastic bone resorption *in vitro*. However, their mechanisms of action remain poorly elucidated. It is well established that the NF-κB signal transduction pathway plays a critical role in osteoclast differentiation. Moreover, we recently demonstrated that NF-κB activity potently antagonizes osteoblastic differentiation and mineralization *in vitro*. In this study, we used transient transfection assays of a NF-κB luciferase reporter to demonstrate that *p*-hydroxycinnamic acid and ß-cryptoxanthin antagonize NF-κB activation in MC3T3 preosteoblastic cells. The data obtained suggest that NF-κB may be a common molecular target by which several bone active agents, including carotenoids, promote osteoblastic bone formation.

Introduction

Bone homeostasis is maintained by a delicate balance between osteoblastic bone formation and osteoblastic bone resorption. Numerous pathological processes have the capacity to disrupt this equilibrium, leading to conditions where the rate of bone resorption surpasses the rate of bone formation. The result is osteoporosis, a devastating bone disease that is widely recognized as a significant public health threat. Post-menopausal osteoporosis is the archetypal osteoporotic condition in women who have passed through menopause, and leads to bone destruction through complex and diverse metabolic and biochemical changes (1).

Pharmacologic and nutritional factors are associated with reduced postmenopausal bone loss, as chemical compounds present in certain foods appear to modulate bone turnover. Isoflavones such as genistein, found in high concentrations in soybeans, have been extensively studied for their estrogen-like properties and their capacity to stimulate bone formation (2) and suppress bone resorption (3,4). However, another group of bioactive phytochemicals, known as carotenoids, also appears to have bone active properties. Found predominantly in fruit and vegetables, carotenoids comprise both carotenes, such as *p*-hydroxycinnamic acid (HCA), and xanthophylls, such as ß-cryptoxanthin (CRP), lutein, lycopene and ß-carotene. Of note, CRP and HCA have anabolic effects on osteoblastic bone formation (5,6) and suppressive effects on osteoclastic bone resorption *in vitro* (5,7). Furthermore, oral administration of CRP (8) and HCA (9) ameliorated the bone loss induced by ovariectomy in rats, in an animal model of postmenopausal osteoporosis. However, the molecular mechanism(s) by which these factors regulate bone homeostasis remain to be elucidated.

The NF-κB signal transduction pathway is critical for osteoclast development and function (10,11). For example, the double knockout of p50 and p52 NF-κB subunits in mice leads to osteopetrosis due to a severe defect in osteoclast differentiation (12). Furthermore, suppression of NF-κB signaling *in vitro* blocks osteoclast activation (13). Recently, we demonstrated the utility of pharmacological NF-κB suppression in inhibiting osteoclastic bone loss in ovariectomized mice (14). In a study on multiple myeloma-induced bone loss *in vitro* (15), and in an animal model of rheumatoid arthritis *in vivo* (16), it was similarly demonstrated that NF-κB modulation is an effective form of therapy.

We recently reported that NF-κB signaling represses basal osteoblast differentiation and mineralization in MC3T3 cells and antagonizes TGFß and BMP-2-mediated MC3T3 mineralization by downregulating SMAD activation (17). Other studies have found that NF-κB signaling antagonizes Smad activation in Saos2 osteosarcoma cells by a mechanism involving the induction of inhibitory Smad7 (18).

In this study, we demonstrated that HCA and CRP have the capacity to suppress NF-κB activity in MC3T3 preosteoblastic cells. HCA, but not CRP, was further found to repress TNFα-

Correspondence to: Dr Masayoshi Yamaguchi, Division of Endocrinology and Metabolism and Lipids, Department of Medicine, Emory University School of Medicine, 101 Woodruff Circle, 1329 WMRB, Atlanta, GA 30322-0001, USA
E-mail: yamamasa1155@yahoo.co.jp

Key words: p-hydroxycinnamic acid, ß-cryptoxanthin, NF-κB promoter, osteoblast, bone formation, osteoporosis

induced NF-κB signaling. These data suggest that the antagonism of NF-κB signaling may be a common mechanism by which anti-osteoporotic carotenoids promote bone formation or offer protection from the inflammatory inhibitors of bone formation, such as TNFα.

Materials and methods

Materials. α-minimal essential medium (α-MEM) and antibiotics (penicillin and streptomycin) were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). ß-cryptoxanthin was obtained from Extrasynthase (Lyon-Nord, France). *p*-Hydroxycinnamic acid was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were obtained from the Sigma Chemical Corporation (St. Louis, MO) unless otherwise stated.

