Symposium

Mechanism of the Cardioprotective Effect of Inhibition of the Renin-Angiotensin System on Ischemia/Reperfusion-Induced Myocardial Injury

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Inhibition of the renin-angiotensin system (RAS) has been shown to be beneficial in providing cardioprotective effects in humans, but the mechanism of these effects is not well understood. In this study, we examined the effects and mechanism of RAS inhibitors on ischemia/reperfusion (IR)-induced myocardial injury in rats. Rats were randomly divided into five groups and treated with vehicle (C), angiotensin converting enzyme inhibitor (ACE-I), angiotensin II type 1 receptor antagonist (AT₁-A), angiotensin II type 2 receptor antagonist (AT₂-A) or ACE-I plus bradykinin B₂ antagonist. Ten minutes after administration, the left main coronary artery was ligated for 45 min, and then reperfused for 120 min. IR-induced cardiomyocyte apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and confirmed by typical DNA laddering. Mitogen-activated protein kinase, extracellular signal-regulated protein kinase (ERK) and *c-Jun* NH₂-terminal protein kinase (JNK) activity in the ischemic zone were measured by an *in vitro* kinase assay. The duration of ventricular tachycardia (VT) during ischemia was reduced by AT₂-A and ACE-I, and increased by AT₁-A and ACE-I+icatibant. ACE-I and AT₂-A reduced apoptosis (by 54% and 53%) and infarct size (by 42% and 41%), while AT₁-A increased apoptosis (by 86%) and infarct size (by 45%). These changes were negatively correlated with the change in ERK activity. The effects of ACE-I on apoptosis and infarct size were abolished by the coadministration of icatibant. Apoptosis was correlated with the occurrence of VT ($r=0.837$, $p<0.001$). These results suggest that both the accumulation of bradykinin and inhibition of AT₂ receptor are cardioprotective against IR injury through the activation of ERK, but not JNK. (*Hypertens Res* 2001; 24: 179-187)

Key Words: angiotensin, ischemia-reperfusion, infarct size, apoptosis, ERK

Introduction

The renin-angiotensin system (RAS) is activated during myocardial ischemia (1, 2) and plays an important role in myocardial ischemia/reperfusion (IR)-induced injury. RAS inhibition is considered to be beneficial in protecting against IR-induced injury (3), but the mechanism of this

effect remains unclear.

It is becoming apparent that some myocardial cell death upon IR is apoptotic (4). The results of both animal experiments and clinical observations indicate that apoptosis is associated with myocardial IR (4-8). Although it has been shown that angiotensin II type 2 (AT₂) receptor is involved in cell apoptosis (9, 10), little is known about the pathophysiological changes mediated

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by AT₂-receptors in myocardial IR.

Extracellular signal-regulated protein kinase (ERK) and *c-Jun* NH₂-terminal protein kinase (JNK) are serine/threonine kinases that belong to the mitogen-activated protein kinase (MAPK) family. ERK and JNK are regulated by different upstream activators and play a central role in cell proliferation and apoptosis (11). It has been shown that MAPKs play key roles in angiotensin II-mediated apoptosis (12, 13), but the role of MAPKs in the pathophysiology of the cardiovascular system *in vivo* is poorly understood.

In this study we examined 1) the mechanism that underlies angiotensin converting enzyme (ACE) inhibitor (ACE-I)-mediated cardioprotection, 2) the effects and mechanism of angiotensin II receptor antagonists in IR injury, and 3) the functions of ERK and JNK in IR injury *in vivo* in rats.

Methods

Animal Model

The present study was conducted under guidelines approved by the Animal Care and Use Committee of Fukuoka University School of Medicine. Male Wistar rats (8–9 weeks of age) were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg). Tracheotomy was performed and an intubating cannula was connected to a ventilator (Harvard rodent ventilator; Harvard Apparatus Inc., South Natick, USA). The animals were ventilated artificially with room air (10 ml kg⁻¹, 50 strokes min⁻¹). Additional doses of pentobarbital (30 mg kg⁻¹) were given when required throughout the experiment. A thoracotomy was performed in the left fourth intercostal space, and the heart was exposed after the pericardium was incised. A 6-0 silk suture was passed around the left coronary artery (LCA). Both ends were threaded through a vinyl tube to form a snare around the vessel. Once hemodynamics had stabilized, coronary occlusion was performed by tightening the loop. Successful coronary artery occlusion was evidenced by regional cyanosis of the heart, a decrease in arterial blood pressure and ischemic ECG changes (ST-segment elevation). Reperfusion was indicated by recovery from cyanosis, an increase in arterial blood pressure and ECG changes, *i.e.*, reversal of ST-segment elevation.

