Activation of Protein Kinase G Up-regulates Expression of 15-Lipoxygenase-1 in Human Colon Cancer Cells

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Abstract

Recent studies indicate that the induction of apoptosis in human colon cancer cells by certain nonsteroidal antiinflammatory drugs involves increased expression of 15-LOX-1 and synthesis of its major product 13-S-hydroxyoctadecadienoic acid (13-S-HODE). Evidence was obtained that this occurs via a cyclooxygenase-2 (COX-2)–independent mechanism, but the actual mechanism of induction of 15-LOX-1 by these compounds is not known. There is extensive evidence that treatment of SW480 human colon cancer cells with sulindac sulfone (Exisulind, Aptosyn) or the related derivative OSI-461, both of which inhibit cyclic GMP (cGMP)-phosphodiesterases but lack COX-2 inhibitory activity, causes an increase in intracellular levels of cGMP, thus activating protein kinase G (PKG), which then activates pathways that lead to apoptosis. Therefore, in the present study, we examined the effects of various agents that cause increased cellular levels of cGMP on the expression of 15-LOX-1 in SW480 human colon cancer cells. Treatment of the cells with Exisulind, sulindac sulfide, OSI-461, the guanylyl cyclase activator YC-1, or the cell-permeable cGMP compound 8-para-chlorophenylthio-cGMP (8-pCPT-cGMP) caused an increase in cellular levels of 15-LOX-1. Exisulind, OSI-461, and 8-pCPT-cGMP also increased mRNA levels of 15-LOX-1, suggesting that the effects were at the level of transcription. The cGMP-phosphodiesterase inhibitors and YC-1 increased the production of 13-S-HODE, which is the linoleic acid metabolite of 15-LOX-1. Treatment of SW480 cells with the PKG inhibitor Rp-8-pCPT-cGMP blocked Exisulind-induced 15-LOX-1 expression. Furthermore, derivatives of SW480 cells that were engineered to stably overexpress wild-type PKG β3 displayed increased cellular levels of 15-LOX-1 when compared with vector control cells. Taken together, these results provide evidence that the cGMP/PKG pathway can play an important role in the induction of 15-LOX-1 expression by nonsteroidal antiinflammatory drugs and related agents.

Introduction

Cyclic guanosine 3’,5’-monophosphate (cGMP) is an important second messenger that mediates the action of several hormones and neurotransmitters, through activation of protein kinase G (PKG), the cGMP-gated channel, and specific phosphodiesterases. There is increasing evidence that it can play an important role in cell proliferation and apoptosis (1, 2). The intracellular level of cGMP is regulated through a dynamic balance between its rate of synthesis by guanylyl cyclases and its degradation by specific cGMP-phosphodiesterases, especially phosphodiesterases 2 and 5. Colon cancers often display decreased levels of guanylin and uroguanylin, which are endogenous activators of guanylyl cyclase (3), and relatively high levels of phosphodiesterases 2 and 5 (4). These findings suggest that cGMP-mediated pathways are suppressed in colon cancer cells, presumably to inhibit downstream signaling pathway related to the activation of PKG. Recent studies indicate that Exisulind, a metabolite of the nonsteroidal antiinflammatory drug (NSAID) sulindac, and two potent derivatives of Exisulind, OSI-486823 (CP-248) and OSI-461, specifically inhibit phosphodiesterases 2 and 5 (5). Evidence has also been obtained that the resulting increase in cellular levels of cGMP in human colon cancer cells leads to activation of PKG and, thereby, the induction of apoptosis (5–8). These novel effects of Exisulind and related drugs may explain why these compounds exert anticancer effects in a variety of biological systems even though, in contrast to conventional NSAIDs, they do not inhibit cyclooxygenase (COX) activity (5). We previously found expression of a mutant form of PKG Iβ that is constitutively activated, is sufficient to inhibit cell proliferation and induce apoptosis in colon cancer cell lines (9). Therefore, it seems that the cGMP/PKG pathway provides a novel molecular mechanism for the induction of apoptosis in human colon cancers and other types of cancer. However, the precise mechanism(s) by which PKG activation induces apoptosis has not been elucidated in detail, but it seems to involve both a decrease in cellular levels of β-catenin and cyclin D1, and activation of c-fos NH2-terminal kinase (6–8).

