Acidification and glucocorticoids independently regulate branched-chain α-ketoacid dehydrogenase subunit genes

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Wang, X., J. M. Chinsky, P. A. Costeas, and S. Russ Price. Acidification and glucocorticoids independently regulate branched-chain α-ketoacid dehydrogenase subunit genes. Am J Physiol Cell Physiol 280: C1176–C1183, 2001.—Acidification or glucocorticoids increase the maximal activity and subunit mRNA levels of branched chain α-ketoacid dehydrogenase (BCKAD) in various cell types. We examined whether these stimuli increase transcription of BCKAD subunit genes by transfecting BCKAD subunit promoter-luciferase plasmids containing the mouse E2 or human E1α-subunit promoter into LLC-PK1 cells, which do not express glucocorticoid receptors, or LLC-PK1-GR101 cells, which we have engineered to constitutively express the glucocorticoid receptor gene. Dexamethasone or acidification increased luciferase activity in LLC-PK1-GR101 cells transfected with the E2 or E1α-minigenes; acidification augmented luciferase activity in LLC-PK1 cells transfected with these minigenes but dexamethasone did not. A pH-responsive element in the E2 subunit promoter was mapped to a region >4.0 kb upstream of the transcription start site. Dexamethasone concurrently stimulated E2 subunit promoter activity and reduced the binding of nuclear factor-κB (NF-κB) to a site in the E2 promoter. Thus acidification and glucocorticoids independently enhance BCKAD subunit gene expression, and the glucocorticoid response in the E2 subunit involves interference with NF-κB, which may act as a trans-repressor.

acidosis; branched-chain amino acids; gene expression; branched-chain ketoacid dehydrogenase

BRANCHED-CHAIN AMINO ACIDS (BCAA) are the most common essential amino acids in protein, and they can influence metabolic pathways in muscle and other tissues. For example, leucine, its ketoacid α-ketoisocaproate, or 3-methylvalerate, the irreversibly decarboxylated product of α-ketoisocaproate, suppresses muscle protein degradation. [Valine, isoleucine, and their metabolites do not influence muscle protein degradation in isolated rat muscles (41).] Amino acids including leucine stimulate protein synthesis in muscle (30, 41, 51) and adipocytes (18, 34) by enhancing translation initiation. Jefferson and colleagues (18, 30, 34, 51) found that leucine stimulates translation initiation by enhancing the activity of eukaryotic initiation factor-4E (eIF-4E) through mammalian target of rapamycin (mTOR)-dependent mechanisms. Leucine also stimulates insulin production, and insulin modulates protein turnover and other metabolic processes (21, 42, 44). Thus it is not surprising that BCAA degradation is tightly controlled in normal adults and in patients with kidney disease (35, 36, 50).

Branched-chain α-ketoacid dehydrogenase (BCKAD) regulates BCAA disposal by catalyzing their irreversible oxidative decarboxylation. The BCKAD complex is located in mitochondria and is composed of unique E1α, E1β, and E2 subunits and an E3 subunit that is also present in pyruvate dehydrogenase. BCKAD activity can be regulated by BCKAD kinase, which phosphorylates the E1α subunit, inhibiting BCKAD activity. In muscle, BCKAD is primarily in the inactive, phosphorylated state (2–5% of enzyme is active), whereas its activity state (the proportion of dephosphorylated BCKAD) is much higher in kidney (60–70% in the active state) and other tissues (23). We and others have documented concurrent changes in total BCKAD activity (a measure of enzyme content), BCKAD subunit proteins, and the mRNAs encoding BCKAD subunits (i.e., E1α, E2) in response to acidosis and other physiological stimuli (e.g., glucocorticoids, insulin) (7, 8, 10, 17, 45, 52). Because acidosis results in higher glucocorticoid production, it is difficult to separate the two signals in vivo (3, 17, 37).