Experimental Protocols

Protocol 1

When the preparation was completed, 10 min were allowed for stabilization. The rats were then randomly assigned to one of the following five groups (*n*=6 each): 1) Control group: 1 ml/kg saline; 2) ACE-I group: 0.2 mg/kg temocaprilat; 3) angiotensin type 1 antagonist

(AT₁-A) group: 0.01 mg/kg RNH6270; 4) AT₂ antagonist (AT₂-A) group: 0.5 mg/kg i.v. + 0.5 mg/kg/min PD123,319; and 5) ACE-I + bradykinin B₂ receptor antagonist group: 0.2 mg/kg temocaprilat + 0.02 mg/kg/min icatibant. The LCA was occluded for 45 min and reperused for 120 min. Drugs were injected into the jugular vein 10 min before occlusion. In coadministration experiments, bradykinin B₂ receptor antagonist was started 15 min before occlusion. At the end of the experiment, hearts were excised and used for terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and to determine the area at risk of ischemia (AAR) and infarct size.

Protocol 2

A second set of experiments were undertaken to examine the effects of ACE-I and angiotensin II receptor antagonist on ERK and JNK activity in the AAR during reperfusion. Rats were randomly assigned to another six groups (*n*=5 each group): a sham, a control, an ACE-I, an AT₁-A, an AT₂-A, and an ACE-I plus icatibant group. The dosage and method for the administration of each drug were the same as in protocol 1. The LCA was occluded for 45 min and reperused for 10 min. The peak activity of ERK and JNK appeared at 10 min after reperfusion (14). The same surgical procedures were performed in sham-operated rats, except that the suture around the coronary artery was not tied. All samples were collected from the AAR.

These drugs have been reported to be effective at the above doses *in vivo* (15–18). Preliminary challenge experiments in our laboratory also showed that these doses of temocaprilat, RNH6270, and icatibant could completely abolish the blood pressure response to exogenous angiotensin I (Ang I) (300 ng/kg), angiotensin II (Ang II) (50 ng/kg) and bradykinin (500 ng/kg), respectively (data not shown).

Hemodynamics and ECG

A 24-gauge angiocatheter was inserted into the right carotid artery to monitor blood pressure. Blood pressure and heart rate (HR) were measured with a pressure transducer (TP-400; Nihon Kohden, Tokyo, Japan) connected to an amplifier and recorded with a polygraph (RM-6000; Nihon Kohden). Mean arterial blood pressure (MAP) and ECG were monitored throughout the experimental period. Arrhythmias were detected during ischemia and diagnosed according to the criteria of the Lambeth Conventions (19). We excluded rats (0 to 1 in each group) that died of sustained ventricular fibrillation from the analysis because of the inadequacy of data collection for hemodynamics and estimation of the area at risk. The incidence of such exclusion was not significantly different among the groups.

Fig. 1. Method of the measurement of AAR and infarct size.

