Altered Expression of P2 Receptor mRNAs in the Basilar Artery in a Rat Double Hemorrhage Model

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Background and Purpose—Extracellular ATP might induce cerebral vasospasm after subarachnoid hemorrhage through P2 receptor. To investigate the roles of P2 receptor subtypes in vasospasm, we examined the changes in mRNA expression of P2 receptor subtypes in basilar arteries from double cisternal blood injection rat models.

Methods—One hundred male Sprague-Dawley rats, each weighing 350 to 400 g, were divided into 2 groups of 50. In the first group (n=50), the autologous arterial blood (0.2 to 0.3 mL) was injected into the cisterna magna on days 0 and 2. The rats were killed on day 3, 5, or 7 (n=10 in each group). In the sham group (n=10), the rats were injected with saline (0.3 mL) instead of blood. Ten rats were killed without blood or saline injection and served as control. The basilar arteries from rats in each group were used for reverse transcription and polymerase chain reaction. In another group of 50 rats, the same experiment was conducted, and the basilar arteries were collected for transmission electron microscopic study.

Results—in the subarachnoid hemorrhage groups, transmission electron microscopy showed the reduction in vessel perimeter on days 5 and 7 to be approximately 30% to 40%. The P2X1 mRNA level was significantly decreased on day 3 and recovered on days 5 and 7. The P2Y1 mRNA level was transiently increased on day 5, and the P2Y2 mRNA level was elevated from day 5 to day 7 (P<0.05).

Conclusions—The differential expression of the P2 receptors indicates that P2X1 subtype might not play an important role in vasospasm. The upregulation of P2Y1 and P2Y2 receptors might enable ATP to produce contraction at low levels of concentration. (Stroke. 2001;32:516-522.)

Key Words: adenosine triphosphate • muscle, smooth • phenotype • receptors, purinergic P2 • rats

Cerebral vasospasm is a leading cause and a frequent complication of the morbidity and mortality of subarachnoid hemorrhage (SAH). Cerebral vasospasm is characterized by a delayed, prolonged constriction, occurring mainly in vascular smooth muscle cells, and by cell proliferation within the arterial wall. The cause of vasospasm might be vasoactive substances (such as oxyhemoglobin, purine, and pyrimidine nucleotides) released into the subarachnoid space by the dissolution of the resultant blood clot or by vasoactive agents released from the vessel wall (such as endothelin). These spasmogens might produce gene expression changes that lead not only to a prolonged constriction but also to cell differentiation, cell proliferation, and cell death.

The P2 receptor (P2 nucleotide receptor) was also referred to as P2 purinergic receptor (purinoceptor) previously. A large number of P2 receptor subtypes can be divided into 2 major families: the ligand-gated ion channel P2X receptors and the G protein–coupled P2Y receptors. More than 7 P2X and 8 P2Y subtypes were identified. P2 receptors play a central role in the functions of extracellular nucleotides in peripheral and central neuronal tissues, in the regulation of lung surfactant secretion, and in the regulation of the cardiovascular system. P2 receptors have been identified in cerebral arteries. Among the many identified P2 receptor subtypes, P2X1, P2Y1, and P2Y2 are the major functional populations expressed in vascular tissues. The P2X1 receptor exists mainly in smooth muscle cells. Activating P2X1 induces Ca2+ influx and cerebral arterial contraction. P2Y1 and P2Y2 are G protein–coupled receptors. Activating P2Y receptors leads to increased intracellular Ca2+, cerebral arterial contraction, and vasospasm in animals. The P2Y1 and P2Y2 subtypes, in particular among the P2 receptors, are also involved in mitogenesis via the mitogen-activated protein kinase pathway.

Since ATP and P2 receptors are believed to be involved in vasospasm, we examined the expression of P2X1, P2Y1, and P2Y2, the most frequently expressed and studied P2 receptors in vascular tissue, in the basilar artery in a rat double hemorrhage model.

