Preventive Effect of *Ginkgo biloba* Extract (GBB) on the Lipopolysaccharide-Induced Expressions of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 via Suppression of Nuclear Factor-κB in RAW 264.7 Cells

Young-Mi PARK, Jong-Heon WON, Kyung-Jin YUN, Jong-Hoon RYU, Yong-Nam HAN, Seung-Ki CHOI, and Kyung-Tae LEE

*Department of Biochemistry, College of Pharmacy, Kyung-Hee University; b Department of Oriental Pharmaceutical Science, College of Pharmacy, Kyung-Hee University; Seoul 130–701, South Korea: c Natural Products Research Institute, College of Pharmacy, Seoul National University; Seoul 110–460, Korea: and d Department of Pharmacology, College of Medicine, Pochon Cha University; Kyonggi-Do 463–712, South Korea.

Received December 22, 2005; accepted January 16, 2006

During our ongoing efforts to identify bioactive natural products with anti-inflammatory activity, we produced an extract from *Ginkgo biloba* (GBB) which contains higher levels of the active principles terpene and flavonoid than EGb, the standard commercially available extract. In the present study, we examined and compared the effects of these two extracts on lipopolysaccharide (LPS)-induced nitric oxide (NO) and prostaglandin E\(_2\) (PGE\(_2\)) production by the RAW 264.7 macrophage cell line. Our data indicate that GBB is a more potent inhibitor of NO and PGE\(_2\) production than EGb 761, and it also significantly decreased tumor necrosis factor (TNF-α) release. Consistent with these observations, the protein and mRNA expression levels of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) were found to be inhibited by GBB in a dose-dependent manner. Furthermore, GBB inhibited the LPS-induced DNA binding activity of nuclear factor-κB (NF-κB), which was associated with the prevention of IκB degradation, and subsequently with decreased p65 protein level in the nucleus. These results suggest that GBB inhibits LPS-induced iNOS, COX-2 and TNF-α expressions through the down-regulation of NF-κB-DNA binding activity.

**Key words** *Ginkgo biloba*; nitric oxide; prostaglandin *E*\(_2\); inducible nitric oxide synthase (iNOS); cyclooxygenase-2 (COX-2); nuclear factor-κB (NF-κB)

Nitric oxide (NO) which is produced by inducible nitric oxide synthase (iNOS) is a pro-inflammatory radical and an important biological mediator.\(^1\) Prostaglandin *E*\(_2\) (PGE\(_2\)), released by arachidonic acid metabolites, is also an important mediator of acute and chronic inflammation, and it is associated with the enzyme, cyclooxygenase-2 (COX-2).\(^2\) In addition to these oxygen and arachidonic acid metabolites, tumor necrosis factor-α (TNF-α) acting as a cytokine or inflammatory mediator plays a major role in various inflammatory diseases, including septic shock and rheumatoid arthritis.\(^3,4\)

Nuclear factor-κB (NF-κB) appears to play a primary role in the transcriptional regulation of these iNOS, COX-2 and TNF-α gene expressions.\(^5\) NF-κB exists as a latent form in the cytoplasm of unstimulated cells and is bound to the inhibitory protein, IκB.\(^6\) Phosphorylation of IκB leads to its degradation and the subsequent translocation of NF-κB to the nucleus where it activates transcriptions of target genes.\(^7,8\)

It is noteworthy that the use of medicinal plants or their crude extracts in the prevention and/or treatment of several chronic diseases has been traditionally practiced in different societies worldwide. Moreover, extracts of *Ginkgo biloba* Linne (Ginkgoaceae) have been used for centuries in traditional Chinese medicine. Today, a standardized extract (EGb 761) is prepared from *Ginkgo biloba* leaves and is prescribed commonly in Europe for the treatment of memory disorders, obstructive arteriosclerosis, Alzheimer’s disease, ischaemic heart disease, cerebral infarction, aging, and age-related macular degeneration.\(^9,10\) Several modes of action of EGb 761 have been described: (1) its effects on the blood circula-tion, such as its vasoregulatory activity and rheological effects on blood (decreased viscosity, anti-platelet activating factor activity)\(^11\); (2) its effects on metabolism changes, such as on neuron metabolism (increased tolerance to anoxia)\(^12,13\); (3) its inhibition of cell membrane damage caused by free radicals\(^11,13–15\); and (4) its gene-regulatory effects, which suggest that it has anticancer activity.\(^16,17\)

EGb 761 contains two groups as its main active constituents: 24% flavonol glycoside (ginkgo-flavonol glycosides; quercetin, kaempferol, isorhamnetin) and 6% terpene (bibilalide and ginkgolides A, B, C).\(^18\) In the present study, we prepared a standardized *Ginkgo biloba* extract (GBB), i.e., an extract of *Ginkgo biloba* leaves standardized in terms of its total terpenes (12±3%), biflavonoid (4.5±1.5%), flavonol glycoside (<8%), and proanthocyanidine (under detection limit) content. The difference between GBB and EGb 761 is that it contains a higher level of terpene, a lower level of flavonol glycoside, and no detectable proanthocyanidine.