Cell culture. The preosteoblastic cell line MC3T3-E1, clone 14 (MC3T3) was purchased from the American Type Culture Collection (Manassas, VA) and has previously been described in detail (19). Cells were cultured at 37˚C in a humidified 5% CO_2 incubator in plastic dishes containing α-MEM supplemented with 10% FBS, with 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were passaged every 3 days using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS).

NF-κB reporter constructs and luciferase assays. The NF-κB responsive reporter pNF-κB-LUC was purchased from BD Biosciences. Reporter plasmids (pNF-κB-LUC) or empty vector controls (pGL3-Basic) were transfected into MC3T3 cells using Lipofectamine 2000 reagent (Invitrogen) as previously described (17). Briefly, MC3T3 cells ($1x10^5$ cells/well) were cultured in 96-well white opaque luminometer plates (Corning Costar, Lowell, MA) for 24 h in α-MEM. Subsequently, the cells were changed to α-MEM without FBS and antibiotics, and transfected with pNF-κB-LUC reporter plasmid or empty vector. After 5 h, the medium was changed to α-MEM containing antibiotics, with or without 10% FBS. Wells were treated with vehicle, HCA (10^{-7} to 10^{-5} M) or CRP (10^{-6} or 10^{-5} M), and/or TNF-α (10 ng/ml), for 24 h. Luciferase activity was measured on a microplate luminometer (Turner Designs, Sunnyvale, CA, USA) following cell lysis in Passive Lysis Buffer (Promega, Madison, WI). Equal transfection efficiency was validated for all plasmids using Renila luciferase reporter plasmid pRL-SV40 (Promega).

Statistical analysis. Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software). Multiple comparisons were performed by one-way ANOVA with the Tukey-Kramer post test. $P<0.05$ was considered statistically significant. Data are presented as the mean ± SD of five replicate wells, and are representative of two or more independent experiments.

Results

HCA and CRP suppress basal NF-κB activity in MC3T3 cells. HCA and CRP are reported to stimulate bone formation *in vivo* and mineralization *in vitro*. However, the mechanisms by which they achieve this action are unknown. We recently reported that NF-κB antagonists stimulate osteoblast differentiation and mineralization (17). Based on this finding, in the present study we investigated the effect of HCA and CRP on basal NF-κB activity in MC3T3 preosteoblastic cells using pNF-κB-LUC. In the absence of FBS, it was found that HCA (Fig. 1A) and CRP (Fig. 1B) dose-dependently (10^{-7} to 10^{-5} M) suppressed basal MC3T3 NF-κB activity.

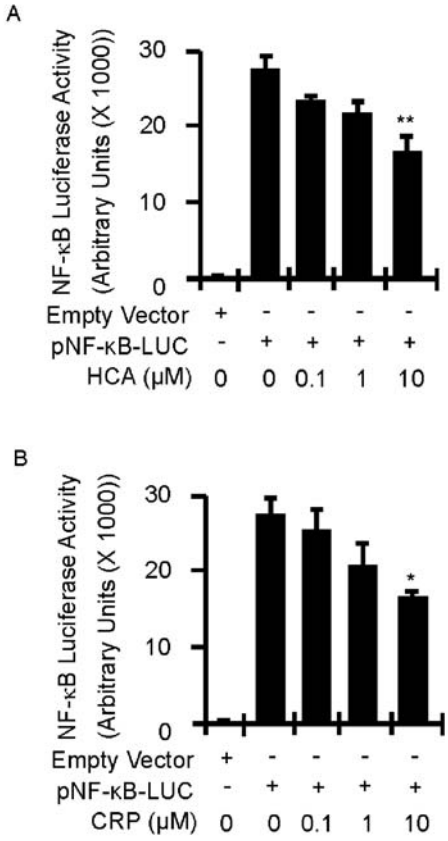

Figure 1. HCA and CRP suppress basal NF-κB activity in MC3T3 preosteoblastic cells. MC3T3 cells were transfected with the NF-κB responsive reporter pNF-κB-LUC and treated with a dose range of HCA or CRP (0.1, 1 or 10 μM) in the absence of FBS. Luciferase activity was quantitated 24 h later. Each data set represents the mean ± SD of five replicate assays. Data are representative of two or more independent experiments. $^{*}p<0.05$, $^{**}p<0.01$ with respect to the no HCA or CRP control.

