Suppression of cyclooxygenase-2 gene transcription by humulon of beer hop extract studied with reference to glucocorticoid

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Abstract In murine osteoblastic MC3T3-E1 cells which produced prostaglandin E2 as a bone resorption factor, the cyclooxygenase-2 induction by tumor necrosis factor α (TNFα) was suppressed by dexamethasone with an IC_{50} of 1 nM. Humulon isolated from hop extract for beer brewing was reported previously as an inhibitor of bone resorption [Tobe, H. et al. (1997) Biosci. Biotech. Biochem. 61, 158–159]. We showed that the compound suppressed the TNFα-dependent cyclooxygenase-2 induction with an IC_{50} of as low as about 30 nM as demonstrated experimentally by catalytic activity assay, Northern blot analysis and promoter analysis. Reporter gene experiments suggested that humulon blocked the cyclooxygenase-2 expression mediated by NFκB and NF-IL6, but the intracellular glucocorticoid receptor was not involved. The catalytic activity of cyclooxygenase-2 was inhibited by humulon with an IC_{50} of as high as 1.6 μM. These results showed that humulon suppressed cyclooxygenase-2 induction at the step of transcription.

Key words: Cyclooxygenase-2; Glucocorticoid; Humulon; Transcriptional regulation; Osteoblast

1. Introduction

It is now well established that there are two isozymes of prostaglandin (PG)-forming cyclooxygenase; constitutive cyclooxygenase-1 and inducible cyclooxygenase-2 [1,2]. MC3T3-E1 cell cloned from newborn mouse calvaria is an osteogenic cell line which differentiates into an osteoblast [3,4]. Much earlier our extensive studies demonstrated the production of E2 as a major arachidonate metabolite in this cell line [5] and the induction of cyclooxygenase by various bioregulators [5–9]. Later the induced enzyme in MC3T3-E1 cells was identified as cyclooxygenase-2 [8–10]. Previously humulon ((*R*)-3,5,6-trihydroxy-4,6-bis(3-methyl-2-butenyl)-2-(3-methyl-1-oxobutyl)-2,4-cyclohexadien-1-one) (Fig. 1) isolated from hop (*Humulus lupulus* L.) extract was found as an inhibitor of bone resorption (IC_{50}: 5.9 nM) [11]. We noted this activity of humulon, and were interested in a possible inhibitory or suppressive effect of humulon on cyclooxygenase-2 leading to a decreased production of PGE2 as a bone resorption factor [12].

2. Materials and methods

2.1. Materials

Humulon was purified from hop paste as described previously [11], and the purified material was dissolved in dimethyl sulfoxide at a concentration of 10 mM. MC3T3-E1 cells were cultured as described previously [5]. Confluent cultures were usually obtained on the 4th day. Then, the medium was changed to α-MEM (Life Technologies, Gaithersburg, MD, USA) supplemented with 2% newborn bovine serum (Irvine Scientific, Santa Ana, CA, USA) and a compound to be tested. At indicated time intervals, the cells were scraped from dishes and subjected to various assays described below.

2.2. Assays of cyclooxygenase-1 and -2 activities

For cyclooxygenase-1 preparation, lyophilized powder of sheep seminal vesicle microsomes (Eldan Technologies, Jerusalem, Israel) was suspended in 20 mM phosphate buffer (pH 7.4) containing 5 mM tryptophan. For cyclooxygenase-2 preparation, 5×10^7 MC3T3-E1 cells were incubated with 100 nM phorbol 12-myristate 13-acetate and 100 nM A23187 for 9 h. The cells were subjected to sonic disruption, and centrifuged at $180\,000\times g$ for 50 min. The resultant pellet was collected as the microsomal fraction, and suspended in 1 ml of 20 mM Tris–HCl (pH 7.4) containing 5 mM tryptophan. The enzyme assay was performed as described previously [9].

