probe-position-specific manner of measurement makes repeated intermittent measurements unreliable. Since LDF generally requires the attachment of a probe onto the skull or the dura, long-term observation would excessively strain the pups. Hence, the monitoring duration with LDF in most cases is short, being less than 1 (Liu et al., 1999; Matsiukevich, et al., 2010; Taniguchi et al., 2007), or 2 h after the end of hypoxic exposure (Fabian et al., 2008; Ioroi et al., 1998). To our knowledge, there is only one report in which the CBF was observed beyond the early perfusion phase in a rodent model of neonatal HIE (Wainwright et al., 2007). Laser Doppler perfusion imaging is a recent development in LDF that extends its power to the two-dimensional measurement of tissue perfusion. However, laser Doppler perfusion imaging cannot be used to dynamically image high frequency blood-flow fluctuations since the temporal separation between the first and last image points within a scan can be several minutes. In addition, its resolution is not very high, ranging from 100 μm/pixel to 1.0 mm/pixel (Forrester et al., 2002; Riyamongkol et al., 2002).

The laser speckle method for imaging vascular structure in a tissue has been available since the 1980s. It has recently been revised to measure CBF, as first described by Dunn et al. (2001). For a duration of several milliseconds to several hours, the speckle imaging method is able to accurately image the cortical blood-flow response over an area ranging from a few millimeters to the whole rodent brain (Dunn et al., 2001). Laser speckle flowmetry (LSF) provides excellent spatial resolution, even through an intact skull. This allows the measurement of CBF changes occurring within the pial vasculature (Ayata et al., 2004). The laser speckle technique primarily measures the velocity of scattering particles (e.g., red blood cells). LSF provides an index of perfusion that has a linear relationship with the absolute CBF value (which is measured by the [^{14}C]iodoamphetamine technique (Ayata et al., 2004) and by the clearance rate of umbelliferone (Strong et al., 2006)). LSF enables the long-term observation of CBF (Fujita et al., 2010). To our knowledge, the use of two-dimensional laser speckle perfusion imaging to observe CBF has never been investigated in immature rodent models of HI insult or stroke.

There is significant inter- and intra-litter variability in the extent of the brain injury in the HI model: a subset of pups suffers no perceivable brain injury, while other pups suffer massive hemispheric infarct (Sheldon et al., 1998). This also holds true in neonatal stroke models (Bonnin et al., 2011; Comi, et al., 2005; Wendland et al., 2008). The variability in animal models resembles the variability seen in human infants with neonatal HIE. In a population-based study, the teenage outcome of children who had been born with moderate neonatal HIE was quite variable: 35% had cerebral palsy or other major neuroimpairments; 46% had cognitive problems without cerebral palsy; and 9% had no problems (Lindström et al., 2006). Hence, it is no wonder that some pups may have no lesion after an HI insult. The variability in animal models, however, makes detailed preclinical analyses difficult to perform. Efforts have been made to offset this hindrance and, in particular, to exclude subjects with no lesion, but there is no widely used method to optimize degree of brain injury. A few laboratories use parameters—such as apparent diffusion coefficient (ADC) obtained by magnetic resonance imaging (MRI) (Derugin et al., 2000; Wendland et al., 2008), or the CBF obtained by color-coded pulsed Doppler ultrasound imaging (Bonnin et al., 2011)—to exclude pups without a lesion at an early stage of brain injury. The objectives of our study are: 1) to show temporal changes in CBF in a mouse model and in a rat model of neonatal HIE and 2) to examine the correlation between CBF during the early stage of HI injury and later morphological brain damage.

Materials and methods

Hypoxia–ischemia procedure

All experiments were performed in accordance with protocols approved by the Experimental Animal Care and Use Committee of the National Cerebral and Cardiovascular Center. Eight-day-old (postnatal day 8, [P8]) male and female CB17 mouse pups (CLEA Japan Inc., Tokyo, Japan) and seven-day-old (P7) male and female Wistar rat pups (Japan SLC Inc., Hamamatsu, Japan) were prepared for surgery. Under isoflurane anesthesia (4.0% for induction and 1.5% to 2.0% for maintenance), the left carotid artery was permanently occluded in the mouse and rat pups. After a one- to two-hour recovery period, the mouse pups were subjected to hypoxia (8% oxygen and 92% nitrogen, at 33.0 °C) for 30 min and the rat pups were subjected to hypoxia for 120 min. After a 60-min recovery period in a temperature-controlled incubator, the pups were returned to their dams and kept in a standard environment.