The addition of FBS blocks HCA and CRP antagonism of NF-κB activity in MC3T3 cells. FBS contains innumerable poorly characterized growth factors and cytokines, as well as other bioactive molecules, that can dramatically impact cellular function. When MC3T3 cells were cultured in the presence of FBS, the unforeseen result was that basal NF-κB activity was ~2.5-fold lower with FBS than in its absence, suggesting the presence of an endogenous inhibitor of NF-κB activation. Interestingly, HCA (Fig. 2A) and CRP (Fig. 2B) had no significant additional suppressive effects on NF-κB activity in the presence of FBS.

HCA suppresses TNFα-induced NF-κB activity in MC3T3 cells. We recently reported that the endogenous serum levels

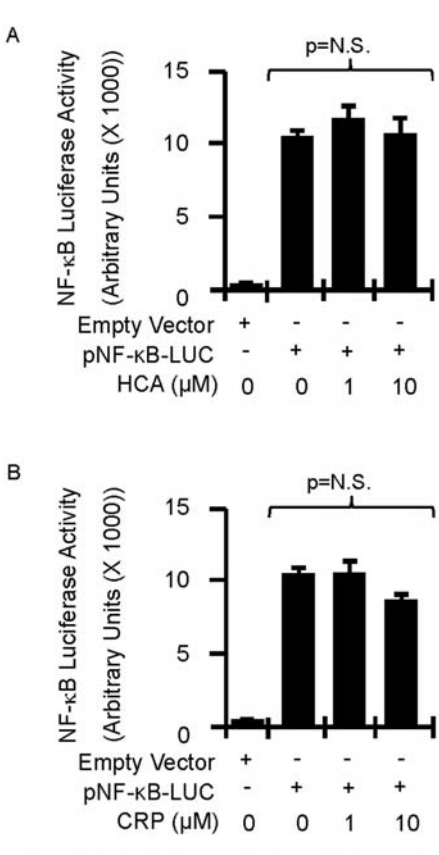

Figure 2. In the presence of FBS, HCA and CRP fail to suppress basal NF-κB activity in MC3T3 preosteoblastic cells. MC3T3 cells were transfected with the NF-κB responsive reporter pNFκB-LUC and treated with HCA or CRP (1 or 10 μM) in the presence of FBS (10%). Luciferase activity was quantitated 24 h later. Each data set represents the mean ± SD of five replicate assays. Data are representative of two or more independent experiments. p = N.S. (not significant) with respect to the no HCA or CRP control.

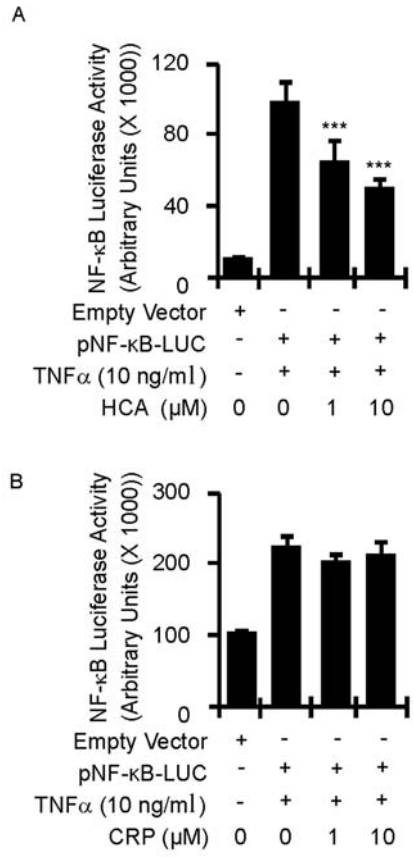

Figure 3. HCA, but not CRP, suppresses TNFα-induced NF-κB activity in MC3T3 preosteoblastic cells. MC3T3 cells were transfected with the NF-κB responsive reporter pNFκB-LUC and treated with HCA or CRP (1 or 10 μM) in the presence of FBS (10%) and in the presence or absence of TNFα (10 ng/ml). Luciferase activity was quantitated 24 h later. Each data set represents the mean ± SD of five replicate assays. Data are representative of two or more independent experiments. ***p<0.001 with respect to the no HCA control.

of TNFα are a potent suppressor of bone formation and lower attainable peak bone mass *in vivo* under basal conditions (17). TNFα is also a well-known inflammatory cytokine that promotes bone destruction under pathological conditions such as estrogen deficiency, as well as in rheumatoid arthritis, by stimulating osteoclastic bone resorption and repressing bone formation (1,20). TNFα is a potent inhibitor of osteoblast differentiation and mineralization *in vitro,* an effect predominantly mediated through NF-κB activation (17,21). Consequently, we examined the effect of HCA and CRP on TNFα-induced NF-κB activity. It was found that HCA potently and dose-dependently (10^{-6} to 10^{-5} M) inhibited TNFα-induced NF-κB activation (Fig. 3A). Interestingly, CRP had no significant effect on TNFα-induced NF-κB activation (Fig. 3B), suggesting a different site of action in the NF-κB pathway than that of HCA.