In the present study, we compared the effects of these *Ginkgo biloba* extracts (GBB and EGb 761) on lipopolysaccharide (LPS)-induced NO and PGE\(_2\) release. To further explore the possible mechanisms of these inhibitions by GBB, we investigated the expression levels of iNOS and COX-2 enzyme and mRNA expression levels via NF-κB activity in the macrophage cell line RAW 264.7.

**MATERIALS AND METHOD**

**Materials** EGb 761 used in this study was obtained from SK Chemical Co. (Seoul, Korea). GBB is an extract of

* To whom correspondence should be addressed. e-mail: ktlee@khu.ac.kr
Ginkgo biloba standardized for the contents of total terpene trilactones (13±3%), biflavone (4.5±1.5%), flavonol glycoside (<8%), and proanthocyanidine (under detection limit).9) Dulbecco's modified Eagle's minimal essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.). COX-2, iNOS, 1xβ, p-1xβ, p65 monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The enzyme immunoassay (EIA) kits for PGE2 and TNF-α were obtained from R&D Systems (Minneapolis, MN, U.S.A.). RNA extraction kit was purchased from Intron Biotechnology. iNOS, COX-2, TNF-α and β-actin oligonucleotide primers were purchased from Bioneer (Seoul, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), aprotinin, leupeptin, phenylmethylsulfonylfluoride (PMSF), dithiothreitol, L-6-(1-iminoethyl)lysine (L-NIL), Escherichia coli LPS, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Culture and Sample Treatment The RAW 264.7 murine macrophage cell line was obtained from the Korean Cell Line Bank (Seoul). Cells were cultured in DMEM medium at 37°C supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO2. The cells were incubated with GBB at various concentrations (40, 80, 120 mg/ml) of GBB or EGb 761. After overnight incubation, cells were treated with LPS (1 μg/ml) of 200 μl containing (final concentration) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, and oligo (dT20) 0.5 μg/ml. Then PCR analyses were performed on the aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α and β-actin (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA, U.S.A.). The reactions were carried out in a volume of 25 μl containing (final concentration) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, ×10 reaction buffer, and 100 pmol of 5’ and 3’ primers. After initial denaturation for 2 min at 95°C, thirty amplification cycles were performed for iNOS (1 min of 95°C denaturation, 1 min of 60°C annealing, and 1.5 min 72°C extension), COX-2 (1 min of 94°C denaturation, 1 min of 60°C annealing, and 1 min 72°C extension) and TNF-α (1 min of 95°C denaturation, 1 min of 55°C annealing, and 1 min 72°C extension). The PCR primers used in this study are listed below and were purchased from Bioneer: sense strand iNOS, 5′-AAATGG-GAAACATCAGTGCCGAACTC-3′; anti-sense strand iNOS, 5′-GCTGT-GTGTCACAGAAAATCG-ACATC-3′; sense strand COX-2, 5′-GGGAGAGACTATC-AAGATGTCAT-3′; anti-sense strand COX-2, 5′-ATGGTGTCAGTAGACTTGTTAC-3′; sense strand TNF-α, 5′-GGATGGCAGCAGAAA-GCATGATC-3′; anti-sense strand TNF-α, 5′-TTACAGCTGTTGACTCAATT-3′; sense strand β-actin, 5′-CTATGAAGGT-GTACGTTGACATCCGG-3′; anti-sense strand β-actin, 5′-CCTAGAAGCATTGG-GGTGCACTGATG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Electrophoretic Mobility Shift Assay (EMSA) Electrophoretic mobility shift assay (EMSA) was performed as previously described.20) Nuclear extracts were prepared as described previously with slight modification.21) Treated and untreated RAW 264.7 cells were lysed with hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM Na orthovannadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated for 30 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions. Forty micrograms of cellular protein from treated and untreated cell extracts were electrophoresed onto a nitrocellulose membrane following separation using 8–12% SDS-polyacrylamide gel electrophoresis (PAGE). The immunoblot was incubated overnight with Tween 20/Tris-buffered saline (TTBS) containing 5% (w/v) nonfat milk at 4°C, followed by incubation for 4 h with a 1:100 dilution of primary antibody (anti-iNOS, anti-COX-2, anti-1xβ, anti-p-1xβ, anti-p65 and β-actin antibody) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). Blots were washed two times with in TTBS and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were again washed three times with TTBS and then visualized by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