The enzyme 15-LOX-1 converts linoleic and arachidonic acid into the biologically active metabolites 13-S-hydroxyoctadecadienoic acid (13-S-HODE) and 15-S-HETE, respectively, through oxidative metabolism (10). This enzyme has become of interest in cancer biology because its expression is often down-regulated in colorectal, pancreatic, and breast cancers (11–14). Furthermore, Shureiqi et al. (15–17) recently found that the induction of apoptosis in colon cancer cells by various NSAIDs was associated with a marked increase in the expression of 15-LOX-1 and its metabolite 13-S-HODE. Because this induction was also seen with Exisulind, which does not inhibit COX-1 or COX-2 (5), and was seen with both the RKO COX-2-positive rectal cancer cell line and the DLD-1 COX-2-negative colon cancer cell line, they suggested that induction of 15-LOX-1 by NSAIDs occurs via a COX-2-independent mechanism (15, 16). However, the actual mechanism of induction of 15-LOX-1 by these compounds is not known. In view of the fact...
that treatment of cells with Exisulind leads to the above-described PKG-mediated pathway of apoptosis (8, 9), in the present study, we examined the possibility that activation of PKG could itself, induce the expression of 15-LOX-1 and the production of 13-S-HODE and 15-S-HETE in colon cancer cells. Using a series of compounds that increase cellular levels of cGMP and cells engineered to stably overexpress PKG Iβ, we have obtained evidence that directly supports this hypothesis.

Materials and Methods

Materials and cell culture. The cell-permeable cGMP compound 8-para-chlorophenylthio-cGMP (8-pCPT-cGMP) and the anti-PKG Iβ antibody were purchased from Calbiochem (San Diego, CA), an anti-VASP antibody from BD Bioscience (San Diego, CA), and an anti-β-actin monoclonal antibody from Sigma (St. Louis, MO). An anti-15-LOX-1 antibody was kindly provided by Imad Shureiqi (University of Texas M.D. Anderson Cancer Center, Houston, TX). Exisulind, sulindac sulfide and OSI-461 were provided by OSI Pharmaceuticals, Inc., Farmingdale, New York. YC-1 was purchased from Alexis Biochemicals (San Diego, CA). The construction and characteristics of plasmids that encode HA-COOH-terminal–tagged wild-type and dominant-negative (KR) forms of PKG Iβ are described in our previous article (8). Similar vector-only plasmids were used as controls. SW480 cells were cultured in DMEM with 10% fetal bovine serum. SMARTpool siRNA for 15-LOX-1 (ALOX15) and control SMARTpool small interfering RNA were purchased from Dharmacon (Lafayette, CO).

Western blot analysis. SW480 cells were harvested and then sonicated in radioimmunoprecipitation assay buffer and extracts examined by Western blot analysis as described previously (8). The lysates were electrophoresed on a 10% polyacrylamide gel, and then electrophoretically transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% dry milk, and incubated with the indicated antibody. After washing, the membrane was incubated with a horseradish peroxidase–conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Protein bands were visualized with the enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech). The relative intensities of the protein bands were quantitated by NIH Image 1.62 software, and normalized to the corresponding β-actin control.

RT-PCR analysis. Total RNA was isolated from SW480 cells using RNasey Mini Kit as recommended by the manufacturer (Qiagen Inc., Valencia, CA). cDNA was amplified from 1 µg of total RNA using a Superscript One-Step RT-PCR with the platinum Taq DNA polymerase system (Invitrogen, Carlsbad, CA). Primer sets were used for 15-LOX-1: 5'-GCT GGA AGA GAG GAA GAT GGT G-3', 15-LOX-1-R, 5'-GAA GTC AGC TTC GAA CAG TGT G-3', giving a 442 bp product. As an internal control, we used GAPDH, 5'-AAT GAC CCC TTC ATT GAC CTC-3', GAPDH-R, 5'GAT TGT CAT GGA TGA CCT TGG-3', giving a 442 bp product. The annealing temperature was 72°C. After 35 cycles, PCR products were analyzed by agarose gel electrophoresis. Fold expression was determined with NIH Image 1.62 software with normalization to the corresponding GAPDH control.