Measurement of Infarct Size

At the end of the experiment, the LCA was re-occluded and 0.5 ml of 1% Evans blue dye (Sigma Chemical Co., St. Louis, USA) was injected into the left ventricle (LV) to delineate the normally perfused area (Evans blue-stained area) and AAR (Evans blue-unstained area). The LV was then separated from the rest of the heart and cut into five separate slices (2-mm thick) parallel to the atrioventricular groove. The middle three slices were weighed separately, and then incubated in 1.5% of triphenyltetrazolium chloride (TTC) for 30 min. After TTC staining, the area of infarction appeared pallid (TTC-negative), whereas viable myocardium appeared red (TTC-positive) (Fig. 1). Both sides of each of the three myocardial slices were photographed. Each area was measured using NIH image software. The area of infarction for each of the three slices was determined by taking the average of the area of infarction on the basal and apical sides of each myocardial slice. Infarct weight was determined as follows: $(A_1 \times WT_1) + (A_2 \times WT_2) + (A_3 \times WT_3)$, where A was the area of infarction for the slice and WT was the weight of the slice. The weight of the AAR was calculated in the same way. Infarct size was then expressed as a percentage of the AAR $[(WT \text{ of infarction} / WT \text{ of AAR}) \times 100]$ and the AAR was expressed as a percentage of the LV $[(WT \text{ of AAR} / WT \text{ of LV}) \times 100]$. These slices were then fixed in 10% neutral-buffered formalin for the TUNEL assay.

DNA Gel Electrophoresis

For detection of DNA laddering, agarose gel electro-

phoresis was performed as described previously (20). Briefly, the hearts of rats ($n=3$ each group) subjected to protocol 1 were excised at 2 h after reperfusion. DNA was extracted from the AAR by GONOMIX (Talent, Follatoio, Italy) according to the manufacturer's instructions. The DNA sample (5 μ g) was then electrophoresed on 1.8% agarose gel. The DNA was visualized with ethidium bromide.

TUNEL Staining

A TUNEL assay for detecting DNA fragmentation *in situ* was performed with a commercially available kit (Apop-Tag kit; Oncor, Gaithersburg, USA) according to the manufacturer's instructions. Cardiomyocytes in the LV were analyzed in 9 separate fields for each tissue section under a light microscope at a magnification of $\times 200$. TUNEL-positive cardiomyocytes from 4 rats per group were averaged.

Measurement of JNK and ERK Activity

The hearts of rats subjected to occlusion and reperfusion in protocol 2 were excised after 10 min of reperfusion. The heart was rapidly removed and washed in pre-cooled phosphate-buffered saline (PBS) (pH 7.4) containing 2.5 mM ethylenediamine- N,N,N',N' -tetraacetic acid (EDTA), 2 mM β -glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride (PMSF). The AAR of the LV was homogenized on ice in cell lysis buffer (20 mM HEPES (pH 7.2), 25 mM NaCl, 2 mM ethylene glycol bis- N,N,N',N' -tetraacetic acid (EGTA), 0.2 mM dithiothreitol (DTT), 60 μ g/ml aprotinin, 2 μ g/ml leupeptin, 1 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4 , 25 mM β -glycerophosphate). After incubation at 4°C for 30 min, the homogenates were sonicated (Ultrasonic processor VP-5, TAITEC, USA) on ice for 1 min, and then centrifuged at 15,000 rpm at 4°C for 30 min. After centrifugation, the supernatants were stored at -80°C until use. Triton (final concentration 1%) was added to protein extracts (500 μ g) followed by incubation with either polyclonal rabbit anti-p46JNK1, which recognizes not only p46JNK but also p55JNK, or polyclonal rabbit anti-p44ERK (ERK-1) immunoglobulin G (IgG) (k-23) and polyclonal rabbit anti-p42ERK (ERK-2) IgG (c-14). All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). The immune complex was precipitated with protein A-agarose at 4°C for a minimum of 1 h. The immobilized immunoprecipitate was washed three times with lysis buffer and once with kinase assay buffer (100 mM Tris-HCl, pH 7, 40 mM magnesium acetate, 0.4 mM NaF, 0.4 mM EGTA), and then incubated with 2.5 μ g GST-c-Jun or 250 μ g myelin basic protein (MBP) (Santa Cruz Biotechnology Inc.), 1 μ Ci (γ - ^{32}P)ATP (Du Pont-New England Nuclear Co.,

Fig. 2. Changes in mean arterial pressure. Values are the means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. control ($n = 6$). ACE-I, ACE inhibitor; AT₁-A, AT₁ antagonist; AT₂-A, AT₂ antagonist.