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Materials and Methods

Rat Double Hemorrhage Model

The protocol for this study complies with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Laboratory Animal Resources (Commission on Life Sciences, National Research Council) and approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center. Thirty Sprague-Dawley male rats (each weighing 350 to 400 g) were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and were allowed to breathe spontaneously in a supine position. The inguinal region was shaved and prepared in a sterile manner. After we exposed the left femoral artery and prepared to obtain arterial blood by cannulation, a midline incision on the dorsal surface of the neck was made from the cranial vertex to the lower cervical spine. The subcuticular and nuchal muscles were divided bilaterally to expose the atlanto-occipital membrane. The rat was then turned over to a supine position again, the blood was drawn, and the rat was placed again in the prone position. With the rat in a prone, head-down position, a 27-gauge needle was inserted into the cisterna magna via an atlanto-occipital membrane puncture. Correct positioning of the needle was determined by successful aspiration of cerebrospinal fluid. After a nonheparinized syringe was used to aspirate 0.3 mL of cerebrospinal fluid, 0.25 to 0.5 mL of autologous arterial blood was withdrawn from the femoral artery and then carefully injected into the cisterna magna over 3 minutes. After injection, the puncture site was immediately sealed with ethyl cyanoacrylate glue. The muscles were sutured layer to layer, and the skin incision was closed. The rats were placed prone in a head-down position at a 30° angle for 20 minutes to ensure rostroventral blood distribution around the basal intracranial arteries. They were kept warm by a heating blanket and monitored closely until they recovered. The day of the first injection was set at day 0. On day 2, the rats received a second injection following the same procedure, but blood was withdrawn from the right femoral artery. One rat from day 3 group died immediately after the second blood injection and was excluded from this study. On days 3, 5, and 7, the rats scheduled for death were euthanatized with an overdose of anesthesia and then decapitated. The basilar arteries were immediately removed under surgical microscope with minimal mechanical manipulation. The vessels were snap-frozen in liquid nitrogen and kept at −80°C until further analysis. On days 0 and 2, these investigators substituted a sterile 0.9% NaCl solution for blood and administered a double injection to a sham group of 10 rats. The sham rats were killed on day 7. Basilar arteries were collected from a control group of 10 rats that underwent no surgical procedure.

Transmission Electron Microscopy and Imaging Analysis

A separate study of 50 rats using this same double hemorrhage model was conducted. These rats were immediately after the second blood injection and were excluded from this study. Rats were euthanatized and killed via left ventricular perfusion of 2% glutaraldehyde-phosphate buffer at physiological blood pressure (100 mm Hg). Basilar arteries were removed and postfixed with 2% glutaraldehyde over a period of 1 week. Ultrathin cross sections of the basilar arteries were stained with uranyl acetate and examined with transmission electron microscopy (TEM). Morphometric determination for lumen perimeter was determined by using a Kodak digital camera and a DigiVision Pro image analysis system (both attached to the LEO 906 TEM). The perimeters of the basilar artery were calculated by imaging analysis. The transverse sections of the basilar artery were scanned by a computer and analyzed as a digital image. The perimeter of the vessels was measured by tracing the entire luminal surface of the intima, and the perimeter of the vessels was calculated. The values from each group were expressed as a percentage of the lumen perimeter of control rat basilar artery.

Reverse Transcription and Polymerase Chain Reaction

Total RNA was isolated from rat basilar arteries with the use of RNAlute B. Total RNA was reverse-transcribed to cDNA for use in polymerase chain reaction (PCR). Amplification was performed with the thermal cycler Power Block II (EPIORC). The thermal cycle profile consisted of denaturation for 1 minute at 92°C, annealing of primers for 1 minute at 56°C, extension for 30 seconds at 72°C, and a final extension step at 72°C for 7 minutes. Reaction conditions and cycle numbers were optimized for each receptor subtype. A relative quantification of the cDNA for each receptor subtype was performed in the logarithmic phase of amplification to obtain a linear relationship between the cycle number and product amplification. An amplification of both the receptor template and an internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was run in parallel. The PCR primers and their expected product size, as previously reported, were as follows: X1 forward primer, 5'-AGAGGGCACTACTACAAGGCAGAA-3'; X1 reverse primer, 5'-GTTAAGGCCTGGGAAAAAG-3' (product size, 434 bp); Y1 fwd primer, 5'-CTGCTGTAGTGGGAGA-3'; Y1 rev primer, 5'-TCCAGTGCCAGAGTGA-3' (663 bp); Y2 fwd primer, 5'-ACCGGCCACCCCTTAATTACT-3'; Y2 rev primer, 5'-CTTAGATGGATTTCCCCA-3' (538 bp); GAPDH fwd primer, 5'-ACCACATGGCATGCATCAC-3'; GAPDH rev primer, 5'-TCCACCCACTGTGCTGTA-3' (452 bp).

