Inhibitory Effect of Chan-Su on the Secretion of PGE2 and NO in LPS-stimulated BV2 Microglial Cells

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Chan-Su (*Venenum bufonis*) has long been for a variety of other purposes including treatment of inflammation in the folk medicine recipe. Since nitric oxide (NO) is one of the major inflammatory parameters, we first studied the effects of Chan-Su on NO production in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. Chan-Su inhibited the secretion of NO in BV2 microglial cells, without affecting cell viability. The protein level of inducible nitric oxide synthase (iNOS) was decreased by Chan-Su. And Chan-Su also inhibited production of prostaglandin E2 (PGE2) and expression of cyclooxygenase (COX)-2. Proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-12, were inhibited by Chan-Su in a dose-dependent manner. And Chan-Su inhibited the degradation of IκB-α, which was considered to be inhibitor of nuclear factor (NF)-κB, one of a potential transcription factor for the expression of iNOS, COX-2 and proinflammatory cytokines. These results suggest that Chan-Su could exert its anti-inflammatory actions by suppressing the synthesis of NO through inhibition of IκB-α degradation.

Key words : Chan-Su(*Venenum bufonis*), nitric oxide, inducible nitric oxide synthase, cyclooxygenase-2, inhibitor kappa B

Introduction

Chan-Su (*Venenum bufonis*) is substance that is dried white intestinal juices secreted from the skin venum gland or postauricular gland of Bufo bufo gargaizans Cantor or Bufo melanostictus Schneider[1]. Chan-Su has long been for a variety of other purposes including treatment of tonsillitis, sore throat, palpitation and pain relief[2].

Microglia are believed to play a major role in inflammatory and immune reactions in the brain, including neurodegenerative disorders such as Alzheimer's disease (AD)[3]. Activated microglia produce inflammatory mediators including cyclooxygenase (COX)-2 (and its product PGs), inducible nitric oxide synthase (iNOS) (and its product NO), or cytokines as well as neurotoxic substances[4], which are thought to be responsible for brain injuries and diseases including trauma, ischemia, Alzheimer's disease, or neural death[5,6].

Nitric oxides (NOs) and prostaglandins (PGs) are well known proinflammatory mediators in the pathogenesis of inflammation[7]. NO plays a major role in the regulation of vascular tone, neurotransmission, platelet aggregation, and other homeostatic mechanisms[8]. PGE2 is synthesized from arachidonic acid by a reaction catalyzed by COX. And PGE2 is overproduced in inflammation and this over-expression is related to pain and inflammation[9].

According to our previous study, Chan-Su induced apoptosis in the human bladder carcinoma T24 cells. But we can't find the study in microglial cells by Chan-Su[10]. Though Chan-Su has been used in treating chronic-inflammation in clinical treatments[11], the regulation mechanism of anti-inflammatory effects by Chan-Su has not been clarified.

Therefore, in this study, we investigated that Chan-Su induces anti-inflammatory effects in BV2 microglia. The results suggest that Chan-Su could exert its anti-inflammatory actions by suppressing the synthesis of NO, COX-2, PGE2 and proinflammatory cytokines through inhibition of NF-κB activity.

Materials and Methods

1. Preparation of extracts

Chan-Su was purchased from a local oriental herb store, Kwang Myoung Dang (Busan, Korea). Chan-Su was identified and authenticated by Professor W.S. Ko, College of Oriental Medicine, Dongeui University (Busan, Korea). A voucher specimen was deposited at the Department of Oriental

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Medicine, Dongeui University, Busan, Korea. Chan-Su (total 2 g) was distilled in phosphate buffered saline (PBS) and filtered through 0.22 μm filter before use.

2. Reagents

Anti-murine iNOS, IκB-polyclonal antibodies and horseradish peroxidase-conjugated anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lipopolysaccharide (LPS), Tween-20, bovine serum albumin (BSA), phosphatase-conjugated goat anti-rabbit IgG, and p-nitrophenyl phosphate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). 24-well, 96-well tissue culture plates and 60 mm culture dishes were purchased from Nunc Inc. (North Aurora Road, IL). DMEM containing L-arginine (200 mg/ℓ), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Life Technologies (Gaithersburg, MD). All reagents and media for tissue culture experiments were tested for their LPS content with use of a colorimetric Limulusamoebocyte lysate assay (detection limit, 10 pg/ml; Whittaker Bioproducts, Walkersville, MD).