Laser speckle blood-flow imaging

In 23 mice and 33 rats, the cortical surface CBF was sequentially measured by a laser speckle flowmetry (LSF) imaging system (Omegazone, Omegawave Inc., Tokyo, Japan) at several time points: pre-surgery; pre-hypoxia (which is post-surgery); and 0 h, 1 h, 2.5 h, 6 h, 9 h, and 24 h after the end of hypoxia (i.e. after the start of reperfusion). The theory and technique for LSF have been described in detail elsewhere (Dunn et al., 2001; Forrester et al., 2002). In brief, the animals were placed in a prone position and spontaneously breathed under isoflurane anesthesia. The animal's skull was exposed by a midline scalp incision and the skull surface was diffusely illuminated by a 780 nm laser light. The penetration depth of the laser is approximately 500 μm. The scattered light was filtered and detected by a charge coupled device (CCD) camera positioned above the animal's head. Raw speckle images were used to compute the speckle contrast, which is a measure of speckle visibility that is related to the number and velocity of moving particles (in this case, CBF). Color-coded blood-flow images were obtained in the high-resolution mode (638 pixels × 480 pixels; 1 image/s). Five consecutive raw speckle images were acquired at 1 Hz, and then averaged. For analytical accuracy in repositioning of the animal's head and regions of interest (ROIs) between imagings, we set the size and the position of an ROI, based on a line drawn from the bregma to the lambda (Fig. 1A). We measured the CBF in three ROIs: the Core (the ischemic core region of the middle cerebral artery (MCA) territory); the Penumbra (the penumbra region of the MCA territory by the sagittal suture); and the MCA region (the broader region covering most of the MCA territory, including the Core and the Penumbra) (Fig. 1A). The same grid was used to set the three matching regions on the contralateral side. The total measuring procedure took approximately 3 min per pup.

Quantitative histological analysis

Seven days after the HI insult, the animals were deeply anesthetized with an overdose of pentobarbital and intracardially perfusion-fixed with 4% paraformaldehyde. After the perfusion, an animal's brain was removed and coronally sectioned in slices 2-mm thick by using a rat brain slicer (Neuroscience Inc., Tokyo, Japan). The area (in mm^2) of the contralateral and ipsilateral hemispheres in each brain section was measured, using NIH Image software (ImageJ, 1.43r, NIH, Bethesda, USA). The hemispheric volume of the brain of each pup was estimated by summing the hemispheric area of the brain slices and multiplying the sum by the section interval thickness. The injury was evaluated by using hematoxylin–eosin-stained sections from four brain regions: the cortex, striatum, hippocampus, and thalamus. We used the system we previously developed for evaluating neuropathologic injury in the present study (Tsuji et al., 2004). Neuropathologic injury in the cerebral cortex was scored on a scale ranging from 0 to 4 points (0, no injury; 4, extensive confluent infarction). Neuropathologic injury in the hippocampus, striatum, and thalamus was scored on a scale ranging from 0 to 6 points. The total score (ranging from 0 to 22 points) was the sum of these ratings. The