We used a semiquantitative PCR rather than a quantitative PCR. We used the housekeeping gene GAPDH as an internal standard. The aliquots of the reverse transcription (RT) products were used with the same amount of cDNA in PCR with primers for GAPDH and with primers for P2 subtypes in each sample. Since GAPDH is a housekeeping gene, the intensity of the resulting GAPDH-PCR product should be the same as if we had used an identical amount of cDNA from control and experimental samples. Thus, the difference between ratios for specific gene/GAPDH is due to the change of the specific gene. Consequently, we can estimate the specific gene mRNA change through the change of this ratio. PCR products were electrophoresed in an ethidium bromide–containing 2% agarose gel in Tris-acetate/EDTA buffer. The gels were analyzed with the use of Gel Doc 1000 and Quantity One software (Bio-Rad). We measured the volume of bands of GAPDH and specific genes in each sample. To correct for any variation in RNA content or cDNA synthesis between samples, each sample was normalized according to its GAPDH content. The ratios of the receptor PCR product/GAPDH product were expressed as a percent increase from those of the control. Because the number of samples of rat basilar arteries was small, 3 to 4 samples from each group were pooled for 1 experiment. Three experiments (from 10 different samples) were averaged for calculation. The linear exponential phases for each gene/GAPDH PCR were 25 to 36 cycles. Thus, we used 32 cycles for P2 subtypes and GAPDH PCR.

Results

Rat Double Hemorrhage Model

All animals were drowsy on days 1 and 3 after cisternal blood injection but resumed normal behavioral patterns and feeding habits the next day. Gross macroscopic observation/inspection of basilar arteries before removal from the brains revealed blood clots on the basal surface of the brains from...
day 3 and 5 samples. The clots were still present but noticeably smaller on samples taken on day 7. Arterial constriction was also observed.

**TEM and Imaging Analysis**

Histological results showed corrugation of the internal elastic lamina and contraction of smooth muscle cells in basilar arteries. Smooth muscle contraction and corrugation of the internal elastic lamina were most severe on days 5 and 7 (Figure 1A and 1B). Imaging analysis demonstrated an overall reduction of the diameter up to 40% in samples collected on days 5 to 7 (Figure 1C).

**Expression of P2 Receptors**

The expression of P2X1 receptors decreased significantly \((P<0.05)\) on day 3 and increased to the normal range on days 5 through 7 in samples from SAH rats. The expression of P2Y1 receptors increased significantly \((P<0.05)\) on day 5 but decreased to the normal range on day 7. The expression of P2Y2 receptors increased on day 5 and remained above normal \((P<0.05)\) in samples taken on day 7. These results are summarized in Figures 2 and 3. In all RT-PCRs of these mRNAs, the results from the sham operation group on day 7 were measured at the same level as the control group.

**Discussion**

The main observations in this study are as follows: (1) double blood injection produced mild to moderate vasospasm on days 5 to 7; (2) mRNA expression of P2X1 receptors in the basilar arteries from the rats assigned to the SAH injection groups was downregulated on day 3 and recovered on days 5 through 7; (3) mRNA expression of P2Y1 receptors was upregulated on day 5 and returned to normal on day 7; and (4) mRNA expression of P2Y2 receptors was upregulated on day 5 and remained above normal on day 7.