2.3. Cyclooxygenase-2 induction

MC3T3-E1 cells (2.2×10^6 cells) were incubated with tumor necrosis factor α (TNFα) (10–20 ng/ml). For determination of PGE2 synthesis, aliquots were removed from the culture medium (1 ml) of 3×10^5 MC3T3-E1 cells, and subjected to radioimmunoassay for PGE2 as described previously [5]. For catalytic activity assay, the cells were scraped from the dishes, suspended in 200 μl of 20 mM Tris–HCl (pH 7.4) containing 5 mM tryptophan, and subjected to sonication. Aliquots (1–30 μl) of the sonicate were incubated with 10 μM [1-^{14}C]arachidonic acid (2.1 GBq/mmol, Amersham Pharmacia Biotech, Bucks, UK) for 2 min at 24°C in a 100-μl mixture containing 100 mM Tris–HCl (pH 8.0), 2 μM hematin, and 5 mM tryptophan. The reaction products were separated by thin-layer chromatography as described previously [9]. Total RNA was extracted from MC3T3-E1 cells using ISOGEN (Nippon Gene, Tokyo, Japan) and subjected to Northern blot analysis for cyclooxygenase-2 and β-actin mRNA as described previously [8].

2.4. Reporter gene assay

A DNA fragment (nucleotides −815 to +123) was prepared from the 5′-flanking region of the mouse cyclooxygenase-2 gene as described previously [9], and was inserted into the luciferase plasmid pGL3 Basic (Promega, Madison, WI, USA). The complementary oligonucleotide with a specific sequence of glucocorticoid response element (5′-AGAGGATCTGTACAGGATGTTCTAGAT-3′; consensus sequence of the glucocorticoid response element is underlined) was synthesized essentially by the same method as described previously [9]. The oligonucleotide of the glucocorticoid response element, NFκB response element [9] or NF-IL6 response element [9] was in-

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Abbreviations: PG, prostaglandin; TNFα, tumor necrosis factor α; LPS, lipopolysaccharide

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serted in front of the herpes simplex virus thymidine kinase gene [13] of the tk-pGL3 vector [14]. For the transfection of luciferase plasmid, 7.8×10^5 confluent cells were treated with pGL3 plasmid DNA (3 μg), standard β-galactosidase DNA (1.5 μg), and 13.5 μl of Trans IT™-LT1 (Takara, Tokyo, Japan) in 2.3 ml of serum-free α-MEM for 4 h at 37°C. After transfection the medium was changed to 5 ml of α-MEM with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD, USA), and the transfected cells were further incubated for 3 days. The medium was changed to α-MEM described above. After 12 h the cells were scraped from the dishes, and the luciferase assay was performed as described previously [9]. The results were normalized with β-galactosidase activity.

3. Results

We investigated the possibility that humulon suppressed the TNFα-mediated induction of cyclooxygenase-2. When MC3T3-E1 cells were incubated with 10 ng/ml TNFα in the presence of humulon for 12 h, the amount of released PGE2 decreased depending on the concentration of humulon with an IC_{50} of about 30 nM (Fig. 2A). As shown in Fig. 2C, the cyclooxygenase activity of the TNFα-treated cells was reduced by humulon dose-dependently. Furthermore, Northern blot analysis showed that the addition of humulon suppressed the TNFα-induced increase of cyclooxygenase-2 mRNA (Fig. 2E). For analysis of the promoter activity, a luciferase vector of cyclooxygenase-2 gene was constructed, and transfected to MC3T3-E1 cells by the lipofection method. The luciferase activity of the cell lysate was measured 12 h after the addition of 10 ng/ml TNFα in the presence of various concentrations of humulon (Fig. 2G). Since the same range of humulon concentration was required for the decrease in the PGE2 release, enzyme activity, mRNA level and promoter activity, we presumed that the induction of cyclooxygenase-2 by TNFα was inhibited at the step of transcription. Then, we examined whether or not humulon inhibited the catalytic activities of cyclooxygenases-1 and -2. As shown in Fig. 3, the cyclooxygenase-1 activity was hardly affected by humulon below 10 μM, whereas the cyclooxygenase-2 activity was inhibited with an IC_{50} value of about 1.6 μM. Thus, the catalytic activity of cyclooxygenase-2 was inhibited by humulon, but its IC_{50} was higher by about two orders of magnitude than the IC_{50} of the cyclooxygenase-2 suppression.

