Effects of cytokine treatment on angiotensin II type 1A receptor transcription and splicing in rat cardiac fibroblasts

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ANGIOTENSIN II (ANG II), the main active peptide of the renin-angiotensin system, plays an important role in regulating the cardiovascular and renal systems during health and in a variety of disease states. Two major receptors exist for ANG II that are designated type 1 and type 2 (AT1 and AT2, respectively). Most of the known biological functions of ANG II can be attributed to AT1 receptors, whereas AT2 receptors tend to act antagonistically when expressed (12, 23). Mice and rats possess two isoforms of AT1 receptors (AT1A and AT1B) that function similarly but are encoded on different chromosomal regions (3). Rat AT1A receptors, which are the most widely studied, are encoded on chromosome 17q12 and consist of three exons interspaced by two large introns. Exons 1 and 2 represent the 5′ untranslated region (UTR), and exon 3 encodes the full open reading frame (ORF; Ref. 34). Encoding of the entire ORF on the final exon is a characteristic of AT1 receptors that is conserved between rats and humans (5, 34).

Postmyocardial infarction (post-MI) remodeling of the extracellular matrix (ECM) includes formation of a replacement scar at the infarct site and deposition of fibrous tissue in noninfarcted myocardium (1). Proper formation of a replacement scar is necessary to maintain structural integrity of the heart. Fibrosis in noninfarcted regions of myocardium, however, can both increase diastolic stiffness and cause contractile dysfunction (2). ANG II is elevated in the hearts of animals with experimentally induced MI (38). Administration of angiotensin-converting enzyme (ACE) inhibitors or AT1 antagonists inhibits adverse ECM remodeling and reduces mortality in these animals (7, 20, 28, 37). ACE inhibitors also increase survival of humans after MI (29). In experimental animals, chronic elevation of circulating ANG II levels alone has been shown to lead to interstitial fibrosis of myocardium (33). In addition, stimulation of cultured rat cardiac fibroblasts with ANG II can lead to increased proliferation, synthesis of ECM proteins, and secretion of profibrotic factors such as transforming growth factor-β (17, 30, 35), which are effects related to activation of AT1A receptors.

Increased AT1 receptor density has been demonstrated near the infarction zone and in noninfarcted regions of myocardium after MI (18, 25). Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are increased in rat heart after experimental MI (15, 26) and have been shown to colocalize with AT1 receptors on nonmyocytes (10). We have demonstrated previously that AT1 receptor mRNA and protein are upregulated in cultured neonatal rat cardiac fibroblasts by TNF-α and IL-1β (11). Because increased AT1 receptor density enhances fibroblast functions that favor fibrosis (27), cytokine-induced upregulation of AT1 receptor mRNA and protein appears to be an important determinant of ECM remodeling after MI. Thus determining the mechanisms and signaling pathways involved in AT1 upregulation by TNF-α and IL-1β would further our understanding of post-MI ECM remodeling and could provide insights toward new approaches for treatment.

We have previously shown (4) that the transcription factor nuclear factor-κB (NF-κB) is necessary for cytokine-induced
AT₁ mRNA upregulation. The present studies were performed to determine how proinflammatory cytokines and NF-κB increase the level of AT₁₅ receptors in rat cardiac fibroblasts. Our findings indicate that cytokine stimulation increases transcription of AT₁₅ through a mechanism that appears to involve two NF-κB binding sites within the 5' flanking region of the gene. Cytokine treatment also alters AT₁₅ mRNA splicing, which results in the preferential increase of a splice variant that is translated more efficiently than the others. Thus cytokines increase AT₁ receptor levels mainly by increasing transcription but also by altering mRNA splicing.

MATERIALS AND METHODS

Cultured cells. Animal protocols were approved and monitored by the Institutional Animal Care and Use Committee of the University of California, San Diego. Rat cardiac fibroblasts were isolated and maintained as described previously (4). The 293A cells used in this study were purchased from Invitrogen and were grown under the same conditions as the fibroblasts. In some experiments that involved general cellular processes (e.g., inhibition of translation by a minicistron), 293A cells were used because they could be transfected more readily than primary fibroblasts.