RNA Preparation and Polymerase Chain Reaction Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology) according to the manufacturer’s instructions. From each sample, 1 μg of RNA was reverse-transcribed (RT) using MuLV reverse transcriptase, 1 mM dNTP, and oligo (dT12–18) 0.5 μg/ml. Then PCR analyses were performed on the aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α and β-actin (as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA, U.S.A.). The reactions were carried out in a volume of 25 μl containing (final concentration) 1 unit of Taq DNA polymerase, 0.2 mM dNTP, ×10 reaction buffer, and 100 pmol of 5’ and 3’ primers. After initial denaturation for 2 min at 95°C, thirty amplification cycles were performed for iNOS (1 min of 95°C denaturation, 1 min of 60°C annealing, and 1.5 min 72°C extension), COX-2 (1 min of 94°C denaturation, 1 min of 60°C annealing, and 1 min 72°C extension) and TNF-α (1 min of 95°C denaturation, 1 min of 55°C annealing, and 1 min 72°C extension). The PCR primers used in this study are listed below and were purchased from Bioneer: sense strand iNOS, 5′-AAATGG-GAAACATCAGTGCCGAACTC-3′; anti-sense strand iNOS, 5′-GCTGT-GTGTCACAGAAAATCGACACTC-3′; sense strand COX-2, 5′-GGGAGAGACTATC-AAGATGTCAT-3′; anti-sense strand COX-2, 5′-ATGGTGTCAGTAGACTTGTTAC-3′; sense strand TNF-α, 5′-GGATGGCAGCAGAAA-GCATGATC-3′; anti-sense strand TNF-α, 5′-TTACAGCTGTTGACTCAATT-3′; sense strand β-actin, 5′-CTATGAAGGT-GTACGTTGACATCCGG-3′; anti-sense strand β-actin, 5′-CCTAGAAGCATTGG-GGTGCACTGATG-3′. After amplification, portions of the PCR reactions were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Western Blot Analysis Cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed in extraction lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol (DTT), 5 mM Na fluoride, 0.5 mM Na orthovanadate) containing 5 μg/ml each of leupeptin and aprotinin and incubated for 30 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions. Forty micrograms of cellular protein from treated and untreated cell extracts were electrophoresed onto a nitrocellulose membrane following separation using 8–12% SDS-polyacrylamide gel electrophoresis (PAGE). The immunoblot was incubated overnight with Tween 20/Tris-buffered saline (TTBS) containing 5% (w/v) nonfat milk at 4°C, followed by incubation for 4 h with a 1:100 dilution of primary antibody (anti-iNOS, anti-COX-2, anti-1xβ, anti-p-1xβ, anti-p65 and β-actin antibody) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). Blots were washed two times with in TTBS and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were again washed three times with TTBS and then visualized by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).
RESULTS

Effects of GBB on LPS-Induced NO and PGE$_2$ Production

To assess the effects of GBB and EGb 761 on LPS-induced NO production in RAW 264.7 cells, cell culture media were harvested and nitrite production was measured using the Griess method. GBB and EGb 761 reduced NO production in a dose-dependent manner with IC$_{50}$ values of 95.8 µg/ml and 230 µg/ml, respectively (Fig. 1A). 1-NIL (10 µM) was used as a positive inhibitor. To examine whether the tested compounds could also inhibit PGE$_2$ production, cells were pre-incubated with GBB or EGb 761 for 1 h, and then activated with 1 µg/ml LPS for 24 h. As shown in Fig. 1B, GBB significantly inhibited the production of PGE$_2$ in a dose-dependent manner, whereas EGb 761 had a lesser effect. The cytotoxic effects of GBB and EGb 761 were evaluated in the presence or absence of LPS using the MTT assay, and these compounds did not affect the cell viability of RAW 264.7 cells even at 500 µg/ml in either the presence or absence of LPS after a period of 24 h (data not shown). Thus, the inhibitory effects observed were not attributable to cytotoxic effects.