As reported preliminarily earlier [15], the TNFα-dependent cyclooxygenase-2 induction in MC3T3-E1 cells was suppressed by dexamethasone which is well known as a potent anti-inflammatory drug and a suppressor of cyclooxygenase-2 induction in most cells [1,2]. We investigated the effect of dexamethasone in MC3T3-E1 cells. When the cells were incubated with 20 ng/ml TNFα for 12 h, the amount of released PGE2 (Fig. 2B) and the cyclooxygenase-2 activity (Fig. 2D) decreased depending on the concentration of added dexamethasone (IC_{50}: 1 nM). As shown in Fig. 2F, the cyclooxygenase-2 mRNA level was also decreased in the presence of dexamethasone. The TNFα-dependent luciferase activity was also decreased by dexamethasone with an IC_{50} of about 1 nM (Fig. 2H). These data indicated that dexamethasone suppressed the cyclooxygenase-2 induction by reducing its mRNA level in MC3T3-E1 cells.

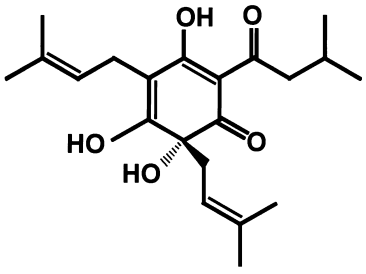

Fig. 1. Structure of humulon.

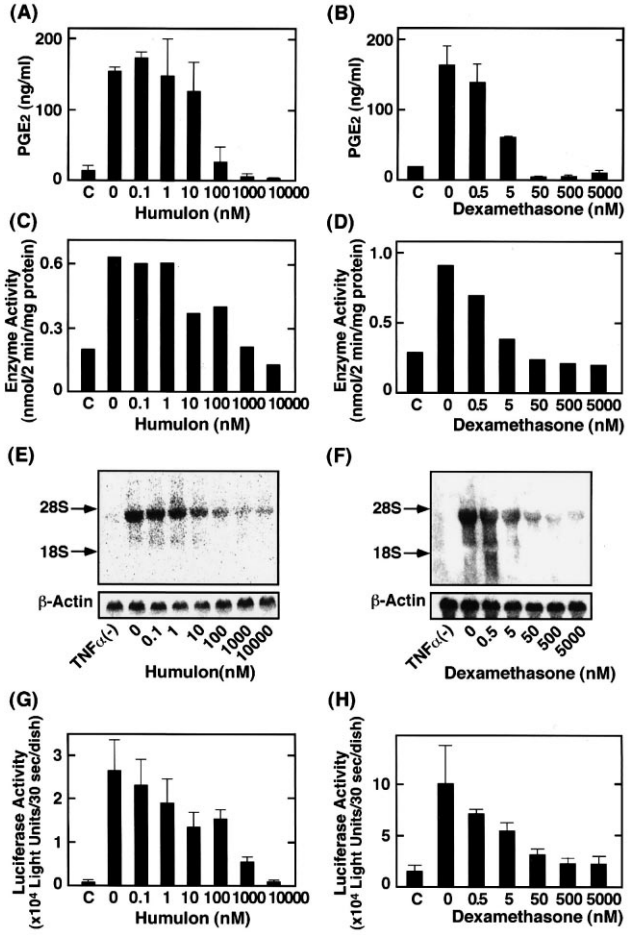

Fig. 2. Effects of humulon and dexamethasone on TNFα-mediated cyclooxygenase-2 induction. TNFα was present at a concentration of 10 ng/ml for humulon and 20 ng/ml for dexamethasone. Humulon (A, C, E and G) or dexamethasone (B, D, F and H) was added at various concentrations. For the PGE2 release assay, 3×10^5 MC3T3-E1 cells were incubated with TNFα for 12 h (A and B). For the cyclooxygenase-2 activity assay, 2.2×10^6 cells were incubated with TNFα for 12 h (C and D). For the cyclooxygenase-2 mRNA assay, 2.2×10^6 cells were incubated with TNFα for 2 h (E and F). Total RNA (10 μg) was applied to Northern blot analysis for cyclooxygenase-2 mRNA and β-actin mRNA. For the luciferase assay (G and H), 7.8×10^5 confluent cells were transfected with luciferase plasmid including promoter regions of mouse cyclooxygenase-2 gene. The cells were incubated with TNFα for 12 h. A, B, G and H are means ± S.D. of triplicate determinations. C, D, E and F are typical results of the experiments each repeated three times giving similar results.