RT-PCR for AT₁₅ heterogeneous nuclear RNA. Total RNA was isolated from confluent neonatal rat cardiac fibroblasts using an RNasea kit (Qiagen) and was then digested with RNase-free DNase I. Concentration of RNA was determined by measuring absorbance at 260 nm. RT-PCR was performed on 100 ng of total RNA using the Titan One Tube RT-PCR System (Roche Applied Science) following the manufacturer’s instructions. Primers were chosen to amplify AT₁₅ heterogeneous nuclear RNA (hnRNA; across the exon 1-intron 1 junction and across the intron II-exon 3 junction), AT₁₅ mRNA (across the exon 2-exon 3 junction), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (see Table 1, nos. 10–17). PCR cycling parameters were as follows: 50°C for 30 min (1 cycle); 94°C for 2 min (1 cycle); 94°C for 30 s, 55°C for 30 s, 68°C for 45 s for 45 s (10 cycles then x cycles, with a 5-s elongation increment per cycle, where x = 5 for mRNA and x = 10 for hnRNA); and 68°C for 7 min. PCR reactions were purified with a QIAquick PCR Purification Kit (Qiagen), brought to 0.4 N NaOH and 10 mM EDTA, and heated at 95°C for 10 min. Denatured samples were slots onto a charged nylon membrane (Magnacharge; GE Osmonics; Minnetonka, MN) using a Bio-Dot SF (slot format) slot-blotting apparatus (Bio-Rad Laboratories) that slotted onto the membrane was labeled with 32P (using a Mega-labelling kit) for 2 min (1 cycle); 94°C for 30 s, 53°C for 30 s, and 68°C for 45 s (10 cycles, with a 5-s elongation increment per cycle); and 68°C for 7 min. PCR reactions were purified with a QiAquick PCR Purification Kit and electrophoresed on a 12% polyacrylamide gel (Mini-Protean II; Bio-Rad) in 40 mM Tris acetate with 1 mM EDTA until the bromophenol blue tracking dye exited the bottom of the gel. The gel was stained for 30 min in 40 mM Tris acetate with 1 mM EDTA and Vistra Green nucleic acid gel stain (Amersham Biosciences) and then visualized on the Storm 860 fluorescent scanner.

Electrophoretic mobility shift assay. Gel-shift assays were performed as described previously (4). Briefly, double-stranded synthetic oligodeoxyribonucleotides were radiolabeled with T4 polynucleotide kinase and [γ-32P]ATP and purified with a QiAquick Nucleotide Removal Kit (Qiagen). Three oligonucleotides were used that correspond to the NF-κB consensus sequence in the B cell κ-light-chain enhancer region and the putative NF-κB binding sequences at −365 and −2540 bp of the rat AT₁₅ gene (for sequence of upper strands, see Table 1, nos. 1–3). Nuclear extract from confluent neonatal rat cardiac fibroblasts (2 μg of protein) was incubated with the radiolabeled probe. Bound probe and free probe were separated by electrophoresis on a nondenaturing polyacrylamide gel. The gel was dried and exposed to film (Kodak BioMax MS) or to a storage phosphor screen.

Cell transfection. The 293A cells (3 × 10⁵ cells/well) were plated in a six-well dish and allowed to grow overnight. Cells were then transfected using FuGENE 6 Transfection Reagent (Roche Applied Science) according to the manufacturer’s instructions. Reagent amounts used per well were 0.25 μg of pIk-lacZ (to normalize transfection efficiencies; kindly provided by Dr. Michael B. Green, University of Ottawa, Ontario, Canada), 0.25 μg of luciferase reporter plasmid, 1.5 μl of FuGENE 6, and 2 ml of serum-containing DMEM. Cells were allowed to transfect for 24 h and then were treated with cytokine if indicated. Neonatal rat cardiac fibroblasts were transfected using essentially the same procedure except that to improve transfection efficiency, 2 × 10⁵ cells/well were plated, and the amounts of plasmid and FuGENE were doubled.

