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Windows of Therapeutic Opportunity on Fetal Growth Retardation Induced by Transient Intrauterine Ischemia in Rats

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Abstract

Objective: To assess the windows of therapeutic opportunity for drugs with various chemical actions on fetal growth retardation induced by transient intrauterine ischemia in rats.

Methods: At 17 days of gestation, ischemia was induced by 30 min of right uterine artery occlusion. The administration of either α-phenyl-N-tert-butyl-nitrone (PBN), FK 506, nifedipine, or MK-801 to pregnant rats was randomly started before occlusion, 1 hour, 3 hours, or 24 hours after recirculation. All of the pups were delivered by cesarean section at 21 days of gestation and were weighed to determine the degree of fetal growth retardation.

Results: The vehicle-treated animals exposed to ischemia showed a significant decrease in fetal body weight compared with the normoxic control animals. The growth disturbances were prevented by nifedipine and MK-801 only when given just prior to ischemia. In contrast, PBN and FK 506 had a protective effect even when given 1 hour and 3 hours after the start of recirculation, respectively.

Conclusions: The present results indicate that treatment with PBN and FK 506 gives relatively wide windows of therapeutic opportunity in fetal growth retardation induced by transient intrauterine ischemia in rats and suggest the possibility of therapeutic intervention after the start of recirculation. (J Nippon Med Sch 2002; 69: 534–541)

Key words: fetal growth retardation, intrauterine ischemia, BPN, FK 506, nifedipine, MK-801

Introduction

Decreased uteroplacental blood flow is commonly associated with intrauterine fetal growth retardation (IUGR), which is a significant cause of perinatal morbidity and mortality in human pregnancies. Recent experimental studies[1–4] have demonstrated that transient uterine artery occlusion in pregnant rats causes an asymmetric retardation of fetal growth. This procedure has also been shown to produce a delayed deterioration of the cellular bioenergetic state[5] and of mitochondrial respiratory activity in the fetal and neonatal rat brain[6–8]. In addition, our preliminary studies suggested the possibility of treatment for these disorders including both IUGR and cellular energy failure with the drugs with various chemical actions, e.g. free radical spin trap agent, α-phenyl-N-tert-butyl-nitrone (PBN)[5], the immunosuppressant drug FK 506[9], the L-type voltage-gated calcium ion channel blocker nifedipine[10], or the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801[11]. However, relatively little is known about the windows of therapeutic opportunity

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for the drugs.

In clinical practice, the timely selection of patients with IUGR for enrollment in a therapeutic trial presents formidable logistic difficulties. Although several risk factors, such as maternal cardiac or renal disease, diabetes, and essential hypertension, have been known to cause IUGR, many patients with these disorders do not develop IUGR. Thus, if such risk factors are used as selection criteria for IUGR treatment, many fetuses will be treated unnecessarily. Possible methods of improving the selection of fetuses that might benefit from treatment include Doppler ultrasound measurements in the uterine artery[12], fetal middle cerebral artery, and umbilical artery[13]. However, these measurements are not yet universally available. At present, most clinicians would prefer to initial treatment only when the fetuses are already revealed to have developed growth restriction. Therefore, it is important to know whether therapy initiated after the onset of the events that may induce growth retardation is effective.

In the present study, we examined the windows of therapeutic opportunity for drugs with various chemical actions, which were previously reported to have a protective effect on fetal growth retardation induced by transient intrauterine ischemia in rats.

Materials and Methods

This study was approved by the Ethics Committee for Animal Experimentation at our university (2000, 10-2). Animal care complied with the "Guidelines for Care of Experimental Animals" issued by the Office of the Prime Minister of Japan.