**Extracellular ATP and Vasospasm**

The source of the extracellular nucleotides is a cellular component of subarachnoid blood clots. Millimolar-level concentrations of ATP and ADP are included in erythrocytes, and a high level of UTP is also in platelets. Some experimental data have supported the role of extracellular ATP in vasospasm. ATP induces \(\text{Ca}^{2+}\) elevation\(^9\) and contraction\(^12\) of cerebral artery. Even though intraluminal application of ATP produced relaxation, extraluminal application of ATP, a situation that more or less resembles SAH, produced vasoconstriction in cerebral arteries.\(^16\) The removal of ATP by incubating erythrocyte lysate with apyrase (an enzyme that breaks down ATP and ADP into AMP) abolished the action of erythrocyte lysate to elevate \(\text{Ca}^{2+}\).\(^5\) Furthermore, ATP...
produced vasospasm in rat femoral arteries and in a monkey model of cerebral vasospasm. Contradictory evidence regarding the role of ATP in cerebral vasospasm also exists. First, an ATP-induced contraction is less than that of erythrocyte lysate. Second, the ATP level in the bloody cerebrospinal fluid from a canine double hemorrhage model is measured at the nanomolar level, which is considered to produce no contraction (Yin, MD, PhD, unpublished data, 2000). However, these contradictory data do not rule out possible molecular events (except contractions) involving smooth muscle cells in which a previously high ATP level is required to induce delayed and prolonged vasocontraction.

Differential Expression of P2 Receptors and Vasospasm

P2 receptors play an important role in regulating cerebral vascular tone. Even though >15 P2 receptor subtypes have been discovered, the most frequently reported P2 receptors in vascular tissue are the P2X1, P2Y1, and P2Y2 subtypes. The P2X1 receptor is a ligand-gated cation channel that exists mainly in smooth muscle cells and mediates vasoconstriction. The P2Y1 and P2Y2 (or P2U) subtypes are G protein–coupled receptors. Activation of P2Y1 receptors, which exist mainly in endothelial cells, leads to vasodilatation. P2Y1 receptors are also found less frequently in smooth muscle cells. The P2Y2 receptor is found in endothelial and smooth muscle cells and is responsible for vasodilatation or constriction, respectively. According to You et al, the relaxant effect of P2Y1 and P2Y2 receptors is endothelium dependent and is related to the generation of nitric oxide or prostacyclin and endothelium-dependent hyperpolarizing factors.

The extracellular nucleotides released from subarachnoid clots are supposed to stimulate all P2 receptor subtypes in cerebral arteries. Although the pathological roles of P2 receptors have been implicated in the development of post-SAH cerebral vasospasm, the differential role of each subtype has not been clearly understood. P2 receptors are involved in contraction or relaxation of cerebral arteries as well as in other cellular functions, such as proliferation and mitogenesis. Differential expression of P2 receptor subtypes occurs when smooth muscle undergoes phenotypic changes.

The P2X1 Subtype

In contractile smooth muscle cells, α,β-methylene-ATP, a potent agonist of P2X1 receptors, induces a transient increase in intracellular Ca2+, indicating the expression of P2X1 receptors. Similarly, a small and sustained P2X1 receptor–mediated intracellular Ca2+ elevation was observed in primary cultures of rat aorta smooth muscle cells. However, this P2X1 receptor–mediated response did not occur in subcultured rat smooth muscle cells, indicating P2X1 receptor downregulation and phenotypic change. Although P2X1 contributes to ATP-induced contraction in rat vascular smooth muscle cells, our data suggest that contractile P2X1 might not be involved in chronic vasospasm: P2X1 mRNA expression was transiently downregulated on day 3 after double hemorrhage. Postsynaptic P2X1 downregulation in the arterial wall might also impair the fine neural regulation of cerebrovascular smooth muscle tone. Because the data of mRNA expression were sampled only on days 3, 5, and 7, this study does not rule out a possible role for P2X1 in “acute vasospasm,” which might occur immediately after the blood injection.

The P2Y1 Subtype

P2Y1 exists mainly in endothelial cells and contributes to endothelium-dependent relaxation. A potent agonist of the P2Y1 receptor (2 MeS-ATP) induces Ca2+ release in cultured rat smooth muscle cells but not in freshly isolated cells, which indicates an upregulation of P2Y1 receptors in cultured smooth muscle cells. Because the P2Y1 receptor is involved in mitogenic effect, the upward regulation of P2Y1 receptors in culture has also been observed to contribute to an increased progression of cell cycles in smooth muscle cells. Even though P2Y1 expression in contractile smooth muscle cells was detected, its role was described in cell mitogenesis, and its role in smooth muscle contraction remains to be determined. In this study the mRNA expression of P2Y1
transiently upregulated and peaked around day 5. This result might be interpreted as an increase in P2Y1 mRNA expression in endothelial cells, as an increase in P2Y1 expression in smooth muscle cells, or as both. Because the nature of multiple layers of smooth muscle cells contrasts with the nature of a thin layer of endothelial cells, we speculate that the altered mRNA P2Y1 expression might reflect an enhanced expression in smooth muscle cells. Endothelial cells were not removed in this study because it is difficult to remove the endothelium from rat basilar arteries, and the procedure followed in this study required the samples to be frozen immediately after euthanasia to avoid possible decay or contamination of the RNA.