3. Cells culture

BV2 microglial cells were used in this study. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % (v/v) heat-inactivated FBS, 100 U/ml Penicillin and 100 μg/ml Streptomycin in a humidified incubator with 5 % CO_2. BV2 cells were incubated for 1 h with the absence or presence of the indicated concentrations of Chan-Su before the stimulus of LPS (1 μg/ml).

4. Measurement of nitric oxide (Griess assay)

NO level in cell cultures were measured by a microplate assay method, as described previously[12]. After cells were stimulated in 24 well for 24 h, 100 μℓ each cultured medium was mixed with the same volume of the Griess reagent (1 % sulfanilamide/ 0.1 % N-(1-naphthyl)-ethylenediamine dihydrochloride/ 2.5 % H_3PO_4). NO concentration was determined by measuring the absorbance at 540 nm with a 96-well microplate spectrophotometer.

5. Cell viability

The cell viability of Chan-Su was assessed using the MTT assay[13] in the remaining cells after Griess reaction. 0.5 mg/ml of MTT solution was added to each well. After incubation for 2 h at 37℃ and 5 % CO_2, the supernatant were removed and the formed formazan crystals in viable cells were measured at 540 nm with microplate reader.

6. Isolation of total RNA from cells and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated as per the manufacture's instructions. Briefly, cells were lysed by the addition of Trizol and the cell lysate was passed through the pipette several times. 0.2 ml of chloroform was added per 1 ml of Trizol reagent. The tubes were shaken vigorously and incubated at room temperature for 2-3 min. The samples were centrifuged at 14,000 g for 20 min. The aqueous phase was transferred to a fresh tube and RNA was precipitated by the addition of 0.5 ml isopropanol. The RNA pellet was air-dried and resuspended in nuclease-free water. The concentration of RNA was estimated spectrophotometrically. Three microgram RNAs were reverse-transcribed using M-MLV reverse transcriptase (Promega). Single stranded cDNA was amplified by PCR with primers (Table 1). PCR amplifications were done in a 20 μℓ PCR PreMix (Bioneer Co. Daejon, Korea) containing 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTP, 1 unit of Taq polymerase. Amplifications were carried out in a PCR machine (ASTEC PC802, Japan) using an initial denaturation at 94 ℃ for 5 min followed by 30 cycles of denaturation for 30 sec at 94 ℃ annealing for 30 sec at 52 ℃ (56 ℃ for GAPDH) and extension for 1 min at 72 ℃ This was concluded with a final extension for 5 min at 72 ℃. Amplicons were separated in 1 % agarose gel in 1× TBE buffer at 100 V for 30 min, stained with ethidium bromide and visualised under UV light. GAPDH was used as an internal control to evaluate relative expression of COX-2 and iNOS, IL-1β, IL-12 and TNF-α.

Table 1. Primers used in this study.

Target gene		Oligonucleotide sequences (5' to 3' direction)	Expected size	Accession Number
iNOS	forward	GACAAGCTGCATGTGACATC	325 bp	NM010927
	reverse	GCTGGTAGGTTCCTGTTGTT		
COX-2	forward	TTGAAGACCAGGAGTACAGC	324 bp	NM011198
	reverse	GGTACAGTTCCATGACATCG		
IL-1β	forward	TGAAGGGCTGCTTCCAAACC	362 bp	NM008361
	reverse	TGTCCATTGAGGTGGAGAGC		
IL-12	forward	ACCTCAGTTTGGCCAGGGTC	559 bp	NM008351
	reverse	GTCACGACGCGGGTGGTGAA		
TNF-α	forward	GCGACGTGGAACTGGCAGAA	341 bp	NM013693
	reverse	TCCATGCCGTTGGCCAGGAG		
GAPDH	forward	GGTGAAGGTCGGTGTGAACG	701 bp	XM001473623
	reverse	GGTAGGAACACGGAAGGCCA		