Table 1. Synthetic oligodeoxyribonucleotides

<table>
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<tr>
<th>No.</th>
<th>Name</th>
<th>5’→3’ Sequence</th>
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<tr>
<td>1</td>
<td>κB EMDA</td>
<td>ATGGAGGGGACCTTCCCCAGGC</td>
</tr>
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<td>AGCAGAGGGGAGTCTCCATATC</td>
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Known or putative nuclear factor (NF)-κB binding elements are underlined.

All synthetic DNA was obtained from Prolog (Boulder, CO).
**Reporter assays.** Cell lysates were assayed for β-galactosidase and luciferase activity essentially as described by Guo et al. (9). Lysates from untransfected cells were also assayed, and the resulting absorbance values were subtracted from all values as background. The β-galactosidase activity was calculated as milliunits per microliter of cell lysate, where one milliunit hydrolyzed 1 nmol 2-nitrophenyl β-D-galactopyranoside per minute at 37°C. Luciferase activity was calculated as relative light units per microliter of cell lysate and was normalized to β-galactosidase activity.

**Construction of reporter plasmids.** Reporters consisting of six tandem NF-κB elements upstream of a minimal SV-40 promoter driving luciferase were derived from the plasmid pGL3-Promoter (Promega; Madison, WI). Complimentary synthetic oligodeoxynucleotides consisting of 6× kB, 6× −365, or 6× −2540 elements (Table 1, nos. 4–9) were annealed and then ligated into the Small/BglII sites of pGL3-Promoter. Chemically competent JM109 Escherichia coli were transformed with ligation mixtures, and plasmid clones were screened for the presence of the EcoRI restriction site arising from the synthetic DNA.

For AT1A 5′ UTR sequences, both a BstXI and an EcoRV site were inserted between the transcriptional and translational start sites of the luciferase gene in the vector pGL3-Promoter by ligation of a synthetic linker adapter (formed by annealing oligonucleotides nos. 20 and 21 from Table 1) into the HindIII and NcoI sites of the vector. AT1A 5′ UTR sequences (from the start of transcription to immediately before the start codon) were amplified from rat RNA using the Titan One Tube RT-PCR System (for primers, see Table 1, nos. 22 and 23). PCR cycling parameters were as follows: 50°C for 30 min (1 cycle); 94°C for 2 min (1 cycle); 94°C for 30 s, 50°C for 30 s, 68°C for 30 s (10 cycles, then 25 cycles with a 5-s elongation increment per cycle); and 68°C for 7 min. The PCR fragments were ligated into the TA cloning vector pCR2.1 (Invitrogen), and three clones were selected that represented the 1-2-3, 1-2-3+6, and 1-3 splice variants (see Fig. 5A for representative structures), all in the forward orientation. The inserts were excised by digestion with EcoRV and partial digestion with BstXI (to minimize cutting at the BstXI site within the AT1A 5′ UTR) and then ligated into the BstXI-EcoRV sites of the modified pGL3-Promoter vector. Thus the entire AT1A 5′ UTR was incorporated into the 5′ UTR of the luciferase reporter gene, and there were three constructs in total, one for each of the AT1A splice variants.

To minimize the effect of lipopolysaccharide on cultured cells, selected plasmid clones were purified using an EndoFree Plasmid Maxi Kit (Qiagen).

**Oligodeoxynucleotide-directed mutagenesis.** Plasmid 1-2-3 (see Fig. 5A) was transformed into E. coli strain JM109 and rendered single stranded by infection with helper filamentous bacteriophage.

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![Fig. 1](http://example.com/image1.png)