To separate the influences of acidification from those of glucocorticoids, we studied how these signals change BCKAD activity in LLC-PK1 pig kidney cells because: 1) the kidney is a major organ for BCAA degradation in humans (49), and proximal tubules constitute the bulk of the kidney mass; and 2) LLC-PK1 cells do not respond to glucocorticoids (25, 52). By generating glucocorticoid-responsive LLC-PK1 cells through stable transfection of LLC-PK1 cells with an expression vector encoding the rat glucocorticoid receptor (LLC-PK1-GR101 cells) (52), we were able to study the effects of

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acidosis and glucocorticoids separately in the same general cell type. We found that either a low extracellular pH or dexamethasone can independently stimulate BCKAD activity and increase the amount of enzyme subunit proteins in these kidney cells. We have extended our findings in kidney cells by showing that a low extracellular pH and/or glucocorticoids stimulate the promoters of the BCKAD E1α and E2 subunit genes. Recently, we showed that nuclear factor-κB (NF-κB) participates in the transcriptional regulation of the proteasome C3 subunit by glucocorticoids in L6 muscle cells (15), and we noted that there are NF-κB-like sites in the mouse BCKAD E2 subunit promoter. Therefore, we evaluated whether NF-κB is involved in the glucocorticoid-dependent regulation of BCKAD E2 subunit transcription. Our results provide evidence of genetic mechanisms that act to regulate BCKAD function in response to catabolic stimuli.

METHODS

BCKAD subunit promoter-luciferase minigenes. Promoter-luciferase chimeric minigenes containing the 5′-flanking region [7,000 to 140 base pairs (bp)] of the murine BCKAD E2 subunit gene have been described (11). To evaluate whether NF-κB is involved in the regulation of BCKAD subunit transcription by glucocorticoids, we changed the 10-base NF-κB-like motif in pE2-0.14 to 5′-TAGTTTAGAT-3′ using substituted-primer PCR. A more conservative alteration was also made by changing the first three bases to 5′-AAAATCTT-TCC-3′ in pE2-0.14. [This should not disrupt the NF-κB p65 subunit interaction site (31)]. These sequence changes were verified by DNA sequencing.

An E1α subunit promoter-luciferase minigene was constructed from a −710 to +83 fragment of the human BCKAD E1α gene (9) amplified from human genomic DNA using PCR. The forward primer (ACGTACGTGGTGTTTAC-CTGATCGCTGATGTA) contained eight random bases and a KpnI restriction site (underlined) followed by the E1α sequence (boldfaced and italicized). The reverse primer (ACGTACGTAGATCTGCTCAAACACCGTTTAG) contained eight random bases and a BglII restriction site (underlined) followed by the E1α DNA sequence (boldfaced and italicized). The PCR conditions consisted of 30 cycles of 95°C for 1 min, 48°C for 45 s, and 72°C for 2.5 min. The amplified E1α DNA fragment was ligated into pGL2 Basic (Promega), and the E1α sequence was confirmed by DNA sequencing.

Cell culture and transfections. LLC-PK1 and LLC-PK1-GR101 cells were maintained and transfected as described (52). Cells were transfected with 3 μg of the E2 subunit minigene plasmids or pE1α-0.8 kb plus 6.5 μg of salmon testis DNA using calcium phosphate; 0.5 μg of a control plasmid DNA (pSVβ) encoding β-galactosidase or pRL-TK encoding renilla luciferase) was cotransfected to correct for differences in transfection efficiency (52). Control cells were cotransfected with pGL2 Basic without a promoter element and a control reporter plasmid. Subsequently, cells were maintained in DMEM with 10% fetal bovine serum (FBS; without hygromycin) for 24 h followed by DMEM with 10% charcoal-treated FBS for 48 h (52). Some cells were incubated with 50 nM dexamethasone and/or acidified (pH 7.0) media for 24 h. The biological potency of this concentration of dexamethasone is ~15 times higher than the circulating level of cortisol in an adult (46). To ensure that dexamethasone responses were mediated through the glucocorticoid receptor, cells were incubated with dexamethasone and an equimolar concentration of the steroid receptor antagonist RU-486 (added 1 h before dexamethasone) (28, 52). The media pH was stable during the 24-h treatment period (52).