Detection of 13-S-HODE and 15-S-HETE. Cellular levels of 13-S-HODE in SW480 cells were measured with an EIA kit (Assay Designs, Inc. Ann Arbor, MI). The cells were grown for 48 hours in the presence of the indicated compounds, then harvested and lysed in lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, 1% SDS, and 400 mmol/L NaCl]. The cell lysate was acidified and 13-S-HODE was extracted with water-saturated ethyl acetate. The level of 13-S-HODE was then measured by EIA assays, according to the manufacturer's protocol. To assay extracellular levels of 13-S-HODE, the cell-free culture medium was collected 48 hours after treating the cells with the indicated compounds. 2 µg of 13-S-HODE-d4 was added and the medium was extracted using a Sep-Pak vac C18 cartridge (Waters Corp., Milford, MA). The eicosanoids were eluted from the cartridge with methanol. The levels of 13-S-HODE were determined by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) as described previously (15). For detecting cellular levels of 15-S-HETE, the cells were grown for 48 hours in the presence of the indicated compounds, and then harvested. The eicosanoids were extracted from the cells thrice with ethyl acetate/hexane (1:1, v/v). The organic phase extracts were collected, dried under nitrogen, reconstituted and prepared for injection and analysis by LC/MS/MS spectrometry, as described previously (15).

Effect of inhibiting 15-LOX-1 expression. SW480 cells were transfected with the SMARTpool siRNA for 15-LOX-1 or nontargeting control siRNA, using LipofectAMINE 2000 (Invitrogen). After 24 hours, the cells were replated at a density of 2 × 10⁴ in 15.6 mm diameter, 24-well plates. The next day, the cells were treated with 0, 100, 300, or 600 µmol/L Exisulind. After 48 hours, the number of cells per well were counted using a Coulter Counter, the mean values of triplicate wells were determined, and the results presented as a percentage of the control.

Statistical methods. Results of replicate assays are reported as mean ± SD. Differences were analyzed by Student's t test. A value of P < 0.05 was regarded as significant. All of the Western blot and PCR analyses are repeated and gave similar results.

Results

Exisulind, OSI-461, and other activators of protein kinase G induce 15-LOX-1 expression in SW480 cells. To examine whether activation of PKG induces increased expression of 15-LOX-1, SW480 cells were treated with the cGMP-phosphodiesterase inhibitors Exisulind, OSI-461, and sulindac sulfide; YC-1 which activates guanylyl cyclase, and 8-pCPT-cGMP. The cells were treated with each of these compounds for 24 hours, and then examined for cellular levels of 15-LOX-1 by Western blotting with a 15-LOX-1 specific antibody. The concentration used for each of these drugs is indicated in the legend to Fig. 1 and is based on our previous studies on the ability of each compound to cause an increase in cellular levels of cGMP, PKG activation, and induce apoptosis in SW480 cells (8). As shown in Fig. 1A, all five of these compounds caused a marked increase in cellular levels of 15-LOX-1. Next, we did a time course study of 15-LOX-1 induction using Exisulind, OSI-461, and 8-pCPT-cGMP. Treatment with each of these compounds caused, within 4 to 8 hours, increased cellular levels of 15-LOX-1. We previously showed that phosphorylated VASP (p-VASP) provides a biomarker of PKG activation (8). One can readily detect p-VASP because it migrates as a slower band than unphosphorylated VASP (8). Western blot analysis indicated that treatment of the cells with each of these three compounds led to the phosphorylation of VASP within 4 hours (Fig. 1B), thus providing evidence that the induction of 15-LOX-1 was associated with PKG activation.

Protein kinase G inhibitor Rp-8-pCPT-cGMPs blocks Exisulind-induced 15-LOX-1 expression. To provide further evidence that the induction of 15-LOX-1 is mediated by PKG activation, we used the PKG-specific inhibitor, Rp-8-pCPT-cGMPs. We found that this inhibitor blocks the induction of apoptosis by Exisulind in SW480 cells (data not shown). SW480 cells were pretreated with Rp-8-pCPT-cGMPs for 4 hours, and then treated with Exisulind (600 µmol/L) for 24 hours. Pretreatment with either 10 or 20 µmol/L Rp-8-pCPT-cGMPs markedly inhibited Exisulind-induced 15-LOX-1 expression (Fig. 2A).