7. Western blot analysis

The cells were collected and lysed with lysis buffer [1 % Triton X-100, 1 % Deoxycholate, 0.1 % NaN_3]. Protein concentrations were determined by the method of Bradford using BSA for the standards. Proteins in whole-cell lysates were separated by 10 % SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked for at least 1 h in 5 % skim milk and then incubated with anti-iNOS (Santa Cruz,

CA), IκB-α (Cell Signaling, Beverly, MA) polyclonal antibodies and anti-COX-2 monoclonal antibody (BD Biosciences, Franklin Lakes, NJ). After washing in 1× TBST three times, incubate with secondary antibody at room temperature and the antibody-specific proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure (ECL, Amersham).

8. Statistical analysis

Data is presented as the mean ± S.E.M. (Std. Err.) of at least three separate experiments. Comparisons between two groups were analyzed using Student's t-test. P values less than 0.05 were considered to be statistically significant.

Results

1. Chan-Su inhibits NO production though the synthesis of iNOS on LPS-stimulated BV2 microglia

We studied the effects of Chan-Su on NO production in order to investigate anti-inflammatory effect of Chan-Su. The accumulated NO, estimated by Griess method, in the culture medium was used as an index for NO synthesis from LPS-stimulated BV2 microglia. Chan-Su suppressed NO release into culture supernatant in a dose-dependent manner (Fig. 1A). Since the expression of iNOS is primarily responsible for the NO over production, we analyzed whether Chan-Su affect the expression of iNOS mRNA. The analysis indicated that LPS-induced expression of the iNOS mRNA was decreased by Chan-Su (Fig. 1B). Especially, the level of iNOS was dramatically reduced by a 100 ng/ml Chan-Su. We also investigated that Chan-Su could affect iNOS protein synthesis by Western blot analysis. The protein level of iNOS was reduced by the Chan-Su in a dose-dependent manner (Fig. 1C). The result of Western blot analysis indicated that Chan-Su inhibits to direct the modulate of the iNOS expression level in the LPS-stimulated BV2 microglia. Cell viability was not affected by Chan-Su as measured by MTT assay (Fig. 2). This results strongly suggests that the inhibitory effect of Chan-Su on NO release is not due to cell viability of Chan-Su but the effect on gene expression level of iNOS.

2. Effects of Chan-Su on the PGE2 synthesis and COX-2 from LPS-induced BV2 microglia

We investigated effect of Chan-Su on LPS-induced PGE2 production. Chan-Su suppressed PGE2 release into culture supernatant (Fig. 3A). Because Chan-Su decreased the PGE2 production, we tried to determine whether it also inhibits expression of COX-2 mRNA. RT-PCR analysis indicated that