Inhibition of LPS-Induced TNF-α Production by GBB

Since GBB was found to be a more potent inhibitor of pro-inflammatory mediators than EGb 761, we further investigated the effect of GBB on LPS-induced TNF-α release by EIA and RT-PCR. Pretreatment of RAW 264.7 cells with 6-(1-iminoethyl) lysine (L-NIL) resulted in a significant reduction of TNF-α release compared to the LPS-treated group; significance of differences between treatment groups was evaluated using the Student’s t-test.

Fig. 1. Effects of GBB and Egb 761 on Nitrite (A) and PGE$_2$ (B) Production by LPS in RAW 264.7 Cells

(A) Cells were treated with LPS 1 µg/ml alone or in the presence of various concentrations (40, 80, 120 µg/ml) of GBB for 24 h. Control (Con) values were obtained in the absence of both LPS and GBB. Ten micromolar of L-NIL was used as a positive control. (B) Samples were treated as described for Fig. 1A. Ten micromolar of NS-398 was used as a positive control in the assay. The values represent mean±S.D. of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. LPS-treatment group; significance of differences between treatment groups was evaluated using the Student’s t-test.

Fig. 2. Effect of GBB on LPS-Induced TNF-α Release in RAW 264.7 Cells

(A) Cells were treated with LPS 1 µg/ml alone or in the presence of various concentrations (40, 80, 120 µg/ml) of GBB for 24 h. Control (Con) values were obtained in the absence of both LPS and GBB. (B) Total RNA was prepared for RT-PCR analysis of TNF-α gene expression from RAW264.7 macrophages stimulated with LPS (1 µg/ml) alone or in combination with increasing concentrations (40, 80, 120 µg/ml) of GBB for 24 h. TNF-α-specific sequences (351 bp) were detected by staining gels (2% agarose) with ethidium bromide. PCR for β-actin was performed to verify that the initial cDNA contents of the samples were similar. The values represent mean±S.D. of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 vs. the LPS-treated group; the significance of differences between treated groups was evaluated using the Student’s t-test.
GBB for 1 h decreased both TNF-α production and mRNA expression in a dose-dependent manner (Figs. 2A, B).

**Effects of GBB on LPS-Induced iNOS and COX-2 Protein and mRNA Expressions** Western blot and RT-PCR analyses were performed to determine whether the inhibitory effects of GBB on these pro-inflammatory mediators (NO and PGE₂) are related to the modulation of the expressions of iNOS and COX-2. In unstimulated RAW 264.7 cells, neither iNOS nor COX-2 protein or mRNA were detectable. In response to LPS, the expression of iNOS was markedly augmented, and GBB significantly inhibited iNOS protein induction in a dose-dependent manner (Fig. 3A). RT-PCR analysis showed that the amount of iNOS mRNA was correlated with its protein level (Fig. 3B). A similar pattern was observed when we examined the effect of GBB on LPS-induced COX-2 expression; densitometric analysis of three independent experiments demonstrated that COX-2 protein expression induced by LPS was inhibited by 64% in cells treated with GBB 80 μg/ml (Fig. 3A). Under the same conditions, COX-2 mRNA levels were found to be similarly decreased (Fig. 3B). GBB did not affect the expression of the housekeeping gene, β-actin. In general, these results are consistent with the profile of the inhibitory effect of GBB on NO and PGE₂ release (Fig. 1).

**Inhibition of NF-κB Activation and the Nuclear Translocation of p65 by GBB** Electrophoretic mobility shift assay (EMSA) experiments indicated that GBB blocked the LPS-induced activation of nuclear factor-κB (NF-κB). Treatment of cells with GBB for 1 h prior to adding LPS inhibited the LPS-induced increase in the band intensity of NF-κB-DNA binding (Fig. 4A). We also measured the protein level of p65 in the nucleus by Western blotting. As was expected, the level of p65 declined in response to LPS treatment with GBB (40, 80, 120 μg/ml) (Fig. 4B). These results suggest that GBB may inhibit NF-κB binding activity by preventing the LPS-induced translocation of p65 to the nucleus.

**Inhibition of 1κBα Degradation and Phosphorylation by GBB** The inhibition of NF-κB activation occurs via the prevention of 1κBα phosphorylation and degradation. We further investigated whether GBB could inhibit the LPS-stimulated phosphorylation and degradation of 1κBα in the cells by conducting a Western blot assay. Figure 5A shows that LPS induced the transient phosphorylation and degradation of 1κBα after 10 to 15 min and that this degradation was markedly blocked by pre-treatment with GBB (80 μg/ml). We also found that GBB exerted an inhibitory effect on the LPS-induced phosphorylation and degradation of 1κBα in a concentration-dependent manner (Fig. 5B). Corresponding with these results, NF-κB activity and the translocation of the p65 subunit in the nucleus were blocked in a concentration-dependent manner (Figs. 4A, B).