Boston, USA), and 50 μ M ATP at 30°C for 60 min. After incubation, the reaction was terminated by adding Laemmli's sample buffer (Sigma Chemical Co.) and boiling for 5 min. The supernatants were electrophoresed on 10% SDS-polyacrylamide gels. The gels were then dried and subjected to autoradiography. To estimate the incorporation of 32 P from (γ - 32 P)ATP into c-Jun or MBP, the intensity of each band was measured by Personal Densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, USA)

Drugs

Temocaprilat and RNH6270 were gifts from Sankyo Co. (Tokyo, Japan). Icatibant was a gift from Hoechst Marion Roussel (Tokyo, Japan). PD123,319 was purchased from Sigma Chemical Co..

Statistical Analysis

Data are expressed as the mean $\pm$ SEM and experimental groups were compared with the use of one-way ANOVA and Fisher's PLSD. MAP and HR were compared among groups by repeated measures of ANOVA. A univariate analysis of the effect of apoptosis on sustained ventricular tachycardia (VT) was performed using a linear regression.

Fig. 3. Duration of ventricular tachycardia (VT) during ischemia. No arrhythmia was observed after reperfusion. Values are the means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. control ($n = 6$). ACE-I, ACE inhibitor; AT₁-A, AT₁ antagonist; AT₂-A, AT₂ antagonist.

Fig. 4. Infarct size (% of LV or % of AAR). There is no difference in AAR among the 5 groups. Values are the means $\pm$ SEM. * $p < 0.05$ vs. control ($n = 6$). ACE-I, ACE inhibitor; AT₁-A, AT₁ antagonist; AT₂-A, AT₂ antagonist.

A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Hemodynamics and ECG

Figure 2 shows the MAP of rats subjected to 45 min of LCA occlusion and 2 h of reperfusion. Regional ischemia evidently causes a sudden decline in MAP after the onset of ischemia. ACE-I and AT₁-A induced a significant reduction in MAP compared with the value in controls.

Fig. 5. TUNEL-positive cardiomyocytes (cell number/field). Values are the means $\pm$ SEM. * $p < 0.05$ vs. control ($n=4$). ACE-I, ACE inhibitor; AT₁-A, AT₁ antagonist; AT₂-A, AT₂ antagonist.

There were no differences in HR among any of the groups. All of the rat hearts that were made ischemic by LCA occlusion showed significant ST segment elevation early after the onset of ischemia; this change was sustained throughout the ischemic period and disappeared within the first 5 min after reperfusion. Changes in MAP and the Lead I ECG waveform were taken as evidence that the artery had been successfully occluded.

Ischemia-Induced Arrhythmia

Arrhythmias were observed from 5 min to 20 min of LCA occlusion. The duration of VT was significantly decreased by ACE-I and AT₂-A, whereas AT₁-A increased the duration of VT (Fig. 3). The antiarrhythmic effect of ACE-I was abolished by the coadministration of icatibant.

Infarct Size

Infarct size in the ACE-I and AT₂-A groups was significantly smaller than that in the controls (Fig. 4), while that in the AT₁-A group was significantly greater. The protective effect of ACE-I was abolished by the coadministration of icatibant.

Apoptosis

There were significantly more TUNEL-positive cardiomyocytes in the AAR in the AT₁-A group, and significantly fewer in the ACE-I and AT₂-A groups, compared to the control group (Fig. 5). The protective effect of ACE-I against apoptosis was abolished by the coadministration of icatibant. No TUNEL-positive cardiomyocytes were detected in sham-operated rats. Furthermore, agarose gel electrophoresis of DNA showed a laddering pattern that was typical of apoptosis, in contrast to that of

(a)

(b)

Fig. 6. (a) A representative photomicrograph of TUNEL-positive cardiomyocytes in control rats; scale bar, 0.2 μ m. (b) Electrophoretic analysis of internucleosomal DNA. ACE-I, ACE inhibitor; AT₁-A, AT₁ antagonist; AT₂-A, AT₂ antagonist.

DNA isolated from sham-operated hearts (Fig. 6).

Activation of ERK and JNK

An increase in ERK activity was seen in the ACE-I and AT₂-A groups, while a decrease was found in the AT₁-A and ACE-I plus icatibant groups (Fig. 7c). JNK activity was increased in the AT₂-A and ACE-I plus icatibant groups and reduced in the ACE-I and AT₁-A groups (Fig. 7d).