Luciferase activity. Firefly luciferase activity was measured as described by Brasier et al. (4). Briefly, cells were harvested in lysis buffer [25 mM Tris, pH 7.8, 4 mM EGTA, 1% Triton X-100, 10% glycerol, and 2 mM dithiothreitol (DTT)]. Samples were diluted (1:8) with assay buffer (25 mM Tris, pH 7.8, 4 mM EGTA, 20 mM MgSO4, 2 mM ATP, and 1 mM DTT), and luminescence was measured with a Turner model TD-20/20 luminometer after the addition of d-luciferin. We assessed transfection efficiencies by either 1) dividing the firefly luciferase activity by the respective β-galactosidase activities after normalizing each activity for protein content (33) or 2) measuring the firefly and renilla luciferase activities in the same sample with the Dual Luciferase Reporter Assay system (Promega).

Electrophoretic mobility shift assays. Nuclear protein extracts were prepared from LLC-PK1-GR101 cells according to Dignam et al. (14). The sense strand of the DNA probes used in the binding assays was 5′-GAGGAGGCTTCCTC- CCAGCTG-3′ for the normal NF-κB motif or 5′-GAGGAGAT- CAAATCTCAAGCTG-3′ for the mutant NF-κB site. Binding reactions were performed as described (15). Reaction products were separated in 4% polyacrylamide, 2.5% glycerol gels with 0.5× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) and visualized with a Molecular Dynamics Storm imaging system.

Statistical analyses. Results (means ± SE) between treatment groups were compared with an unpaired Student’s t-test, but for three or more groups, significance was evaluated by one-way ANOVA followed by Tukey’s t-test. In all cases, a value of P < 0.05 was considered significant.

RESULTS

Glucocorticoids induce BCKAD E1α and E2 transcription. To evaluate the influence of glucocorticoids on BCKAD subunit promoters, LLC-PK1-GR101 cells were transfected with a BCKAD E1α or E2 subunit promoter-luciferase reporter plasmid. These cells respond to dexamethasone (52). Basal luciferase activity (without dexamethasone) in cells transfected with either the pE2-7.0 or pE1α-0.8 kb subunit-promoter minigenes was higher than in cells transfected with the control pGL2 Basic luciferase expression plasmid with no promoter (P < 0.05; Fig. 1A). After dexamethasone (50 nM) was added for 24 h, firefly luciferase activity in cells transfected with pE2-7.0 was increased by 127% (P < 0.05 vs. no dexamethasone). Dexamethasone stimulated pE1α-0.8 minigene luciferase activity 259% (P < 0.01 vs. no dexamethasone; Fig. 1B). When cells transfected with either pE2-7.0 or pE1α-0.8 were incubated with equimolar concentrations of dexamethasone plus RU-486, luciferase activity was not increased (Fig. 1B). In a preliminary study, RU-486 alone did not change luciferase activity in cells transfected with pE2-7.0 (data not shown), whereas renilla luciferase activity in cells transfected with pRL-TK (controlled by the thymidine kinase promoter) was not increased by dexamethasone (37.3 ± 5.4 light U/mg without dexamethasone vs. 36.8 ± 3.8 with dexamethasone; P, not significant). Thus glucocorticoids specifically...
cally stimulate the E1α and E2 subunit promoters by mechanisms requiring the glucocorticoid receptor.

The E2 subunit glucocorticoid-responsive element is an NF-κB binding site. E2 promoter-luciferase reporter gene plasmids with progressively shorter fragments of the proximal 5′-flanking region (Fig. 2A) were transfected into LLC-PK1-GR101 cells to localize the region of the promoter that responds to glucocorticoids. Mini-genes containing ≥140 bp of E2 promoter were stimulated by dexamethasone but to a variable degree (i.e., between a 2- and 4-fold increase; Fig. 2B). Maximal activation by dexamethasone was measured in cells transfected with the E2 minigene containing 900 bases of 5′-flanking region (pE2-0.9 kb). With additional distal promoter sequence, induction of luciferase activity by dexamethasone was less effective (Fig. 2B).

**Fig. 1.** Dexamethasone increases branched-chain α-ketoacid dehydrogenase (BCKAD) subunit promoter activities in LLC-PK1-GR101 cells. **A:** LLC-PK1-GR101 cells were transfected with the following promoter-luciferase plasmids: pE2-7.0 kb, pE1α-0.8 kb, or pGL2 Basic (lacking a promoter). pSVβ was cotransfected with the firefly luciferase plasmids to normalize results for transfection efficiencies. Results are means ± SE (n = 5 for each plasmid) of the firefly luciferase activity corrected for transfection efficiency and protein content. **B:** cells were cotransfected with pE2-7.0 kb or pE1α-0.8 kb and pRL-TK. Some cells were incubated (24 h) with dexamethasone alone (50 nM; open bars) or equimolar (50 nM) dexamethasone and RU-486 (solid bars). Results are means ± SE (n = 5 for each plasmid) of the normalized firefly luciferase activity expressed as the percentage of the activity from cells incubated without dexamethasone.