**Fig. 1.** Treatment with TNF-α (T) or IL-1β (IL) upregulates both angiotensin (ANG) II type 1A (AT1A) heterogeneous nuclear RNA (hnRNA) and messenger RNA (mRNA) in rat cardiac fibroblasts. *A*: schematic (not to scale) of rat AT1A hnRNA (top) and a spliced, mature mRNA (bottom). Exons 1, 2, and 3 are depicted as shaded boxes that are connected by straight lines representing introns I and II. Regions amplified by RT-PCR are indicated as i, ii, and iii. *B*: fibroblast cultures were stimulated with 1 ng/ml IL-1β or 10 ng/ml TNF-α. Treatment times were chosen to maximize RNA increases (i.e., IL-1β: 3 h for hnRNA, 8 h for mRNA; TNF-α: 6 h for hnRNA, 24 h for mRNA). Total RNA was isolated from cells and subjected to RT-PCR as described (see MATERIALS AND METHODS). Primers that amplified across the exon 1-intron I junction (amplimer i; primer nos. 10 and 11 in Table 1) and the intron II-exon 3 junction (amplimer ii; primer nos. 12 and 13 in Table 1) were utilized for hnRNA, whereas primers that amplified from exon 2 to exon 3 (amplimer iii; primer nos. 14 and 15 in Table 1) were used for mRNA. AT1A RNA levels are plotted as fold increases over the corresponding “no cytokine treatment” value (means ± SE; n = 3). No, no cytokine treatment.
R408 (Promega). Approximately 50 ng of single-strand DNA was annealed to oligonucleotide no. 24 (Table 1) and then treated with 5 U of T4 DNA polymerase and 1 U of T4 DNA ligase in 20 mM Tris-HCl (pH 7.5), 0.5 mM dNTPs, 1 mM ATP, 2 mM DTT, 7 mM MgCl2, and 30 mM NaCl at 37°C for 90 min. The mixture was transformed into competent BMH 71-18mutS E. coli (Promega) and grown overnight in Luria-Bertani broth with 100 μg/ml ampicillin. The plasmid mixture was isolated from the culture, transformed into competent TOP10 E. coli (Invitrogen), and plated on Luria-Bertani agar with 100 μg/ml ampicillin. Single colonies were selected and screened for the presence of the newly generated Pmel restriction site. Thus both start codons in AT1A exon 2 were eliminated by point mutation, and the resulting mutant was termed Mut-1-2-3 (see Fig. 5A). Successful mutagenesis was verified by sequencing (DNA Sequencing Shared Resource; University of California, San Diego Cancer Center).

RESULTS

Cytokines IL-1β and TNF-α increase transcription of rat AT1A gene. We have previously demonstrated that the proinflammatory cytokines TNF-α and IL-1β upregulate AT1A mRNA in neonatal rat cardiac fibroblasts (10, 11), a process that involves the transcription factor NF-κB (4). To provide additional evidence that this upregulation resulted from increased transcriptional activity, RT-PCR of AT1A mRNA was performed (8, 14). Consistent with our previous findings, AT1A gene.

mRNA levels, as measured by amplifying from exon 2 to exon 3 (Fig. 1B, bottom). Cytokine treatment also increased AT1A mRNA levels as measured by amplification across the exon 1-intron I junction and across the intron II-exon 3 junction (Fig. 1B, top). Amplimers were not detected in the absence of reverse transcription, and thus did not originate from contaminating DNA (data not shown). Cytokine treatment failed to alter GAPDH mRNA levels (using primers no. 16 and 17 from Table 1; data not shown), which indicates that the observed upregulations of AT1 RNA were not the result of a global alteration of RNA levels. Therefore, the finding that proinflammatory cytokines increase AT1A mRNA stability (11) collectively indicate that AT1A upregulation occurs transcriptionally.

Identification of rat AT1A start of transcription and upstream NF-κB binding sequences. To determine the region of the AT1A promoter used in rat neonatal cardiac fibroblasts, RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE; GeneRacer Kit; Invitrogen) was performed on total RNA from cultured cells. All 5′ RACE PCR products started with the sequence 5′-TCCAGGCAGTTGGGA-3′, which indicates that the start of transcription occurred 3 bp upstream of that previously reported for rat vascular smooth muscle, glial, and liver cells (21, 34). Therefore, the promoter region used in neonatal rat cardiac fibroblasts was identical to that published previously (GenBank accession no. S66402).