Fig. 7. In-vitro assay of ERK and JNK activity. (a) and (b) show SDS-polyacrylamide gel. (c) and (d) show representative autoradiograms of ERK and JNK activity. ACE-I, ACE inhibitor; AT₁-A, AT₁ antagonist; AT₂-A, AT₂ antagonist.

Fig. 8. Relation between apoptotic cardiomyocytes and duration of ventricular tachycardia (VT) ($n=20$, $r=0.837$, $p<0.001$). ACE-I, ACE inhibitor; AT₁-A, AT₁ antagonist; AT₂-A, AT₂ antagonist.

Apoptosis and VT

Myocardial apoptosis was significantly correlated with the duration of ischemia-induced sustained VT (Fig. 8).

Discussion

In this study, both ACE-I and AT₂-A reduced IR injury *in vivo*. ERK activation by the accumulation of bradykinin and by AT₂ receptor inhibition may be responsible for these positive effects. The finding that ACE-I and AT₁-A had opposite effects on IR injury, and that coadministration of icatibant led to a decrease in the cardioprotective effect of ACE-I, strongly suggest that ACE-I exerts its beneficial effects independent of an AT₁ receptor-mediated pathway. That AT₁-A and AT₂-A had opposite effects on ERK activation may account for their different effects on the parameters examined in this study. No relation was found between JNK activity and IR injury. Myocardial apoptosis was shown to be positively correlated with the occurrence of VT.

It has been suggested that ACE-I are effective for preserving myocyte viability in the setting of acute ischemia and infarction (21) beyond treating cardiac remodeling and heart failure induced by myocardial infarction (22). The present study demonstrates that short-term treatment with ACE-I is effective for reducing IR injury. The parameters of myocardial apoptosis, infarct size and ventricu-

lar arrhythmia were all reduced in the present study. Therefore, it can be postulated that Ang II may directly mediate pathways involved in IR, with signaling through AT₁ receptor. To verify this assumption, we tested whether inhibition of AT₁ receptor would result in a similar reduction in IR injury. Interestingly, short-term treatment with AT₁-A had the opposite effect—*i.e.*, it enhanced IR injury, as evidenced by increased myocardial cell death and arrhythmia. Furthermore, the cardioprotective effects of ACE-I were abolished by the coadministration of icatibant, and this strongly indicates that a kinin-dependent pathway is involved in the cardioprotective effect of ACE-I. This notion is supported by a recent study showing that kallikrein gene delivery attenuates myocardial infarction and apoptosis after myocardial IR (23). Stimulation of bradykinin B₂ receptor has been reported to activate the ERK pathway in *in vitro* experiments (24, 25). Thus, we speculated that the activation of ERK is also an important mechanism underlying the cardioprotective effect of ACE-I *in vivo*. As expected, ERK was activated by ACE-I in the ischemia-reperfused myocardium, and this effect was completely abolished by the coadministration of icatibant. This is the first report to indicate that the accumulation of bradykinin by ACE-I can activate the ERK pathway and mediate the cardioprotective effect *in vivo*.

AT₁ and AT₂ receptors play an important role in regulating cardiovascular hemodynamics and structure. Both receptors are widely distributed throughout the rat heart, with each receptor subtype accounting for approximately 50% of the specific binding (26). Accumulating evidence demonstrates that the function and signaling mechanisms of these receptor subtypes are quite different (27). The classic view that most of the known effects of Ang II are attributable to AT₁ receptor has been challenged by a growing body of evidence that AT₂ receptor also mediates responses to Ang II in adult tissue and acts as an antagonistic receptor against AT₁ receptor (28–30). Our results suggest that AT₁ and AT₂ receptors counter-regulate apoptosis, and consequently the size of myocardial infarct. This supports an earlier report indicating that short-term treatment with a selective AT₁-A had different effects, in contrast to such treatment with AT₂-A, on the recovery of mechanical function after IR; *i.e.*, AT₂-A was cardioprotective, whereas AT₁-A was not (30).