LPS-induced expression of the COX-2 mRNA was decreased significantly by Chan-Su in dose-dependent manner (Fig. 3B). The protein level of COX-2 was reduced by Chan-Su (Fig. 3C). The result of Western blot analysis indicated that Chan-Su inhibits PGE2 release through modulating the COX-2 expression level in the LPS-stimulated BV2 microglia.
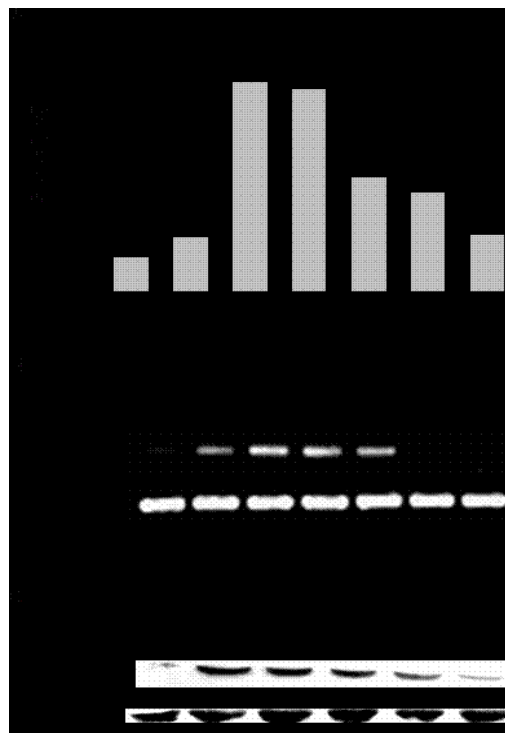
Fig. 1. Inhibitory effect of Chan-Su on the iNOS and the nitrite production on the LPS-stimulated BV2 microglia. (A) The cells (2×10^5 cells/well) were stimulated with LPS (1 μg/ml). After cells were incubated for 24 h, the production of nitrite concentration was determined. Values are mean ± S.E.M. of three individual experiments performed in duplicate. * P < 0.05, significantly different from the LPS. (B) The cells were stimulated with or without LPS (1 μg/ml) and incubated for 6 h. After, iNOS mRNA was analyzed by RT-PCR. GAPDH was used as control genes. (C) The cellular proteins were collected and then Western blot was probed with a monoclonal antibody to iNOS.
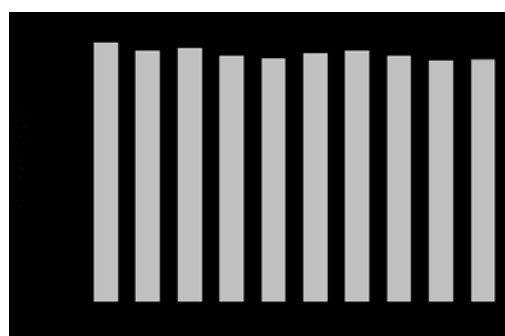

Fig. 2. Effect of Chan-Su on the cell viability of LPS-stimulated BV2 microglia. Cells (2×10^5 cells/well) were treated with 0, 25, 50, 100, 200 ng/ml of Chan-Su in the presence or absence of LPS (1 μg/ml). After cells were incubated for 24 h, the cell viability wasdetected in the tested concentration as measured by the MTT assay. Results are presented as the mean ± S.E.M. of three individual experiments performed in duplicate.
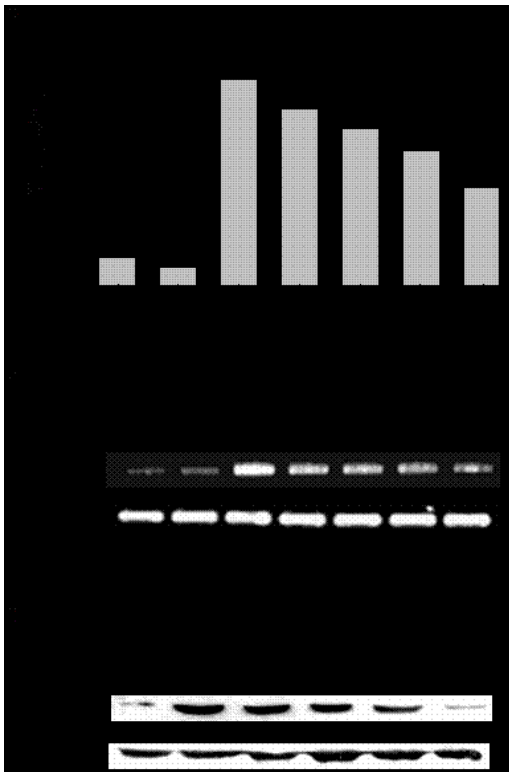
Fig. 3. Effects of Chan-Su in the PGE2 synthesis and expression COX-2 on the LPS-stimulated BV2 microglia. (A) The cells (2×10^5 cells/well) were incubated for 24 h, the production of PGE2 concentration was determined by ELISA. Values are mean ± S.E.M. of three individual experiments performed in duplicate. * P < 0.05, significantly different from the LPS. (B) The cells were incubated for 6 h. After, iNOS mRNA was analyzed by RT-PCR. GAPDH was used as control genes. (C) The cellular proteins were collected and then Western blot was probed with a monoclonal antibody to COX-2.