Exisulind, OSI-461, and 8-pCPT-cGMP increase mRNA levels of 15-LOX-1. Because we found that Exisulind, OSI-461, and 8-pCPT-cGMP increased the cellular level of the 15-LOX-1 protein, we then examined the effects of these compound on mRNA levels of 15-LOX-1. Treatment of SW480 cells with Exisulind, OSI-461, or 8-pCPT-cGMP caused an increase in cellular levels of 15-LOX-1 mRNA within 4 hours (Fig. 2B), suggesting that the increased levels of the 15-LOX-1 protein induced by these drugs is due to increased transcription of the 15-LOX-1 gene (see Discussion).
Stable overexpression of protein kinase G I\(^\beta\) in SW480 cells increases cellular levels of 15-LOX-1. In a previous study, we showed that stable overexpression of wild-type PKG I\(^\beta\) inhibited the growth of SW480 cells, whereas expression of a dominant-negative mutant of PKG I\(^\beta\) (PKG\(^\beta\)KR) enhanced the growth of these cells. Therefore, it was of interest to use these derivatives as an additional approach to examine the effects of PKG on the expression of 15-LOX-1. Protein extracts were obtained from exponentially growing cultures of a vector control clone (vec\#3), two clones that stably overexpress PKG I\(^\beta\) WT (I\(^\beta\)KR\#3 and I\(^\beta\)KR\#9). Western blot analysis indicated that the I\(^\beta\)KR\#3 and I\(^\beta\)KR\#9 cells displayed ~3-fold increase in the cellular level of 15-LOX-1 when compared with the vec\#3 cells, whereas the I\(^\beta\)KR\#3 and I\(^\beta\)KR\#9 cells had ~20% lower level of 15-LOX-1 than the vec\#3 cells (Fig. 3). Analysis of the same blot with a PKG I\(^\beta\) antibody confirmed the increased expression of the wild-type or mutant forms of PKG I\(^\beta\) (Fig. 3). The increased expression of 15-LOX-1 in the PKG I\(^\beta\) wild-type cells may explain, at least in part, why derivatives that stably overexpress wild-type PKG I\(^\beta\) grow slower than vector control cells (9).

Phosphodiesterase inhibitors and a guanylyl cyclase activator increase the production of 13-S-HODE and 15-S-HETE. The 15-LOX-1 enzyme converts linoleic acid and arachidonic acid into the biologically active lipid metabolites, 13-S-HODE and 15-S-HETE, respectively. These lipid metabolites are ligands for the peroxisome proliferator-activated receptor-\(\gamma\), which when activated causes increased expression of antiproliferative molecules, including p21\(^{CIP1}\), PTEN, and spermidine/spermine N1-acetyltransferase (18). Because we found that cGMP-phosphodiesterase inhibitors and the guanylyl cyclase activator YC-1 increased the expression of 15-LOX-1 in SW480 cells (Figs. 1 and 2), we assayed the effects of these compounds on cellular levels of 13-S-HODE using an EIA assay. We found that treatment of SW480 cells with Exisulind, OSI-461, sulindac sulfide, or YC-1 for 48 hours caused ~2- to 3-fold increase in cellular levels of 13-S-HODE (Fig. 4A). To extend this finding, we also collected medium from the treated cells and then assayed extracts for extracellular levels of 13-S-HODE. These assays were done using a LC/MS/MS spectrometry method (15) to assure the specificity of the assay. We found that treatment with these four compounds caused ~3- to 6-fold increase in extracellular levels of 13-S-HODE (Fig. 4B). We also assayed the cell extracts by LC/MS/
MS spectrometry for cellular levels of 15-S-HETE, the arachidonic acid metabolite of 15-LOX-1. Treatment with Exisulind or sulindac sulfide caused a 2-fold increase, and treatment with OSI-461 or YC-1 caused a 3-fold increase in cellular levels of 15-S-HETE (Fig. 4C).