There is some evidence of linkage between the activation of MAPKs and stimulation of the Ang II receptor signaling system in cardiomyocytes (31, 32). Furthermore, *in vitro* experiments have shown that AT₁ and AT₂ receptors exert counteracting effects on ERK and JNK, and consequently apoptosis (28, 33), and that the AT₂ receptor has growth inhibitory and proapoptotic effects through the inactivation of ERK activity, which results in Bcl2 dephosphorylation and up-regulation of Bax (34). Three major mammalian mitogen-activated protein kinases,

ERK, p38, and JNK, have been reported to be activated in the ischemia-reperfused myocardium (20, 35). Activated ERK has been shown to play a protective role against apoptosis *in vitro* (14, 36), while JNK activation in cardiomyocytes contributes to apoptosis (37, 38). Therefore, ERK and JNK may be important molecular components involved in myocardial injury following ischemia/reperfusion. In our experiments, increased ERK activity was observed in the ACE-I and AT₂-A groups accompanied by decreased apoptosis and infarct size. This is the first report that both ACE-I and AT₂-A can attenuate ischemia-reperfused myocardial apoptosis and infarct size by increasing ERK activity *in vivo*. However, the underlying mechanisms are different. The increase in ERK activity by ACE-I is kinin-dependent, whereas the increase in ERK activity by AT₂-A may be due to stimulation of AT₁ receptor by AT₂-A or blockage of AT₂ receptor-mediated inactivation of ERK activity (33). Changes in JNK activity upon IR were found to be independent of myocardial injury.

Both ACE-I and AT₂-A were also effective at attenuating sustained VT, whereas VT was frequently seen in the AT₁-A- and ACE-I plus icatibant-treated groups. Myocardial apoptosis was positively correlated with the occurrence of VT. Although the causative relation between apoptosis and arrhythmia was not established in our experiment, myocardial apoptosis may play some role in cardiac electrical instability.

The signaling pathways that determine cell death *vs.* cell survival *via* AT₁ are poorly understood. In addition to the activation of ERK, the third cytoplasmic loop of the AT₁ receptor has also been shown to be effective in the activation of protein kinase B, which is known to promote cell survival (12). Binding of Ang II to AT₁ inhibits the apoptosis of vascular smooth muscle cell (39). Although a previous report showed that p53 induces myocyte apoptosis *via* the activation of RAS *in vitro* (40), another found that myocyte apoptosis occurred independently of p53 *in vivo* during acute myocardial infarction (41). In contrast, other investigators reported that the inhibition of TUNEL-positive cardiomyocytes did not significantly change the infarct size (42). This may be because intervention in a post-mitochondrial process fails to protect the ischemic myocardium (43).

In sharp contrast to an earlier report (44) that suggested AT₁-A was cardioprotective against IR injury, we did not find any improvement in myocardial salvage in our animal model. Consistent with a recent report (45), we observed increased cellular damage in the AT₁-A-treated group after IR injury. Differences in the collateral blood system and Ang II receptor status among different animal models and the use of different drug concentrations could contribute to these conflicting results. It is likely that the short-term benefits of Ang II in inhibiting apoptosis may be lost if myocardial Ang II expression be-

comes sustained and excessive. It is not surprising that Liu and colleagues demonstrated that AT₁-A had a cardioprotective effect in the chronic phase after myocardial infarction, in which condition a severe degree of hypertrophy and remodeling may determine the prognosis (22).

It can be argued that the effect of AT₁-A on IR injury is secondary to the decrease in blood pressure induced by AT₁-A. However, this is unlikely because a marked reduction in blood pressure was also observed in ACE-I-treated rats in which cardioprotection against IR injury was shown.

In conclusion, both the accumulation of bradykinin and the inhibition of AT₂ receptor were suggested to be cardioprotective against IR injury through the activation of ERK, rather than JNK. Inhibition of AT₁ receptor was suggested to increase IR injury with acute ischemia-reperfusion. Our study paves the way for a complete understanding of the effects and mechanisms of RAS blockers on IR injury, and provides the important implication that short-term treatment with AT₂-A, rather than AT₁-A, may be beneficial in the management of IR injury with acute ischemia-reperfusion.

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