**DISCUSSION**

In the present study, we prepared a fraction from *Ginkgo biloba* extract (GBB) and examined the effects of GBB and standardized *Ginkgo biloba* extract (EGb 761) on the LPS-induced pro-inflammatory molecules NO and PGE₂. We
found that GBB had a much stronger inhibitory effect on these inductions than EGb 761. To further explore the mechanism of these inhibitions, the expressions of iNOS and COX-2 were examined at the protein and mRNA levels. The inhibition of the LPS-stimulated expressions of these molecules in RAW 264.7 cells by GBB was not attributable to cytotoxicity, as determined by the MTT assay and β-actin expression. The inhibitions of the expressions of iNOS, COX-2 and TNF-α genes were evidenced by reductions in their mRNA levels in a parallel concentration-dependent manner. Thus, the inhibitions of NO and PGE₂ release by GBB can be attributed to the suppressions of iNOS and COX-2 mRNA transcription followed by protein expressions.

The expressions of iNOS, COX-2 and TNF-α in murine macrophages has been shown to be dependent on NF-κB activity. 22) Thus the possibility that GBB might inhibit NF-κB activation was examined. Our results indicate that the expression inhibitions of iNOS and COX-2 at the protein and mRNA levels by GBB were probably due to the suppression of NF-κB. This is consistent with the findings of previous studies, namely that NF-κB response elements are present on the promoters of the iNOS, COX-2 and TNF-α genes. 23,24) NF-κB is primarily composed of two proteins, p50 and p65, which are also referred to as RelA and cRel, respectively. 22) In the resting state, NF-κB is present in the cytosol and is bound to the inhibitory protein, IκB. Following the induction of NF-κB by a variety of agents, such as LPS, TNF-α, or tissue plasminogen activator, IκB is phosphorylated which triggers its proteolytic degradation via 26s proteosome. 22) NF-κB is then released from IκB and is translocated into the nucleus, where it binds to the κB binding sites in the promoter regions of target genes. In the present study, we found that GBB blocks the LPS-induced activation of NF-κB by inhibiting the degradation of IκB. These results suggest that

the inhibition by GBB of the LPS-induced expressions of the iNOS and COX-2 genes occurs by blocking NF-κB activation, although the inhibition of other transcription factors such as AP-1, interferon response element, and the γ-activated site may also be involved.

Ginkgo biloba standardized extract (EGb 761) has been shown to possess antioxidant and free radical scavenging activities 25) and to have vasodilatory, 26) rheological, 27,28) blood flow promoting and anti-edematogenic properties. 29—31) The cellular mechanisms underlying these multiple effects can be attributed to different components in the extract, which may act independently or synergistically. Although some of the cellular mechanisms have been found to involve terpenes such as ginkgolides or bilobalide, 32,33) many remain unexplained, despite the large number of experiments performed on these materials. Moreover, the anti-inflammatory effects of the water soluble portion of EGb 761 on inflammation caused by Candida albicans revealed that the terpene, rather than the flavone portion of this material is responsible for these therapeutic effects. 34) EGb 761 contains ca. 24% ginkgo-flavonol glycosides and 6% terpenes (ginkgolides, bilobalide). However, GBB contains more terpene and a lower level of flavonol glycosides than EGb 761. Although it has yet to be determined whether the effects of EGb 761 or GBB are caused by a single active ingredient or by the combined action of several components, we suggest that the terpene in GBB may be responsible for its enhanced physico-chemical properties and anti-inflammatory action.

The results of the present study indicate that GBB is a potent inhibitor of LPS-induced NO, PGE₂, and TNF-α production and that it acts at the gene level. Moreover, this inhibition is caused by blocking NF-κB activation in RAW 264.7 macrophages. These findings suggest that GBB is a potential therapeutic for the treatment of LPS-induced sepsis syndrome and rheumatoid arthritis.

Acknowledgments This research was supported by a grant (PF002104-07) from the Plant Diversity Research Center of the 21st Frontier Research Program funded by the Korean Ministry of Science and Technology and by the Korean Science & Engineering Foundation (No. R13-2002-020-01002-0).

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