Animal preparation and transient intrauterine ischemia

Ten-week-old pregnant Wistar rats (Sankyo Lab Service Tokyo, Japan) weighing 250～300 g were used. The rats were housed separately with a 12-hour light cycle, and were allowed free access to water and food. At 17 days of gestation (term 21.5 days), the animals were anesthetized with 3% halothane in a mixture of N_2O: O_2 (70: 30) after overnight fasting. They were then intubated and artificially ventilated on 1.0% to 1.5% halothane during the operation. The maternal tail artery was cannulated to measure arterial blood gases, arterial pH, blood glucose, and blood pressure. The maternal tail vein was also cannulated for the drug injections. A midline abdominal incision was performed and the two uterine horns were exposed and kept moist with saline. Transient uterine artery occlusion was induced according to the technique of Tanaka et al [1]. Briefly, two microvascular clips were used to occlude the uterine vessels near the lower and upper ends of the right uterine horn. The clips were removed after 30 min of ischemia. During the operation a core temperature was regularly maintained at 37.0℃ with a heating pad. For each experiment the fetuses in the right uterine horn served as the ischemia group and those in the left horn as the non-ischemia group. At 21 days of gestation the animals were re-anesthetized, tracheotomized, and artificially ventilated. After the physiological parameters had been stabilized for at least 10 min, the fetuses were delivered by cesarean section and weighed to determine the degree of intrauterine fetal growth retardation. The fetal brains and livers and placenta were also weighed.

Drug administration and experimental protocol

FK 506 was kindly provided us by Fujisawa Pharmaceutical (Osaka, Japan). The other drugs were purchased from Sigma Chemical Co. (St. Louis, MO). PBN dissolved in saline (10 $mg \cdot ml^{-1}$) was administered intraperitoneally to pregnant rats at a dose of 100 $mg \cdot kg^{-1}$. FK 506 and MK-801 diluted ten times with saline was injected at a dose of 1.0 $mg \cdot kg^{-1}$ and 0.5 $mg \cdot kg^{-1}$ into the maternal tail vein, respectively. Nifedipine was dissolved in a mixture of 10% polyethylenglycol-400 and 10% ethanol in physiological saline and injected subcutaneously at a dose of 1 $mg \cdot kg^{-1}$. The doses and methods for drug administrations were determined according to the previous reports[5,9-11], which demonstrated the fetal neuroprotective effects of each drug in this experimental paradigm.

Studies were carried out in the following five groups of pregnant rats (total, n = 102) : the control group (n = 6 pregnant rats), which underwent

transient intrauterine ischemia due to 30 min uterine artery occlusion and received vehicle (saline) injections; the PBN treated group, which underwent transient intrauterine ischemia and started PBN injections 10 minutes before occlusion (n=6 pregnant rats), 1 hour (n=6 pregnant rats), 3 hours (n=6 pregnant rats), or 24 hours after recirculation (n=6 pregnant rats). A similar experimental protocol with the same number of subjects was used for FK 506 (n=24 pregnant rats), nifedipine (n=24 pregnant rats) and MK-801 (n=24 pregnant rats).

After the initial treatments, the subsequent administrations of half the dose of each drug were given, with an interval of 24 hours, until 20 days of gestation.

Statistical analysis

All data were expressed as mean±standard deviation (SD). One-way ANOVA followed by Scheffé's *F*-test was used to compare the values within each experimental group. Unpaired *t*-test was used to compare the values between ischemia and non-ischemia uterine horns. Differences with a *P* value of <0.05 were considered to be statistically significant.

Results

The physiological parameters of the pregnant animals during and after 30-minute uterine artery occlusion are shown in **Table 1**. There were no significant differences in blood pressure, arterial PO_2, PCO_2, pH, glucose concentrations, or core temperatures among the experimental groups.

The weights of the neonatal body, organs, and placenta of the PBN treated group are shown in **Table 2**. In the non-ischemic uterine horn, there were no significant differences in the weights of the neonatal body, organs or placenta among the experimental groups. The vehicle-treated animals exposed to ischemia showed a significant decrease in neonatal body and liver weights compared with the normoxic control animals. The growth disturbances in the neonatal body and liver were prevented by pretreatment with PBN. A similar beneficial effect was obtained when the drug was given at 1 hour of recirculation, but not at 3 hours or 24 hours of recirculation. There were no significant differences in the weights of the neonatal brain or placenta of the ischemic uterine horn among the experimental groups, either compared to each other or to the non-ischemic uterine horn.

Table 3 shows the neonatal body, organs, and placenta weights of the FK 506 treated group. FK 506 had a protective effect for the growth disturbances in the neonatal body and liver when the drug was given before occlusion, 1 hour, and 3 hours following recirculation. However, there was no protective effect when the drug was given at 24 hours of recirculation.