**Fig. 2.** Localization of the glucocorticoid-responsive element in the BCKAD E2 promoter. **A:** a schematic representation of the BCKAD mouse E2 or human E1α-subunit promoter luciferase plasmids depicting the approximate length of the respective subunit promoter in each minigene. **B:** LLC-PK1-GR101 cells were cotransfected with BCKAD E2 promoter minigenes and the control plasmid pRL-TK. Dexamethasone (Dex, 50 nM) was added to some cells for 24 h before measuring firefly luciferase activity. Results (means ± SE; n = 5 for each plasmid) are normalized luciferase activity (i.e., luminescence) in Dex-treated cells expressed as a percentage of the activity in transfected cells not treated with Dex. *P < 0.05 vs. pH 7.4 without Dex.
ACIDOSIS, GLUCOCORTICOIDS, AND BCKAD GENE EXPRESSION

With E2 subunit promoter-luciferase minigenes containing <140 bp of flanking sequence, basal luciferase activity in transfected LLC-PK1-GR101 cells decreased significantly (data not shown). In this region, there is a pyrimidine-rich region (−132 to −120) and two inverted CCAAT-like motifs (−85 to −79 and −42 to −48) that could influence basal transcription activity (12). There is also an NF-κB-like sequence at position −28 to −19 (5′-GCGTCTTTC-3′) that has similarities to a site in the proteasome C3 subunit that we found was critical for its transcription activation by glucocorticoids (15). Using two strategies, we tested whether this E2 subunit NF-κB site is important for its transcription activation by glucocorticoids.

Acidification stimulates transcription of BCKAD E2 and E1α-subunits. To determine if extracellular acidification activates the BCKAD E2 or E1α-promoters, we transfected steroid-receptor null LLC-PK1 cells with the BCKAD subunit promoter-luciferase minigenes (25, 52). These cells make it possible to distinguish responses to a reduced extracellular pH from those of the glucocorticoid receptor (25, 52). Acidification (24 h) increased luciferase activity from pE2-7.0 kb by 87% (P < 0.05 vs. pH 7.4) but not in cells transfected with pE2-0.3 kb (Fig. 4A). Likewise, acidification increased luciferase activity from pE1α-0.8 kb by 60% (P < 0.05 vs. pH 7.4; Fig. 4A). Dexamethasone did not increase luciferase activity in any of the transfected cells. Similar responses were obtained when LLC-PK1-GR101 cells were transfected with the pE2-7.0 or pE1α-0.8 kb minigenes; however, acidification did not increase luciferase activity in cells transfected with E2 minigenes containing ~4,000 bp or less of promoter sequence (Fig. 4B). Thus acidification acts independently of glucocorticoids to stimulate E2 and E1α gene expression.

Acidification and glucocorticoids act cooperatively to stimulate BCKAD subunit transcription. To evaluate if extracellular acidification and glucocorticoids can act cooperatively to increase E2 or E1α transcription, LLC-PK1-GR101 cells were transfected with pE2-7.0 or pE1α-0.8 kb and then were subjected to one or both stimuli together. With either subunit minigene, dexamethasone plus acidification increased luciferase activity more than in cells incubated with either dexamethasone or a low pH alone (Fig. 5). In cells transfected with the pE2-0.3-kb minigene, dexamethasone plus acidification increased luciferase activity to a level similar to that measured with glucocorticoids alone (Fig. 5).