3. Effects of Chan-Su on the IL-1β, IL-12 and TNF-α mRNA expressions

We investigated whether iNOS protein reduction is correlated with pro-inflammatory cytokines, IL-1β, IL-12 and TNF-α. IL-1β, IL-12 and TNF-α mRNA level is gradually inhibited by the Chan-Su respectively (Fig. 4).
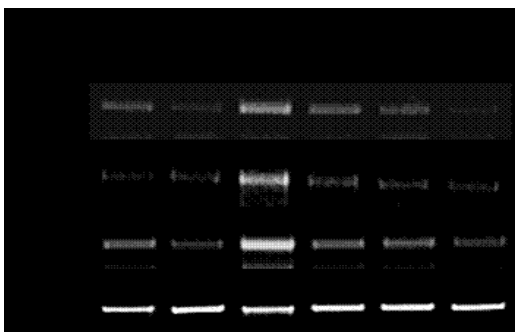
Fig. 4. Inhibitory effects of Chan-Su on IL-1β, IL-12 and TNF-α mRNA expression in the LPS-stimulated BV2 microglia. Cells (5×10^5 cells/well) were incubated for 1 h in the presence of the indicated concentrations of Chan-Su. Then, the cells were stimulated with or without LPS (1 μg/ml) and incubated for 6 h. After, IL-1β, IL-12 and TNF-α mRNA expressions level were determined by RT-PCR. GAPDH was used as control genes.

4. Chan-Su prevented degradation of IκB-α on LPS-induced BV2 microglia

To clarify the effect of Chan-Su on the early stages of proinflammatory, iNOS and COX-2 expression, the activation of NF-κB in BV2 microglia. The translocation of NF-κB into the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκB-α[14]. We examined the effect of Chan-Su on degradation the IκB-α in order to investigate whether Chan-Su prevents NF-κB signaling pathway in LPS-stimulated BV2 microglia. Our results show that LPS treatment caused degradation of IκB-α at 15 min and 30 min. However Chan-Su inhibited degradation of IκB-α in proportion to time (Fig. 5).
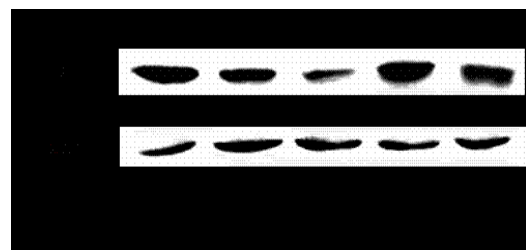
Fig. 5. Effects of Chan-Su on LPS-induced IκB-α degradation. Cells (5×10^6 cells/well) were incubated for 1 h in the presence 200 ng/ml Chan-Su, and then stimulated with 1 μg/ml of LPS for 15 and 30 min. Cells were lysed and subjected to anti-IκB-immunoblot analysis.

Discussion

NO is an important regulatory molecule in diverse physiological functions such as vasodilation, neural communication, and host defense[15,16]. High levels of NO produced by iNOS, however, have been defined as a cytotoxic molecule in inflammation and endotoxemia[17]. There are at least three isoforms of NO synthase (NOS): a constitutive, calcium-dependent endothelial form (eNOS), a constitutive, calcium-dependent neuronal form (nNOS), and an inducible, calcium-independent form (iNOS)[18]. iNOS isoform is an important enzyme involved in the regulation of inflammation[19] and can produce high, persistent concentrations of NO on induction with endotoxin alone or incombination with cytokines in many cell types, potentially resulting in cytotoxicity, tissue damage, or DNA damage. Therefore, the reduction of these harmful effects is seemed to be important in inflammation therapy.

In this study, Chan-Su significantly inhibited LPS-induced NO production without appreciable cell viability. These results suggest that Chan-Su could do potent anti-inflammatory action via inhibition of NO release. But, it is not known whether the reduction in nitrite accumulation by Chan-Su is a result of

inhibition of iNOS expression. Therefore, it was investigated whether Chan-Su could affect iNOS mRNA and protein levels. To determine whether the decreased NO synthesis is correlated with iNOS expression, iNOS mRNA and protein level was analyzed by RT-PCR and Western blotting. The expression of iNOS mRNA and amount of protein was gradually decreased by treatment of Chan-Su in a dose dependent manner. Especially, expression of iNOS mRNA is dramatically inhibited by 100 ng/ml Chan-Su. This result implicates that a significant decrease in NO release by Chan-Su is linked to the expression level of iNOS gene.