Tables 4 and 5 show the values for the nifedipine and the MK-801 treated animals, respectively. Both the drugs had a protective effect for the growth disturbances in the neonatal body and liver only when given just prior to ischemia.

Discussion

Insufficient uteroplacental blood flow with a reduction in oxygen supply and nutrients to the fetus is considered to be one of the major factors associated with IUGR. In a clinical setting, a late pregnancy insult such as placental insufficiency associated with hypertension would primarily affect cell size. Moreover, because placental insufficiency may result in diminished glucose transfer and hepatic storage, fetal abdominal circumference, which reflects liver size, would be reduced. Simultaneously, it is proposed that there is preferential shunting of oxygen and nutrients to the brain, which allows normal brain and head growth. As reported recently[1–4], transient uterine artery occlusion in pregnant rats causes the asymmetric retardation of fetal growth. In agreement with those reports, our results on the growth retardation of vehicle treated animals demonstrated significant differences in the weights of the neonatal body and liver between the non-ischemic and ischemic uterine horns. The average weights of the fetal body and liver measured at 21 days of gestation were 18% and 25% lower in the vehicle-treated animals

Table 1 Physiolosical parameters of the pregnant rats used in this study

	Vehicle	pretreatment	posttreatment		
			1 hour	3 hours	24 hours
PBN treated animals	(n = 6)	(n = 6)	(n = 6)	(n = 6)	(n = 6)
Blood pressure (mmHg)	98.2 ± 11.1	101.2 ± 12.4	95.6 ± 7.8	96.1 ± 8.9	105.7 ± 14.5
PO_2 (mmHg)	156.3 ± 16.1	150.8 ± 13.3	149.0 ± 18.8	163.3 ± 18.1	157.8 ± 17.7
PCO_2 (mmHg)	39.8 ± 2.2	40.1 ± 4.0	42.1 ± 4.1	39.1 ± 3.3	39.6 ± 2.8
pH	7.41 ± 0.03	7.39 ± 0.04	7.38 ± 0.06	7.41 ± 0.04	7.40 ± 0.05
Blood glucose (mg/dl)	76.1 ± 9.8	80.0 ± 15.5	84.4 ± 13.9	77.5 ± 13.2	79.4 ± 13.4
Core temperature (℃)	36.6 ± 0.3	36.5 ± 0.4	36.4 ± 0.4	36.3 ± 0.5	36.5 ± 0.4
FK506 treated animals		(n = 6)	(n = 6)	(n = 6)	(n = 6)
Blood pressure (mmHg)		96.9 ± 14.8	105.1 ± 9.5	94.1 ± 14.5	102.0 ± 13.2
PO_2 (mmHg)		159.8 ± 19.1	161.8 ± 20.1	159.7 ± 19.7	152.2 ± 18.2
PCO_2 (mmHg)		40.0 ± 3.2	40.2 ± 2.0	38.9 ± 3.7	40.1 ± 1.8
pH		7.40 ± 0.04	7.40 ± 0.03	7.38 ± 0.05	7.39 ± 0.03
Blood glucose (mg/dl)		78.8 ± 8.7	85.2 ± 11.1	79.4 ± 8.7	80.2 ± 11.7
Core temperature (℃)		36.3 ± 0.5	36.7 ± 0.3	36.6 ± 0.3	36.4 ± 0.5
Nifedipine treated animals		(n = 6)	(n = 6)	(n = 6)	(n = 6)
Blood pressure (mmHg)		95.5 ± 10.1	110.2 ± 16.5	108.2 ± 12.0	96.6 ± 10.8
PO_2 (mmHg)		154.9 ± 17.0	150.0 ± 11.8	160.3 ± 19.1	157.0 ± 17.8
PCO_2 (mmHg)		39.5 ± 3.4	41.2 ± 3.2	40.1 ± 1.8	41.2 ± 2.5
pH		7.41 ± 0.05	7.38 ± 0.04	7.40 ± 0.03	7.39 ± 0.04
Blood glucose (mg/dl)		82.6 ± 15.0	75.3 ± 9.2	80.7 ± 16.7	78.1 ± 13.0
Core temperature (℃)		36.5 ± 0.3	36.6 ± 0.3	36.5 ± 0.3	36.4 ± 0.4
MK-801 treated animals		(n = 6)	(n = 6)	(n = 6)	(n = 6)
Blood pressure (mmHg)		99.7 ± 10.3	97.6 ± 12.2	92.3 ± 14.1	101.0 ± 10.7
PO_2 (mmHg)		158.1 ± 19.1	158.2 ± 18.6	153.6 ± 13.8	160.3 ± 14.4
PCO_2 (mmHg)		39.2 ± 2.9	38.9 ± 3.9	40.0 ± 2.8	41.1 ± 2.2
pH		7.41 ± 0.05	7.42 ± 0.04	7.39 ± 0.03	7.39 ± 0.05
Blood glucose (mg/dl)		78.9 ± 10.5	80.2 ± 13.7	76.8 ± 8.1	77.8 ± 12.9
Core temperature (℃)		36.7 ± 0.3	36.5 ± 0.4	36.4 ± 0.5	36.5 ± 0.3