Our previous work with MC3T3-E1 cells demonstrated the involvement of NFκB and NF-IL6 as transcription factors in the cyclooxygenase-2 induction by TNFα [9]. We investigated whether or not humulon and dexamethasone functioned with the aid of NFκB (nucleotides −401 to −393) and NF-IL6 (nucleotides −138 to −130) response elements. As shown in Fig. 4, we constructed a luciferase vector containing thymi-

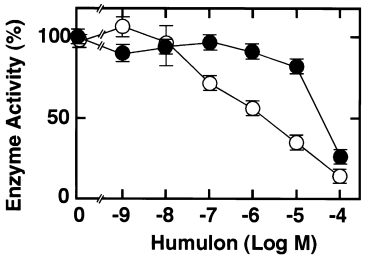

Fig. 3. Effects of humulon on cyclooxygenase-1 and -2 activities. The standard cyclooxygenase assays were performed with [1-^{14}C]-arachidonic acid (10 μM) incubated with the cyclooxygenase-1 of sheep seminal vesicle microsome (4.3 μg protein) (closed circle) or the cyclooxygenase-2 of MC3T3-E1 cell lysate (50 μg protein) stimulated by phorbol 12-myristate 13-acetate and A23187 (open circle). Humulon was added at various concentrations. Data are means ± S.D. of duplicate determinations.

dine kinase gene and NFκB or NF-IL6 response element. Each vector was transfected to MC3T3-E1 cells, and the luciferase activity was measured. Both humulon and dexamethasone decreased the luciferase activity induced by TNFα. Thus, the suppressive effect of humulon and dexamethasone on the cyclooxygenase-2 induction was attributable presumably to NFκB or NF-IL6 or both.

To investigate whether or not humulon bound to glucocorticoid receptor, we used RU486 as a glucocorticoid receptor antagonist [16]. The TNFα-dependent PGE2 production decreased by dexamethasone was reversed by RU486 dose-dependently (Fig. 5A). However, the decreased PGE2 production by humulon was not affected by RU486 (Fig. 5A). Then, we constructed a luciferase vector containing thymidine kinase gene and glucocorticoid response element (Fig. 5B). The vector was transfected to MC3T3-E1 cells, and the luciferase activity of the cell lysate was measured 12 h after the addition of TNFα plus dexamethasone or humulon. As shown in Fig. 5B, the addition of dexamethasone increased luciferase activity of MC3T3-E1 cells by 16–18 fold. Therefore, dexamethasone bound to the glucocorticoid receptor and then to the glucocorticoid response element. In contrast, when the transfected cells were incubated with humulon, the luciferase activity was not increased. These data suggested that the humulon-dependent suppression of cyclooxygenase-2 induction was not mediated by glucocorticoid receptor.

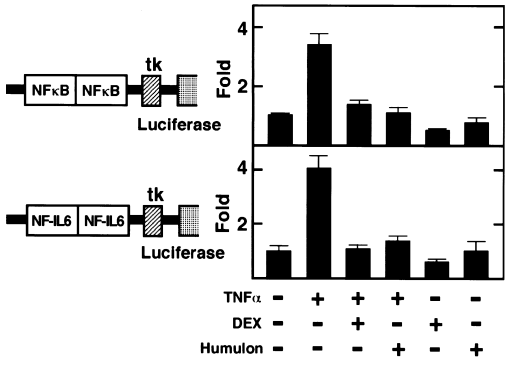

Fig. 4. Effect of humulon and dexamethasone on NFκB and NF-IL6 response elements as examined by the luciferase analysis. Confluent MC3T3-E1 cells (7.8×10^5) were transfected with luciferase plasmid including NFκB or NF-IL6 response element. The cells were incubated for 12 h in the presence or absence of 10 ng/ml TNFα, 5 μM dexamethasone (DEX) or 10 μM humulon as indicated. Data are means ± S.D. of triplicate determinations.