DISCUSSION

In patients or rats with uremia or metabolic acidosis, the plasma levels of BCAA are reduced (2, 19, 22, 37, 39). In humans, the tissues that contribute the most to whole body leucine oxidation are skeletal muscle and kidney (49). In muscle, catabolic stimuli (e.g., acidosis treated or dexamethasone-treated LLC-PK1-GR101 cells. A protein was present in control cell extracts that binds to the NF-κB probe (Fig. 3B). Dexamethasone reduced the amount of protein-DNA complex (Fig. 3B). The binding of the protein to the NF-κB sequence was specific, because no protein-DNA complex was detected when an excess (100-fold) of unlabeled NF-κB DNA probe was present in the binding reaction or when a mutant NF-κB site E2 probe was used as the target DNA (data not shown). We also tested whether the protein that binds to the DNA probe is a member of the NF-κB protein family by including antibodies against the NF-κB p65 subunit in the binding reaction. These antibodies competed with the DNA probe for protein binding and prevented DNA-protein complex formation (Fig. 3B). Thus glucocorticoids act to antagonize a suppressive effect of NF-κB on E2 subunit transcription.

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or glucocorticoids) increase the steady-state levels of subunit mRNAs and total BCKAD activity concurrently (7, 8, 10, 17, 20, 45, 48). In intact rats, it is difficult to separate the effects of acidosis from those of glucocorticoids because glucocorticoid production is stimulated by acidosis (38). Therefore, we studied the regulation of BCKAD activity in LLC-PK1 and LLC-PK1-GR101, because these cells allowed us to determine the separate influences of acidosis and glucocorticoids in the same general cell type. We found that acidification and glucocorticoids independently increase BCAA catabolism and the amounts of mitochondrial BCKAD subunit proteins (52). Our current findings demonstrate that acidosis or glucocorticoids stimulate the transcription of the E2 and E1α BCKAD subunit genes through unique cis-acting elements under conditions that also increase BCKAD activity.

We were surprised to find that stimulation of the mouse E2 subunit promoter by glucocorticoids involves a binding site for the transcription factor NF-κB. First, we found that dexamethasone-induced stimulation of the pE2-0.14 kb reporter gene was prevented by changing the base sequence of the NF-κB binding motif at position 28 to 19. When the first three bases of the NF-κB site were changed, basal luciferase activity increased 12-fold, suggesting that NF-κB suppresses E2 subunit promoter activity. Second, an abundant nuclear protein in untreated LLC-PK1-GR101 cells binds to the NF-κB binding site in the E2 subunit promoter, and incubating cells with dexamethasone reduced the binding of this nuclear protein. Formation of the protein-DNA complex was blocked by polyclonal antibodies against NF-κB p65, suggesting that protein epitopes recognized by the p65 antibodies are in close proximity to the DNA binding domain of the protein. Thus, glucocorticoids stimulate E2 subunit transcription by reducing the binding of NF-κB and, hence, its suppressor action on the E2 subunit promoter in LLC-PK1-GR101 cells. This is a different mechanism of glucocorticoid stimulation than occurs in cultured hepatic cells (12). In H4-II-E-C3 hepatoma cells, E2 subunit transcription is increased by dexamethasone, but the response involved the −140 to −70 region of the E2 subunit promoter, which is upstream of the NF-κB site that is important for the response to glucocorticoids in LLC-PK1-GR101 cells. Notably, we found that dexamethasone increased the transcription of the proteasome C3 subunit gene in L6 muscle cells by a mechanism that is similar to the one we identified for the glucocorticoid-mediated induction of the BCKAD E2

Fig. 4. Acidification stimulates BCKAD subunit promoter activities. A: LLC-PK1 cells were transfected with pE2-7.0, pE2-0.3, or pE1α-0.8 kb minigenes and pSVβ as a control plasmid. Cells were incubated at pH 7.4 (control), pH 7.4 with 50 nM dexamethasone (open bars), or pH 7.0 (solid bars) for 24 h. Results (n = 5 for each plasmid) are expressed as described in Fig. 2. B: LLC-PK1-GR101 cells were transfected with the BCKAD E2 promoter minigenes pE2-7.0, pE2-4.0, pE2-2.3, pE2-0.9, pE2-0.3, or pE1α-0.8 kb and pRL-TK as a control plasmid. Cells were incubated at pH 7.4 or 7.0 for 24 h. Results (n = 5 for each plasmid) are as described in Fig. 2. *P < 0.05 vs. pH 7.4.