PGE2 is overproduced in inflammation and this over-expression is related to pain and inflammation[9]. PGE2 is converted from arachidonic acid by COX. Two isoforms of this enzyme have been identified[20]. The COX-1 gene has properties commonly associated with housekeeping genes and is constitutively expressed. COX-2 has been shown to be induced through molecules such as adenosine[21], inflammatory cytokines[22,23], bacterial LPS and mitogens[4,24], and during insults such as hypoxia, ischemia and in various neurodegenerative disorders[25,26]. Chan-Su inhibited PGE2 production in the BV2 microglial cells. PGE2, a major metabolite of the COX-2 pathway and immunoregulatory processes. PGE2 has been implicated in the pathogenesis of acute and chronic inflammatory disease states[27]. But, it is not known whether the reduction in PGE2 production by Chan-Su is a result of inhibition of COX-2 expression or inhibition of its enzymatic activity. Therefore, it was investigated whether Chan-Su could affect COX-2 mRNA and protein levels. The expression of COX-2 mRNA and an amount of protein was gradually decreased by treatment of Chan-Su. This result implicates that a significant decrease in PGE2 release by Chan-Su is linked to the expression level of COX-2 gene. This suppression was correlated with down-regulation of iNOS and COX-2 expression. It has been demonstrated that NO plays a pivotal role as neurotransmitter, vasodilator, and immune regulator in a variety of tissues at physiological concentrations[15]. However, high levels of NO produced by iNOS have been defined as a cytotoxic molecule in inflammation and endotoxemia[17]. Thus, potential inhibitors of iNOS and COX-2 have been considered effective therapeutically for preventing inflammatory reaction and disease.

TNF-α has been found to act synergistically on NO production and blocking of TNF-α by a specific antibody to indirectly decreases NO synthesis, impairing cell's defense against parasites[28,29]. Therefore, Chan-Su may inhibit the production of NO by suppressing TNF-α secretion. It may be suggest that these results imply that NO production was decreased indirectly by suppressing expression of TNF-α. IL-12 is a heterodimeric cytokine which is produced by activated antigen-presenting cells (APC), such as dendritic cells, monocytes/macrophages and microglia, in response to bacterial products and immune signals[30]. The expression of proinflammatory cytokines, such as IL-1β and IL-12 also suppressed by the Chan-Su.

The promoter of the iNOS and COX-2 genes contains two major discrete regions synergistically functioning for binding of transcription factors[31-33], NF-κB, which is mainly activated by LPS. NF-κB is a mammalian transcription factor that controls a number of genes that are important for immunity and inflammation such as iNOS and TNF-α genes[32]. Since deregulation of NF-κB function is associated with inflammation, a development of a drug that controls NF-κB is one of promising candidates for the therapeutic strategy in the treatment of inflammatory disease[34]. In most cells, NF-κB is sequestered in the cytoplasm in an inactive form, because of a tight association with its inhibitors, the IκBs. Activation of NF-κB is achieved through signal-induced phosphorylation of IκB at specific amino-terminal serine residues by the IκB kinase (IKK) complex. This phosphorylation triggers IκB degradation via the ubiquitin-proteasome pathway, resulting in NF-κB translocation into the nucleus[35]. Chan-Su suppressed LPS-stimulated degradation of IκB-α at 15 min and 30 min and these results suggest Chan-Su can block activation of NF-κB by blocking the degradation of IκB-α.

In summary, the results of the present study demonstrated that Chan-Su inhibited LPS-stimulated NO, COX-2, and proinflammatory cytokines production. Moreover, effects of Chan-Su in the cell signal pathway may involve the inhibition of NF-κB through regulation of the IκB-α pathway. Therefore, this suggests that Chan-Su could be used as an anti-inflammatory herbal drug.

Acknowledgements

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