To identify putative NF-κB binding sites in the rat AT1A promoter region, we screened its known sequence (GenBank accession no. S66402) against the vertebrate matrix of the TRANSFAC database (13) using the TFSEARCH computer program (available at http://www.cbrc.jp/research/db/TRANSFAC.html) at a threshold score of 85.0. This screening identified two putative sites at −365 (5′-GGGAGTTCCC-3′) and −2540 (5′-GGGAAAGCCC-3′) bp. To verify qualitatively that these putative sites could bind NF-κB, electrophoretic mobility shift assays (EMSAs) were performed. Similar to that observed with the classical κB element (5′-GGGACATTCCC-3′), a band shift was observed with either putative sequence after treatment of cardiac fibroblasts with TNF-α or IL-1β (Fig. 2A). To provide additional evidence of NF-κB binding, band shifts were competed with excess nonradiolabeled κB probe (Fig. 2B, κB, lane 3; −365, lane 8; and −2540, lane 9).
revealed the expression of three splice variants (Fig. 4A, top, lane 1). DNA sequencing of the two major PCR products demonstrated that the smaller amplimer represented exon 1-3, whereas the larger represented exon 1-2-3. In addition, there was a minor amplimer representing exon 1-2-3, where the splice-acceptor site in exon 3 had been shifted 6 bp upstream (Fig. 4A, top, exon 1-2-3+6). The variants differed in size by ±89 bp (approximately the size of exon 2), which explains why they appeared as a single band on Northern blots. All rat tissues examined expressed the three variants with exon 1-2-3 predominating; the notable exception was brain tissue, where expression levels were low but exon 1-3 did appear more prevalent (Fig. 4A). The sequences of the splice variants were submitted to GenBank (accession nos. AY585652, AY585653, and AY585654).

We next addressed whether expression levels of the splice variants were altered by cytokine treatment. Treatment of fibroblasts with 1 ng/ml IL-1β for 8 h or 50 ng/ml TNF-α for 24 h increased the levels of all three variants (Fig. 4B). However, quantification of band intensities revealed that cytokine treatment increased the amount of the exon 1-3 variant (IL-1β, 3.6×; TNF-α, 3.2×) more than the exon 1-2-3 variants (IL-1β, 2.8×; TNF-α, 2.7×). When expressed as a ratio to minimize variations between different explants of primary fibroblasts, this effect was significant with IL-1β but not quite significant with TNF-α (Fig. 4C). Because of its minor contribution and the difficulty of cleanly separating it, the exon 1-2-3+6 variant was amalgamated with exon 1-2-3 in these quantifications. Therefore, in addition to increasing transcription of all variants, IL-1β treatment (and TNF-α to a lesser extent) altered posttranscriptional splicing of AT1A, with the smaller variant increasing more than the larger variants.

**Fig. 3. Sequences at −365 and −2540 bp of rat AT1A gene increase transcript from a minimal promoter upon NF-κB activation.** Luciferase reporter plasmids that contain six tandem NF-κB binding elements upstream of a minimum SV-40 promoter were constructed as described (see MATERIALS AND METHODS). Cells (293A cells and neonatal rat cardiac fibroblasts) were cotransfected with the indicated luciferase reporter and a vector encoding LacZ to control for variations in transfection efficiency. At 24 h posttransfection, cells were stimulated without (open bars) or with (solid bars) 50 ng/ml of either recombinant human or rat TNF-α for 4 h. A: 293A cells stimulated with recombinant human TNF-α. B: neonatal rat cardiac fibroblasts stimulated with rat TNF-α. Cell extracts were prepared and assayed for luciferase and β-galactosidase as described (see MATERIALS AND METHODS). Luciferase light units were normalized to β-galactosidase activity and plotted as fold increases over “no cytokine” values (means ± SE; n = 3). Minimal SV-40 is the parent vector, pGL3-Promoter (Promega), from which the other constructs were derived.

Identification of AT1A splice variants and differential up-regulation by cytokines. While we performed RACE to identify the promoter region, it became apparent that more than one transcript size of AT1A mRNA existed. A higher-resolution separation of RT-PCR products spanning exon 1 to exon 3 revealed the expression of three splice variants (Fig. 4A, top, lane 1). DNA sequencing of the two major PCR products demonstrated that the smaller amplimer represented exon 1-3, whereas the larger represented exon 1-2-3. In addition, there was a minor amplimer representing exon 1-2-3, where the splice-acceptor site in exon 3 had been shifted 6 bp upstream (Fig. 4A, top, exon 1-2-3+6). The variants differed in size by ±89 bp (approximately the size of exon 2), which explains why they appeared as a single band on Northern blots. All rat tissues examined expressed the three variants with exon 1-2-3 predominating; the notable exception was brain tissue, where expression levels were low but exon 1-3 did appear more prevalent (Fig. 4A). The sequences of the splice variants were submitted to GenBank (accession nos. AY585652, AY585653, and AY585654).