Note. Values are mean ± SD. All results are not significant.

Table 2 Neonatal body weight and organ weight in PBN treated animals

	Vehicle	PBN treatment			
		pretreatment	posttreatment		
			1 hour	3 hours	24 hours
Non-ischemia uterine horn	(n = 24)	(n = 27)	(n = 22)	(n = 23)	(n = 25)
Neonatal body weight (mg)	3,294 ± 229	3,338 ± 296	3,198 ± 217	3,310 ± 308	3,281 ± 190
Brain weight (mg)	153 ± 14	155 ± 13	150 ± 18	159 ± 20	154 ± 16
Liver weight (mg)	131 ± 17	129 ± 15	125 ± 15	128 ± 14	127 ± 11
Placenta weight (mg)	538 ± 70	542 ± 86	552 ± 90	544 ± 99	532 ± 107
Ischemia uterine horn	(n = 26)	(n = 22)	(n = 23)	(n = 19)	(n = 20)
Neonatal body weight (mg)	2,701 ± 364†	3,013 ± 356*	3,050 ± 368*	2,810 ± 510†	2,788 ± 412†
Brain weight (mg)	149 ± 13	151 ± 16	153 ± 13	151 ± 15	146 ± 13
Liver weight (mg)	97 ± 24†	119 ± 20*	124 ± 21*	101 ± 16†	103 ± 19†
Placenta weight (mg)	502 ± 55	564 ± 69	528 ± 68	513 ± 63	510 ± 71

Values are mean ± SD.

*Against vehicle treated animal in ischemia uterine horn, one-factor ANOVA followed by Scheffe's *F* test ($P<0.05$).

†Against non-ischemia uterine horn, unpaired *t*-test ($P<0.05$).

Table 3 Neonatal body weight and organ weight in FK506 treated animals

	Vehicle	FK506 treatment			
		pretreatment	posttreatment		
			1 hour	3 hours	24 hours
Non-ischemia uterine horn	(n = 24)	(n = 24)	(n = 19)	(n = 22)	(n = 23)
Neonatal body weight (mg)	3,294 ± 229	3,288 ± 250	3,200 ± 286	3,307 ± 322	3,342 ± 314
Brain weight (mg)	153 ± 14	160 ± 19	154 ± 12	159 ± 17	162 ± 20
Liver weight (mg)	131 ± 17	125 ± 15	130 ± 20	129 ± 18	133 ± 18
Placenta weight (mg)	538 ± 70	552 ± 80	551 ± 74	530 ± 90	544 ± 81
Ischemia uterine horn	(n = 26)	(n = 25)	(n = 25)	(n = 18)	(n = 23)
Neonatal body weight (mg)	2,701 ± 364†	3,128 ± 184*	3,080 ± 388*	3,003 ± 461*	2,880 ± 342†
Brain weight (mg)	149 ± 13	154 ± 12	150 ± 11	154 ± 12	151 ± 15
Liver weight (mg)	97 ± 24†	126 ± 19*	127 ± 19*	120 ± 20*	109 ± 21†
Placenta weight (mg)	502 ± 55	532 ± 66	511 ± 64	524 ± 62	514 ± 66

Values are mean ± SD.
*Against vehicle treated animal in ischemia uterine horn, one-factor ANOVA followed by Scheffe's *F* test ($P<0.05$).
†Against non-ischemia uterine horn, unpaired *t*-test ($P<0.05$).