The P2Y2 Subtype
The mRNA expression of P2Y2 was upregulated, and the time course of its upregulation was consistent with the time course of cerebral vasospasm. This P2Y2 upregulation might have led to a contractile response (even to a low concentration of extracellular ATP) and might have contributed to cerebral vasospasm. According to some investigators, P2Y2 also contributes to mitogenesis by cooperating with other growth factors, such as serum or platelet-derived growth factor. After balloon injury in the endothelium-damaged intimal lesions, the neointimal subpopulation of smooth muscle cells on luminal surface demonstrates a P2Y2 subtype overexpression. Extracellular ATP also regulates this P2Y2-specific upregulation process through the mitogen-activated protein kinase pathway and plays an important role in atherosclerotic intimal hyperplasia. Thus, P2Y2 mRNA upregulation might be involved in spastic arterial mitogenesis. In a recent article outlining the P2 receptor subtype changes that occurred during the switching of smooth muscle phenotypes, P2X1 and P2Y1 were expressed in freshly isolated rat aortas. If rat aortic smooth muscle cells were cultured, however, the P2X1 subtype disappeared, and P2Y1 and P2Y2 were overexpressed. However, in this rat model the endothelial cells did not detach markedly, nor did subintimal cells proliferate. It is speculated that the upregulation of P2Y2 receptor in this study might be related to the long-term contractile response of the basilar artery instead of tissue proliferation.

The cause of the upregulation of P2Y2 receptor in smooth muscle cells undergoing cerebral vasospasm is unclear. The most likely cause is blood clot and its lysate (such as erythrocyte lysate, hemoglobin, ATP, and their degenerative products). Perhaps these spasmogens directly or indirectly (by releasing other factors such as endothelin or growth factors and together with these factors) stimulate smooth muscle cells. In addition, hemoglobin and other factors cause cytotoxicity and possibly trigger inflammatory response and generate cytokines. Platelet or leukocyte infiltration might lead to the production of 5-hydroxytryptamine, UTP, thromboxanes, and cytokines. In turn, the conditions fostered by these growth factors might stimulate the basilar artery, resulting in changes of P2Y2 receptors.

Rat Double Hemorrhage Model of SAH
Developing a reliable model for rat SAH that closely parallels the pathogenesis of vasospasm in humans has proven difficult. Single hemorrhage rat models do not closely resemble the biphasic phenomena characteristic of SAH-induced vasospasm in large-animal models. Additionally, most single injection models have been conducted in acute studies—ie, those investigating only the initial acute constriction—while excluding the characteristic delayed spasm resulting in cerebral ischemia and the concomitant morbidity and mortality. Other problems with previous models include the indirect evaluation of vasospasm (via angiogram or cerebral blood flow determination) or direct evaluation by gross observation. The illustrations of the small rat cerebral arteries proved to be difficult in these studies. Investigating the molecular changes associated with SAH-induced vasospasm requires a reliable rat model because most documented genes are from either rats or mice. A double hemorrhage model in rats has created vasospasm that resembles angiographically the time course of vasospasm in large-animal models. Although massive single injections (0.5 mL) of blood have also been found to generate a similar time course for constriction, we found this method to be undesirable because it led to increased mortality (ie, high incidence of respiratory arrest due to extremely high inspiratory capacity, as noted in unpublished observations [R.C. Carpenter, BS, et al, unpublished data, 1999]).