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Promoter. Thus the AT1A 1-3 variant is expected to produce more AT1A protein per unit of mRNA than the 1-2-3 or 1-2-3-6 variant. In addition, the 1-2-3 variant is expected to produce more protein per unit of mRNA than the 1-2-3+6 variant.

**DISCUSSION**

These studies were designed to investigate the mechanisms involved in cytokine-induced upregulation of AT1A receptors. Our results demonstrating increased hnRNA levels suggest that mRNA upregulation occurs transcriptionally. To further elucidate the role of NF-κB in transcriptional regulation of AT1, we searched the known AT1A promoter region for potential NF-κB binding sites. Two potential sites were identified and were verified by luciferase reporter assays. Therefore, NF-κB can increase via enhanced nuclear stability, increased hnRNA synthesis in cardiac fibroblasts treated with either IL-1β (IL; 1 ng/ml for 8 h) or TNF-α (T; 50 ng/ml for 24 h) or no cytokine treatment (No) and subjected to RT-PCR. Positions of the three splice variants are indicated (right). C: smallest AT1A splice variant, exon 1-3, increases to a greater extent with cytokine treatment. The 1-3 and amalgamated (1-2-3 + 1-2-3+6) signals from B were quantified on the Storm 860 fluorescent scanner using ImageQuant software. The 1-3 value was multiplied by 1.392 to correct for its smaller mass. Ratio of the corrected 1-3 to the amalgamated 1-2-3 signal was plotted (mean ± SE; n = 6). Values were compared using one-way ANOVA and subsequent Dunnett’s multiple-comparison testing. *P < 0.01, significantly different from “No” value; NS, not significantly different from “No” value (P > 0.05).
Previously as unique transcripts. The human AT1 gene also possesses a single coding exon that is preceded by multiple exons comprising the 5' UTR. In humans, four alternatively spliced mRNAs have been identified (19). Human exon 3 has an inframe start codon that can produce a long receptor isoform with a greater than threefold diminished affinity for ANG II (6, 19), since it possesses the exon 2 minicistron and hairpin.

We have shown previously that the inflammatory cytokines TNF-α and IL-1β upregulate AT1A mRNA and protein in cultured neonatal rat cardiac fibroblasts (11). Both inflammatory cytokines and increased AT1 receptor density are found in the post-MI hearts of animal models and humans (10, 15, 18, 25, 26). These findings suggest that TNF-α and/or IL-1β present in vivo in post-MI hearts are responsible for the observed upregulation of AT1 receptors. Receptor upregulation induced by these cytokines in cultured fibroblasts enhances [3H]proline incorporation and tissue inhibitor of metalloproteinase-1 (TIMP-1) protein production and reduces matrix metalloproteinase-2 (MMP-2) levels upon ANG II stimulation (27). These findings suggest that increased AT1 receptor levels enhance ANG II-mediated functions favoring ECM deposition. Inflammatory cytokines such as TNF-α can directly stimulate ECM degradation (27, 32), but through AT1 upregulation, for example, they may facilitate subsequent events that lead to ECM deposition. Studying the mechanism(s) of cytokine-induced AT1 upregulation in cultured neonatal rat cardiac fibroblasts should help improve understanding of how fibroblasts modulate their activity within the environment of post-MI hearts. The present studies show that proinflammatory cytokines increase AT1 receptor expression mainly by increasing transcription but also by altering mRNA splicing. These results extend our previous findings that NF-kB activation is necessary for cytokine-induced AT1 transcription by demonstrating binding sites in the promoter region. Inhibition of NF-kB activation either by interruption of binding to these sites or by disruption of signaling pathways (4) could be exploited as a therapeutic approach to modify AT1 receptor expression in certain circumstances.
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GRANTS
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