Table 4 Neonatal body weight and organ weight in nifedipine treated animals

	Vehicle	Nifedipine treatment			
		pretreatment	posttreatment		
			1 hour	3 hours	24 hours
Non-ischemia uterine horn	(n = 24)	(n = 29)	(n = 27)	(n = 25)	(n = 25)
Neonatal body weight (mg)	3,294 ± 229	3,155 ± 237	3,321 ± 290	3,295 ± 288	3,190 ± 216
Brain weight (mg)	153 ± 14	152 ± 13	160 ± 18	157 ± 12	150 ± 14
Liver weight (mg)	131 ± 17	122 ± 16	129 ± 19	126 ± 16	125 ± 16
Placenta weight (mg)	538 ± 70	545 ± 86	552 ± 77	539 ± 89	562 ± 90
Ischemia uterine horn	(n = 26)	(n = 22)	(n = 21)	(n = 23)	(n = 20)
Neonatal body weight (mg)	2,701 ± 364†	2,991 ± 246*	2,740 ± 380†	2,726 ± 384†	2,698 ± 378†
Brain weight (mg)	149 ± 13	147 ± 15	155 ± 13	149 ± 11	146 ± 16
Liver weight (mg)	97 ± 24†	117 ± 22*	97 ± 20†	99 ± 19†	95 ± 21†
Placenta weight (mg)	502 ± 55	527 ± 64	506 ± 75	507 ± 65	512 ± 79

Values are mean ± SD.
*Against vehicle treated animal in ischemia uterine horn, one-factor ANOVA followed by Scheffe's *F* test ($P<0.05$).
†Against non-ischemia uterine horn, unpaired *t*-test ($P<0.05$).

exposed to ischemia, respectively, when compared with those in the non-ischemic uterine horns. The growth retardation observed in this study is not as severe as in many other models, where corticosteroid treatment[14], chronic undernutrition[15], temporary starvation[16], and hypoxia[17] may induce IUGR of up to 30% before term.

In contrast to the vehicle treated animals, pretreatment with PBN, FK 506, nifedipine, or MK-801 showed a protective effect on the growth retardation. The protective effect may be implicated in the restoration of intracellular bioenergetic failure in fetal rats. Recirculation following 30 minutes of intrauterine ischemia due to uterine artery occlusion has previously been found to be accompanied by a delayed deterioration of cellular bioenergetic state [5] and of mitochondrial respiratory activity in fetal and neonatal rat brain[6–8]. Because our preliminary studies demonstrated that the deterioration was prevented by PBN[5], FK 506[9], nifedipine[10], or MK-801[11], these drugs may act at the intracellular mitochondrial level.

Although all the drugs had a protective effect on the growth retardation when the drugs were given prior to ischemia, there was an obvious difference in the windows of therapeutic opportunity between

Table 5 Neonatal body weight and organ weight in MK-801 treated animals

	Vehicle	MK801 treatment			
		pretreatment	posttreatment		
			1 hour	3 hours	24 hours
Non-ischemia uterine horn	(n = 24)	(n = 23)	(n = 27)	(n = 26)	(n = 24)
Neonatal body weight (mg)	3,294 ± 229	3,212 ± 199	3,222 ± 284	3,312 ± 230	3,325 ± 291
Brain weight (mg)	153 ± 14	151 ± 16	150 ± 18	159 ± 19	160 ± 19
Liver weight (mg)	131 ± 17	129 ± 20	132 ± 15	128 ± 18	133 ± 21
Placenta weight (mg)	538 ± 70	544 ± 98	526 ± 91	551 ± 108	565 ± 96
Ischemia uterine horn	(n = 26)	(n = 22)	(n = 20)	(n = 23)	(n = 22)
Neonatal body weight (mg)	2,701 ± 364†	3,007 ± 272*	2,827 ± 337†	2,794 ± 418†	2,727 ± 391†
Brain weight (mg)	149 ± 13	149 ± 13	146 ± 13	150 ± 13	152 ± 15
Liver weight (mg)	97 ± 24†	121 ± 21*	101 ± 18†	103 ± 25†	92 ± 20†
Placenta weight (mg)	502 ± 55	532 ± 61	508 ± 78	514 ± 63	522 ± 79

Values are mean ± SD.
*Against vehicle treated animal in ischemia uterine horn, one-factor ANOVA followed by Scheffe's *F* test ($P<0.05$).
†Against non-ischemia uterine horn, unpaired *t*-test ($P<0.05$).

these drugs. Our results indicate that the treatment with PBN and FK 506 offer relatively wide windows of therapeutic opportunity. In contrast, nifedipine and MK-801, given after the start of recirculation, have no protective effects for the growth disturbances. The differences may be due to various phases that each drug acts in the cascade of ischemia and reperfusion resulting the growth disturbance.