According to our results, the profile of the cerebral vasospasm produced in this rat double hemorrhage model of SAH falls in the mild to moderate range, and we observed few severe vasospasms. The morphological changes as observed by TEM are consistent with those observed in most animal models (such as corrugation of the elastic lamina, which indicates vasoconstriction). Other features that have been described in humans (such as endothelial damage, smooth muscle migration, proliferation, necrosis, and apoptosis) were not observed to any appreciable extent. Therefore, this rat double hemorrhage model is more or less an experimental albeit atypical SAH model designed to parallel cerebral vasospasm observed in humans or in larger-animal models. Because this model did not successfully produce severe cerebral vasospasm, the molecular changes in P2 receptor expression can only be analyzed within the context of an SAH model.

By examining the mRNA expression of P2 receptors, this study demonstrates an upregulation of P2Y receptors that might enhance a contractile response to extracellular ATP even at lower levels. The P2X1 receptor might not be involved in chronic vasospasm, even though its role in acute vasospasm cannot be excluded. For several reasons, we recommend additional studies using larger animals (such as canines or primates) as models. First, these models replicate human vasospasm more closely (eg, severe contraction, endothelial damage). Second, separating the endothelial cells from the relatively larger cerebral arteries of canines or primates (as opposed to those of rodents) might prove useful (removal of endothelial cells is a crucial step in identifying the sources of different P2 receptor mRNAs). Third, the protein expression of different P2 receptors and the functional activities of these receptors during cerebral vasospasm need further examination.

The mRNA expression of P2Y2 was upregulated, and the time course of its upregulation might have led to a contractile response (even to a low concentration of extracellular ATP) and might have contributed to cerebral vasospasm. According to some investigators, P2Y2 also contributes to mitogenesis by cooperating with other growth factors, such as serum or platelet-derived growth factor. After balloon injury in the endothelium-damaged intimal lesions, the neointimal subpopulation of smooth muscle cells on luminal surface demonstrates a P2Y2 subtype overexpression. Extracellular ATP also regulates this P2Y2-specific upregulation process through the mitogen-activated protein kinase pathway and plays an important role in atherosclerotic intimal hyperplasia. Thus, P2Y2 mRNA upregulation might be involved in spastic arterial mitogenesis. In a recent article outlining the P2 receptor subtype changes that occurred during the switching of smooth muscle phenotypes, P2X1 and P2Y1 were expressed in freshly isolated rat aortas. If rat aortic smooth muscle cells were cultured, however, the P2X1 subtype disappeared, and P2Y1 and P2Y2 were overexpressed. However, in this rat model the endothelial cells did not detach markedly, nor did subintimal cells proliferate. It is speculated that the upregulation of P2Y2 receptor in this study might be related to the long-term contractile response of the basilar artery instead of tissue proliferation.

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References

Editorial Comment
The authors describe an interesting piece of vascular physiology, applied to subarachnoid hemorrhage in the rat. They are careful to point out the differences in (especially) morphology between vasospastic arteries in humans and in rats, but on balance this model appears to be at least as valid as the widely used dog double-hemorrhage model. This is useful, not only because rats are cheaper, easier to handle, and have less “pet appeal,” but also because most of the genes known to play a role in vascular physiology have not been described in dogs, cats, or monkeys, but rather in rats and mice. Obviously, having less “pet appeal” does not absolve us from treating rats as humanely as possible, but the authors have
clearly adhered to the guidelines described in Materials and Methods.

One of the vexing problems in this type of work is that practically every agonist and every receptor modulate both vasodilation and vasoconstriction, depending not only on the various contributions of the agonist and receptor but also on the doses of the former. This, then, makes this work more suitable for learning about vascular physiology than about vasospasm. Nevertheless, the switch in phenotype rather than change in sensitivity to agonists with the upregulated expression P2Y2 mRNA is an interesting clue in understanding the phenomenon of “vasospasm.”

The authors note that “Converting a phenotype to synthetic smooth muscle during a prolonged contraction (such as cerebral vasospasm) might reduce smooth muscle sensitivity to contractile or more likely relaxant stimulation, and lead to resistance to vasodilators.” The reverse may be true as well (see above), and this possibly explains some of the effects of prophylactic balloon dilation through its endothelium-modulating effects, in which vessels were found to become unresponsive to vasodilator and vasoconstricting agents after SAH!

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Reference
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