Treatment of SW480 cells with 15-LOX-1 siRNA partially protects the cells from growth inhibition by Exisulind. We next examined whether induction of 15-LOX-1 is essential to the growth-inhibitory effects of Exisulind in SW480 cells by using a siRNA to 15-LOX-1. Western blot analysis indicated that when SW480 cells were transfected with a siRNA to 15-LOX-1, this markedly inhibited the induction of 15-LOX-1 protein expression by either 300 or 600 μmol/L Exisulind, whereas Exisulind caused marked induction of 15-LOX-1 in cells transfected with nontargeting siRNA (Fig. 5A). Cell proliferation assays indicated that with the latter control cells, 300 μmol/L Exisulind caused 45% inhibition of growth (Fig. 5B), which is similar to the growth-inhibitory effect of 300 μmol/L Exisulind with parental SW480 cells (data not shown). On the other hand, this concentration of Exisulind caused only 8% inhibition of growth in the cells transfected with the 15-LOX-1 siRNA. When the cells were treated with 600 μmol/L Exisulind, the respective values were 60% and 40% (Fig. 5B). Thus, it seems that induction of 15-LOX-1 can play an important role in mediating the growth-inhibitory effects of Exisulind in SW480 cells. The appreciable inhibition of growth seen with 600 μmol/L Exisulind in the 15-LOX-1 siRNA cells suggests that, at this higher concentration, other mechanisms play a more important role in mediating the growth-inhibitory effects of this compound (see Discussion).

Discussion

The present studies provide the first evidence that activation of the cGMP-dependent enzyme PKG can induce the expression of the lipid-metabolizing enzyme 15-LOX-1 in mammalian cells. Thus, we found that compounds that increase intracellular levels of cGMP, either by inhibiting cGMP–phosphodiesterases (sulindac sulfide, Exisulind, OSI-461), by increasing the synthesis of cGMP by cellular guanylyl cyclase (YC-1), or by increasing the uptake of extracellular cGMP (8-pCPT-cGMP), cause a marked increase in the expression of 15-LOX-1 in SW480 human colon cancer cells (Fig. 1). In this study (Fig. 1) and in a previous study (8) we showed that the increase in intracellular cGMP evoked by all of these agents leads to activation of PKG because there was increased phosphorylation of the protein VASP, a marker of PKG activation (Fig. 1B). Furthermore, although these agents may induce other effects that lead to induction of 15-LOX-1, the specific role of PKG in this process was established by using the specific PKG inhibitor Rp-8-pCPT-cGMP (Fig. 2A). In addition to this pharmacologic approach, we used a genetic
Unpublished studies. A percentage of the respective untreated cells. The cells for 48 hours with Exisulind, using a Coulter counter, and expressed as $< 0.005$).

Bars, nontargeting siRNA cells. Cancer Res 2005; 65: (18). September 15, 2005 8446 www.aacrjournals.org

Activating PKG. Phosphodiesterase, thereby increasing cellular levels of cGMP and because various NSAIDs including Celecoxib can inhibit cGMP-LOX-1 expression via a COX-2-independent mechanism (15–17), may explain how NSAIDs and related compounds can induce 15-LOX-1 expression. These findings are sufficient to induce expression of 15-LOX-1. These findings may explain how NSAIDs and related compounds can induce 15-LOX-1 expression via a COX-2-independent mechanism (15–17), because various NSAIDs including Celecoxib can inhibit cGMP-phosphodiesterase, thereby increasing cellular levels of cGMP and activating PKG.