Fig. 5. Dex and acidification cooperatively increase BCKAD subunit promoter activities in LLC-PK1-GR101 cells. LLC-PK1-GR101 cells were transfected with pE2-7.0, pE2-0.3, or pE1α-0.8 kb and pRL-TK as a control plasmid. Cells were incubated at pH 7.4, pH 7.4 plus 50 nM Dex (open bars), pH 7.0 (gray bars), or pH 7.0 plus 50 nM Dex (black bars) for 24 h. Results are expressed as described in Fig. 2 (n = 5 for each plasmid). *P < 0.05 vs. pH 7.4 with no Dex.
subunit promoter (15). In both cases, dexamethasone interferes with the binding of NF-κB, which acts as a transcriptional suppressor for both promoters. The results of our studies with the BCKAD E1α-subunit promoter suggest that glucocorticoids stimulate this promoter by a NF-κB-independent mechanism, because a NF-κB binding site was not present in the segment of the human E1α promoter that we used in the transfection experiments.

We did not determine how glucocorticoids interfere with NF-κB binding to the E2 subunit promoter, but activated glucocorticoid receptors can block NF-κB-induced transcription of inflammatory genes (e.g., interleukin-6, intercellular adhesion molecule-1) by directly interfering with their binding to DNA response elements and, hence, transcription (13, 32, 40). In contrast to the these reports, NF-κB appeared to be a transrepressor of the E2 subunit gene in our studies. Other potential mechanisms include a glucocorticoid-induced increase in the amount of inhibitory protein of NF-κB (IkB) leading to sequestration of NF-κB in the cytosol (15, 47).

Acidification has been shown to increase transcription of a few specific genes, including renal glutaminase, sodium-hydrogen exchanger-3, and most notably, phosphoenolpyruvate carboxykinase (PEPCK) in rat kidney and proximal tubule cells (5, 6, 24, 26). In the kidney, PEPCK expression is stimulated by either acidosis or glucocorticoids, but the location in the PEPCK promoter where acidification exerts its effects and the identity of the acidification-induced transactivating factor(s) binding to the PEPCK promoter have not been conclusively identified (6, 24, 43). Cassuto et al. (6) reported that a HNF-1 (P2) recognition motif is required for pH sensitivity, whereas Holcomb et al. (24) identified other promoter regions that appear to be crucial. In both cases, the upstream elements that were reported to be responsive to acidification were located close to the transcription initiation site.

How could acidification stimulate E2 or E1α promoters? The intracellular pH in cultured muscle and LLC-PK1 cells decreased when the extracellular pH was reduced (16, 29). In acidicem rats, including those with chronic uremia, the intracellular pH in muscle was not reduced (16, 29). In acidemic rats, including those with chronic acidosis and renal failure. In rats that were treated identically, BCKAD activity and the levels of BCKAD subunit mRNAs were increased (1, 22). Thus it is plausible that extracellular acidification stimulates an intracellular signaling pathway with effectors that can regulate the transcription of genes including the BCKAD E1α and E2 subunits. In one case, acute acidification increased the transcription of a number of immediate early genes (e.g., Egr-1, c-fos, c-jun) in vivo in rat renal cortical cells and in simian virus 40-transformed, mouse proximal tubule cells (MCT cells) (53). Our current results indicate that there is an acidification-responsive element in the E1α and E2 BCKAD subunit promoter regions. In the E2 subunit gene, this cis-acting element does not require a position close to the transcription initiation site as it is >4,000 bp upstream. In contrast, the acidification-response element in the E1α gene is located in a position more proximal (<710 bp) to the transcription start site. If the acidification-responsive elements in the regions upstream of the E1α and E2 genes are identical, we conclude that they do not require a specific location in the gene (e.g., adjacent to the transcription start site). Alternatively, the response elements responding to acidification in the E1α and E2 genes may be different.

In summary, we have shown that two BCKAD subunit promoters are activated by glucocorticoids or acidified media. The stimuli also increased BCKAD subunit protein and total enzyme activity in LLC-PK1-GR101 cells (52). The concurrent nature of these responses suggests that increased BCKAD subunit transcription could be a mechanism for the increase in BCKAD subunit proteins in these renal proximal tubular cells. Such a mechanism, coupled with activation/inactivation of the BCKAD enzyme by a kinase/phosphatase system, would allow cells to precisely regulate the activity of BCKAD, the key enzyme responsible for the degradation of the essential BCAA in different catabolic conditions.

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