The reactions elicited by sudden ischemia can be regarded as a cascade in which the first event is a fall in phosphorylation potential, and the next is membrane depolarization with an ensuing loss of ion homeostasis, including uncontrolled calcium influx into cells through voltage-gated calcium ion channels and ionotropic glutamate receptors[18,19]. The increased intracellular calcium ion is considered as an important mediator for several cascades which lead to ischemic tissue damage[20]. However, our results in the nifedipine and MK-801 treated animals, which demonstrated short therapeutic windows against transient intrauterine ischemia, suggest that the L-type voltage-gated calcium ion channel and ionotropic glutamate (NMDA) receptor play only a transient role in the development of growth retardation in fetal rats.

Recirculation or reoxygenation acts as a trigger of a second series of reactions that lead to the production of potentially toxic metabolites, and to a critical perturbation of membrane structure and function. The delayed damage can be defined as cell injury which is not obviously present immediately after recirculation but which develops after a delay of hours or days. Although the mechanisms that lead to the secondary damage following transient ischemia have not yet been elucidated, most evidence indicates that free radicals are implicated in the pathogenesis of this damage[20,21].

Because PBN is one of the classical spin trapping agents, it seems likely that it acts by scavenging free radicals[22]. Theoretically, the beneficial effects could reflect accumulation of PBN in mitochondria, where it could conceivably prevent free radical damage to components of the respiratory chain. Indeed, it has been shown that delayed treatment with PBN attenuates cellular bioenergetic failure and mitochondrial dysfunction in adult[23,24] and immature ischemic animal models [5,7]. These findings, together with our results, indicate a wide therapeutic window for nitrones of the PBN type of potentially large clinical importance.

FK 506 is presently used in clinical practice for prevention of allograft rejection[25,26]. Recent studies suggest that FK 506 is of use in the treatment of stroke[9,27-31], as supported by evidence of marked neuroprotection observed against excitotoxic neuronal death in cell culture systems[27] and ischemic brain damage in adult[28-31] and neonatal animal

models[9]. In the present study, FK 506 showed the widest window of therapeutic opportunity for the growth disturbances in the neonatal body and liver. This wide therapeutic window is in agreement with results reported in adult animals[28-31]. However, the mechanisms by which FK 506 ameliorates ischemia-reperfusion injury have been unclear. The inhibition of free radical production may be involved, particularly that related to leukocyte adhesion; in animal models with myocardial infarction, FK 506 reduces ischemic damage by preventing superoxide free radical production in polymorphonuclear (PMN) leukocytes[32]. In addition, Dawson et al.[27] showed that FK 506 prevents the calcineurin-mediated dephosphorylation of neuronal nitric oxide synthase (nNOS) and reduces NO production. As discussed, NO and O_2^- react to yield peroxynitrite, which in turn causes neurotoxicity by inhibiting mitochondrial respiration and DNA synthesis[33,34]. Indeed, our previous report demonstrated that relatively late phase recirculation following transient intrauterine ischemia enhances the production of NO in the term fetal brain[35]. Thus, FK 506 may act in the relatively late phase of recirculation, resulting in the wide therapeutic window.

Another possible explanation for the beneficial effect is that all the drugs used in this study improved the perfusion of the whole fetoplacental unit after 30 min of ischemia. However, there have been, as far as we know, no comparable studies which demonstrated the effect of the drugs on the fetoplacental perfusion during the recirculation period after transient intrauterine ischemia.

In summary, our results indicate that treatment with PBN and FK 506 offer relatively wide windows of therapeutic opportunity in fetal growth retardation induced by transient intrauterine ischemia in rats and suggest the possibility of therapeutic interventions after the start of recirculation.

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