The fact that treatment of SW480 cells with either Exisulind, OSI-461, or 8-pCPT-cGMP led to a rapid increase (within 4 hours) in cellular levels of 15-LOX-1 mRNA suggests that PKG induces expression of this gene at the level of de novo transcription. However, further studies are required to determine the precise mechanism. The 5'-flanking promoter region of the human 15-LOX-1 gene contains putative binding sites for the STAT-6, AP-2, GATA, NF-1, and SP-1 transcription factors. The transcription factor GATA-6, which binds to the GATA element, is expressed at a higher level in the proliferating region of the intestinal crypt than in the villus tips, where cells undergo differentiation and apoptosis (19). GATA-6 is also expressed in proliferating colon cancer cells but is down-regulated during their differentiation (19, 20). Furthermore, colon cancers often display decreased expression of 15-LOX-1 and the induction of apoptosis in these cells by various NSAIDs in associated with decreased expression of GATA-6 (21). These and other findings suggest that GATA-6 may be a negative regulator of the expression of 15-LOX-1. Therefore, in future studies, it will be of interest to determine whether activation of PKG affects cellular levels or the function of GATA-6, or other transcription factors that act on the promoter region of 15-LOX-1. Alternatively, activation of PKG may enhance the expression of 15-LOX-1 by producing changes in histone acetylation or other localized changes in chromatin structure, because several HDAC inhibitors can enhance the promoter activity of the 15-LOX-1 gene and increase the expression of 15-LOX-1 (22, 23). In addition, Shankaranarayanan et al. (24) obtained evidence that acetylation of both histones and STAT6 seem to be necessary for the transcriptional activity of 15-LOX-1 in A549 cells. Furthermore, recent studies indicate that the 15-LOX-1 promoter is hypermethylated in several types of cancer (25). Therefore, in future studies, it will also be of interest to determine whether PKG activation enhances the expression of 15-LOX-1 by producing localized changes in chromatin structure. Our findings may also be relevant to the mechanisms by which activation of PKG can inhibit growth and induce apoptosis in human colon cancer cells, and also other types of cancer cells (8, 9). Previous studies have implicated a decrease in cellular levels of β-catenin and cyclin D1, activation of c-Jun NH2-terminal kinase, induction of p21CIP1, VASP phosphorylation, and activation of caspases (7–9). In the present study, we found that the induction of 15-LOX-1 expression caused by PKG activation was associated with increased synthesis of the bioactive metabolites 13-S-HODE and 15-S-HETE (Fig. 4). Because both of these lipids have been implicated in growth inhibition and apoptosis in colon cancer cells (11, 26), they may also play roles in the process by which PKG activation induces growth inhibition and apoptosis in colon cancer cells. Indeed, previous investigators have found that addition of 13-S-HODE to the growth medium can inhibit proliferation in RKO, Caco-2, and DLD-1 colorectal cancer cells (11, 12, 16), and we have seen similar effects in SW480 cells. We should stress, however, that these effects were seen with only high extracellular concentrations of 13-S-HODE, in the range of 50 to 100 μmol/L. The actual intracellular concentrations achieved in these studies were not determined. Therefore, it is not apparent that the relatively low levels of 13-S-HODE and 15-S-HETE seen in the present study (Fig. 4) play critical roles in the growth inhibition and apoptosis induced by the activation of PKG.

Previous studies indicated that an antisense cDNA for 15-LOX-1 blocked Celecoxib-induced apoptosis in RKO cells (17). We found that inhibition of the expression of 15-LOX-1 with siRNA in SW480 cells could markedly reduce the growth-inhibitory effects of Exisulind (Fig. 5). Because this did not completely protect the cells, especially when we used a high concentration of Exisulind (Fig. 5B), other factors seem to play a role in the growth-inhibitory effects of Exisulind in SW480 cells. Presumably, these include decreased expression of cyclin D1 and β-catenin, c-Jun NH2-terminal kinase activation, and induction of p21CIP1 (5–8). Nevertheless, the present approach by demonstrating that stable overexpression of wild-type PKG Iα, but not a dominant-negative mutant of this protein, causes increased expression of 15-LOX-1 in SW480 cells (Fig. 3). Taken together, these findings provide strong evidence that activation of PKG is sufficient to induce expression of 15-LOX-1. These findings may explain how NSAIDs and related compounds can induce 15-LOX-1 expression via a COX-2-independent mechanism (15–17), because various NSAIDs including Celecoxib can inhibit cGMP-phosphodiesterase, thereby increasing cellular levels of cGMP and activating PKG.


7 Unpublished studies.
study provides definitive evidence that activation of PKG in colon cancer cells is sufficient to induce 15-LOX-1 expression (Figs. 1–3).

We should emphasize that in the present study, we used high concentrations of Exisulind (300-600 μmol/L), but the plasma levels in humans is 20 to 50 μmol/L (27). However, in vivo Exisulind undergoes enterohepatic recirculation and with chronic exposure, the tissue levels in the intestine and intestinal tumors may be higher. Nevertheless, it remains to be determined whether Exisulind and other compounds that can cause activation of PKG induce 15-LOX-1 in vivo.

References

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