Discussion

It has been established that the carotenoids CRP and HCA mediate anti-osteoporotic properties *in vivo* (8,9). These two factors have the capacity to stimulate bone formation *in vivo* and to promote osteoblastic differentiation and mineralization *in vitro* (5-7). However, the mechanisms by which these anabolic actions are achieved are unclear. In this study, we demonstrated that the NF-κB signal transduction pathway, which antagonizes osteoblastic differentiation and function, is significantly suppressed by the two factors, thereby providing a potential partial explanation for their anabolic actions.

TNFα is an inflammatory cytokine that antagonizes bone formation *in vivo* and osteoblastic differentiation *in vitro*. These effects are larely mediated through NF-κB signaling. Inflammatory levels of TNFα are known to impact bone formation. However, we recently reported that basal TNFα levels *in vivo* also dramatically lower the basal bone formation rate. We therefore speculated that HCA and CRP may promote the accumulation of basal bone mass and forestall bone loss during osteoporotic states, in part by antagonizing TNFα-induced NF-κB. Interestingly, only the carotenoid HCA was found to significantly reduce TNFα-induced NF-κB activation. In contrast, CRP, which was capable of regulating basal NF-κB activity, had no effect on TNFα-induced NF-κB. The reason for the difference in response between the two carotenoids remains unclear. However, given the diverse nature of the NF-κB signaling pathway, it is possible that these agents regulate different NF-κB species or pathways. The five members of the mammalian NF-κB family, RelA/p65, RelB, c-Rel, NF-κB1/p50 and NF-κB2/p52, are activated through one of

two specific pathways: the canonical NF-κB pathway or an alternative pathway. In the canonical pathway, activation of the inhibitor of IκB kinase (IKK) complex leads to the phosphorylation of NF-κB-associated IκBα. This catalyzes IκBα ubiquitination and proteasomal degradation and, in the process, releases active NF-κB dimers that translocate to the nucleus and enhance the transcription of target genes. In the alternative NF-κB pathway, NF-κB-inducing kinase (NIK) and IKKα target p100 for proteolytic processing, thereby releasing active RelB-containing dimers (22). The differential regulation of the two pathways by HCA and CRP provides a possible explanation for the differences observed in TNFα-induced NF-κB activation.

FBS contains a large number of poorly characterized growth factors and cytokines, proteins, and bioactive molecules that can dramatically alter cell function. Interestingly, our data demonstrate that factors contained in FBS have a suppressive effect on basal NF-κB activation. The relevance of this phenomenon *in vivo* remains unclear. Moreover, whether this factor is present in neonatal or adult serum is presently unknown. However, it worth noting that NF-κB is a significant initiator of inflammatory and immunological responses. During pregnancy, tolerance is essential to prevent fetal rejection. However, the mechanisms for maintaining tolerance are multifactorial and poorly understood (23). Nonetheless, it can be speculated that fetal production of an immunological suppressor that antagonizes maternal host NF-κB activation could play a role in desensitizing the maternal immune response to fetal antigens.

In conclusion, our data suggest that NF-κB antagonism may be a common mechanism by which several diverse bone anabolic agents act to stimulate osteoblast differentiation and activity. Some of these agents may also act to suppress TNFα-induced NF-κB, while others may target basal NF-κB levels. The identification of further novel NF-κB antagonists may be an important strategy for the future development of bone anabolic agents.

Acknowledgements

This study was supported in part by a grant from the University Research Committee of Emory University. M.N.W. is supported in part by grants from NIAMS (AR053607 and AR056090).