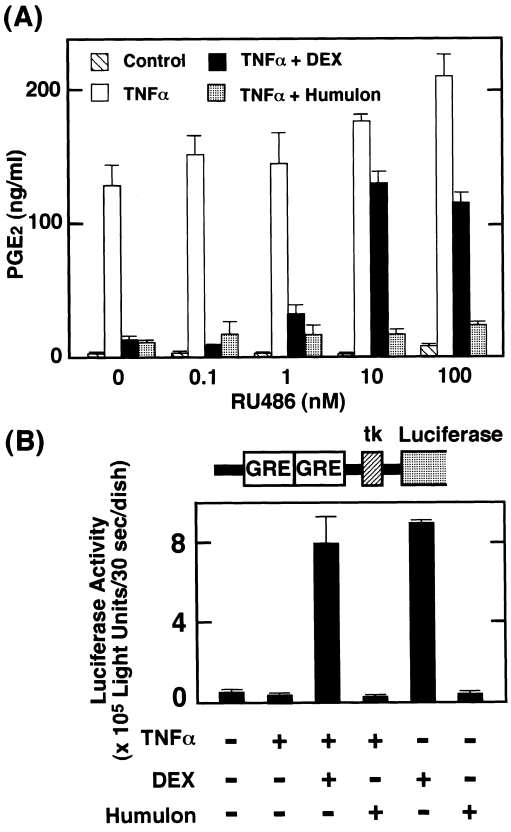

Fig. 5. Possible binding of humulon and dexamethasone to glucocorticoid receptor. A: MC3T3-E1 cells (3×10^5) were incubated with 10 ng/ml TNFα for 12 h in the absence or presence of 5 μM dexamethasone (DEX) or 10 μM humulon. Various concentrations of RU486 were added. Produced PGE2 was measured using radioimmunoassay. Data are means ± S.D. of triplicate determinations. B: Confluent cells (7.8×10^5) were transfected with luciferase plasmid including two glucocorticoid response elements (GRE) and thymidine kinase gene (tk). The cells were incubated for 12 h in the presence or absence of 10 ng/ml TNFα, 5 μM dexamethasone (DEX) or 10 μM humulon. Data are means ± S.D. of triplicate determinations.

4. Discussion

As for the inhibitors of cyclooxygenase induction other than glucocorticoid, a recent paper reported a cyclooxygenase-2 transcription in phorbol ester-treated human epithelial cells inhibited by resveratrol, which is a phytoalexin found in grapes and other foods [17]. The compound reduced both the enzyme activity and the gene transcription at a concentration in the order of 10 μM. Resveratrol also blocked cyclooxygenase-2 activation by protein kinase C-α, ERK1, and c-Jun [17]. In addition, sodium salicylate and aspirin at pharmacological concentrations (0.1–100 μM) inhibited cyclooxygenase-2 transcription induced by phorbol ester, interleukin-1β, and lipopolysaccharide (LPS) [18].

It is established that glucocorticoid binds to its intracellular receptor migrating into nucleus and the steroid-receptor complex binds to a glucocorticoid response element of the promoter region of target gene [19]. Inoue and others reported

that the activation of the cyclooxygenase-2 gene promoter by LPS was suppressed by glucocorticoid, and the suppression was enhanced by transfection of the glucocorticoid receptor cDNA to vascular endothelial cells [20]. However, there is no typical sequence for glucocorticoid response element within 1.2 kb upstream in cyclooxygenase-2 gene promoter [1,2]. One of the two NFκB sites (nucleotides –223 to –214) was involved in both the LPS-induced human cyclooxygenase-2 gene expression in U937 cells and its suppression by dexamethasone and herbimycin A [21].

In conclusion, our results demonstrated that humulon has a glucocorticoid-like suppression activity in TNFα-induced cyclooxygenase-2 transcription and its signal transduction may be independent from glucocorticoid receptor. However, these results required further investigations of a detailed molecular mechanism and of in vivo anti-inflammatory action of humulon.

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