References

1. Weitzmann MN and Pacifici R: Estrogen deficiency and bone loss: an inflammatory tale. J Clin Invest 116: 1186-1194, 2006.
2. Sugimoto E and Yamaguchi M: Anabolic effect of genistein in osteoblastic MC3T3-E1 cells. Int J Mol Med 5: 515-520, 2000.
3. Yamaguchi M and Gao YH: Inhibitory effect of genistein on bone resorption in tissue culture. Biochem Pharmacol 55: 71-76, 1998.
4. Gao YH and Yamaguchi M: Suppressive effect of genistein on rat bone osteoclasts: involvement of protein kinase inhibition and protein tyrosine phosphatase activation. Int J Mol Med 5: 261-267, 2000.
5. Uchiyama S and Yamaguchi M: ß-cryptoxanthin stimulates cell differentiation and mineralization in osteoblastic MC3T3-E1 cells. J Cell Biochem 95: 1224-1234, 2005.
6. Lai YL and Yamaguchi M: Phytocomponent p-hydroxycinnamic acid stimulates bone formation and inhibits bone resorption in rat femoral tissues in vitro. Mol Cell Biochem 292: 45-52, 2006.
7. Lai YL and Yamaguchi M: Phytocomponent p-hydroxycinnamic acid inhibits osteoclast-like cell formation in mouse bone marrow cultures. Int J Mol Med 19: 123-128, 2007.
8. Uchiyama S and Yamaguchi M: Oral administration of ß-cryptoxanthin prevents bone loss in ovariectomized rats. Int J Mol Med 17: 15-20, 2006.
9. Yamaguchi M, Lai YL, Uchiyama S and Nakagawa T: Oral administration of phytocomponent p-hydroxycinnamic acid prevents bone loss in ovariectomized rats. Mol Cell Biochem 311: 31-36, 2008.
10. Franzoso G, Carlson L, Xing L, *et al*: Requirement for NF-kappaB in osteoclast and B-cell development. Genes Dev 11: 3482-3496, 1997.
11. Boyce BF, Xing L, Franzoso G and Siebenlist U: Required and nonessential functions of nuclear factor-kappa B in bone cells. Bone 25: 137-139, 1999.
12. Iotsova V, Caamaño J, Loy J, Yang Y, Lewin A and Bravo R: Osteopetrosis in mice lacking NF-kappaB1 and NF-kappaB2. Nat Med 3: 1285-1289, 1997.
13. Hall TJ, Schaeublin M, Jeker H, Fuller K and Chambers TJ: The role of reactive oxygen intermediates in osteoclastic bone resorption. Biochem Biophys Res Commun 207: 280-287, 1995.
14. Strait K, Li Y, Dillehay DL and Weitzmann MN: Suppression of NF-κB activation blocks osteoclastic bone resorption during estrogen deficiency. Int J Mol Med 21: 521-525, 2008.
15. Feng R, Anderson G, Xiao G, *et al*: SDX-308, a nonsteroidal anti-inflammatory agent, inhibits NF-kappaB activity, resulting in strong inhibition of osteoclast formation/activity and multiple myeloma cell growth. Blood 109: 2130-2138, 2007.
16. Dai S, Hirayama T, Abbas S and Abu-Amer Y: The IkappaB kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks osteoclastogenesis and bone erosion in inflammatory arthritis. J Biol Chem 279: 37219-37222, 2004.
17. Li Y, Li A, Strait K, Zhang H, Nanes MS and Weitzmann MN: Endogenous TNFalpha lowers maximum peak bone mass and inhibits osteoblastic Smad activation, through NF-kappaB. J Bone Miner Res 22: 646-655, 2007.
18. Eliseev RA, Schwarz EM, Zuscik MJ, O'Keefe RJ, Drissi H and Rosier RN: Smad7 mediates inhibition of Saos2 osteosarcoma cell differentiation by NFkappaB. Exp Cell Res 312: 40-50, 2006.
19. Wang D, Christensen K, Chawla K, Xiao G, Krebsbach PH and Franceschi RT: Isolation and characterization of MC3T3-E1 preosteoblast subclones with distinct in vitro and in vivo differentiation/mineralization potential. J Bone Miner Res 14: 893-903, 1999.
20. Kimble RB, Matayoshi AB, Vannice JL, Kung VT, Williams C and Pacifici R: Simultaneous block of interleukin-1 and tumor necrosis factor is required to completely prevent bone loss in the early postovariectomy period. Endocrinology 136: 3054-3061, 1995.
21. Nanes MS: Tumor necrosis factor-alpha: molecular and cellular mechanisms in skeletal pathology. Gene 321: 1-15, 2003.
22. Vaira S, Alhawagri M, Anwisye I, Kitaura H, Faccio R and Novack DV: RelA/p65 promotes osteoclast differentiation by blocking a RANKL-induced apoptotic JNK pathway in mice. J Clin Invest 118: 2088-2097, 2008.
23. Mellor AL and Munn DH: Immunology at the maternal-fetal interface: lessons for T cell tolerance and suppression. Annu Rev Immunol 18: 367-391, 2000.