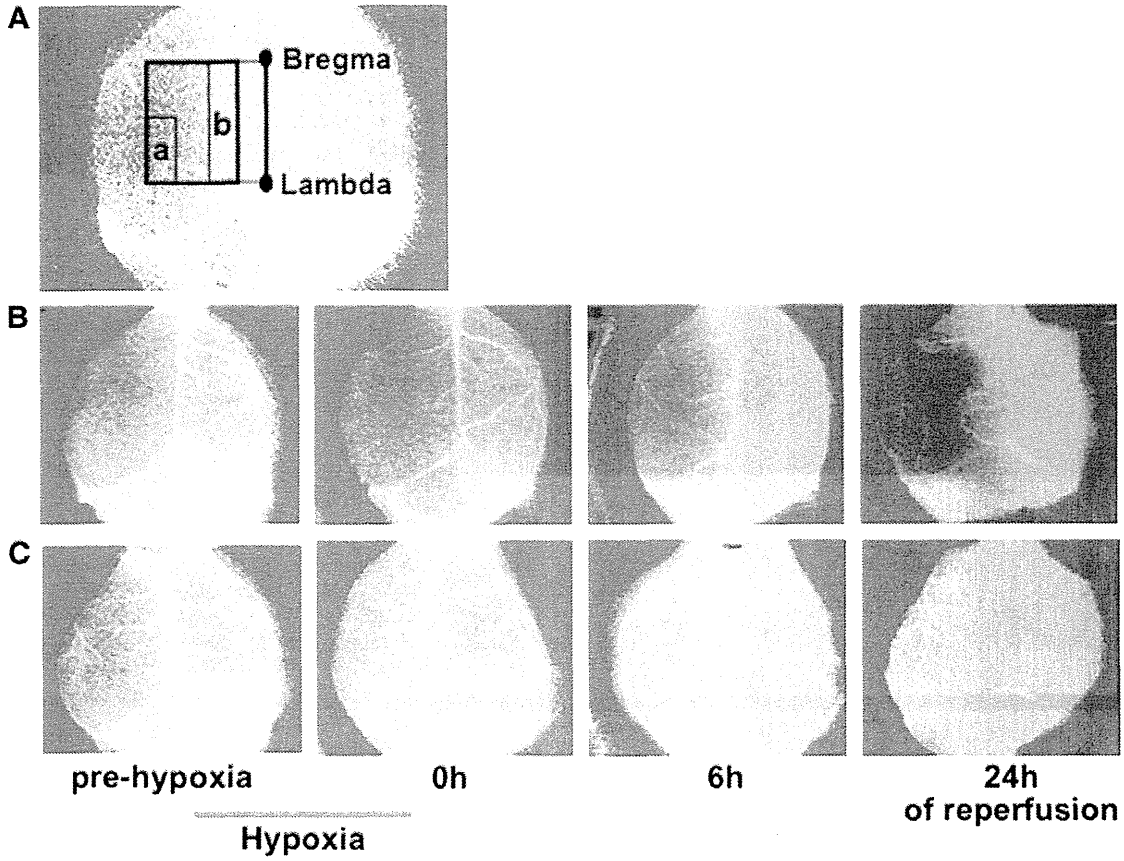

Fig. 1. We measured the cerebral blood flow (CBF) in three regions of interest (ROIs) (A): the Core (the ischemic core region of the middle cerebral artery [MCA] territory) (a); the Penumbra (the penumbra region of the MCA territory by the sagittal suture (b); and the MCA region (a broad region covering most of the MCA territory including the Core and the Penumbra) (represented by a square with bold lines). We set the grid to standardize the size and position of the ROIs. A line is drawn from the bregma to the lambda. Using this line, a square is drawn. The line perpendicular to the bregma–lambda line is divided into four equal segments, establishing four quarter-rectangles. The second quarter-rectangle from the center (b) is the Penumbra. The posterior half of the most lateral quarter-rectangle (a) is the Core. The whole square, excluding the most medial quarter-rectangle, is the MCA region. Representative laser speckle images were sequentially taken at several time points in two mouse pups. One pup (B) shows progressive reduction of CBF on the ipsilateral hemisphere of the carotid artery ligation, while the other pup (C) shows restoration of CBF.

hemispheric volume measurement and neuropathological scoring were assessed blindly.

Drug administration

In a different cohort, we used dexamethasone to examine how drug treatment affects the correlation between CBF and brain damage. Dexamethasone is a known neuroprotective drug, if it is administered before a neurologic insult in rodents (Tuor, 1995). On postnatal day 7, mouse littermates were randomly assigned to either a dexamethasone-treated group (n = 17) or a vehicle-treated group (n = 16). In the former group, 0.5 mg/kg of dexamethasone dissolved in normal saline (40 μl) was injected intraperitoneally 24 h before the HI insult. The vehicle was administered in the same manner.

Statistics

Hemispheric differences in CBF were assessed using repeated-measures two-way analysis of variance (ANOVA), followed by the Bonferroni–Dunn test. The temporal changes in CBF were analyzed by the Friedman test, followed by the Bonferroni–Dunn test. Pearson's productmoment correlation coefficient analysis was performed to determine the correlation between CBF and brain injury. Linear regression analysis and the Student *t*-test were performed to assess the effects of dexamethasone. Differences were considered significant at $P<0.05$. The results are presented as the mean ± standard error of the mean (SEM).

Results

Temporal profile of CBF in mice

LSF imaging through the intact mouse skull demonstrated clear two-dimensional images of the cortical surface blood flow (Figs. 1B, C). The surface blood flow during the pre-hypoxia period (i.e., after carotid artery occlusion but immediately before hypoxic exposure) decreased in the MCA territory on the ipsilateral side of the carotid artery occlusion in all mouse pups. In some pups, the CBF on the ipsilateral side of the carotid artery occlusion remained decreased or decreased further during the reperfusion phase (Fig. 1B). In other pups, the CBF on the ipsilateral side was nearly restored to the same level as on the contralateral side (Fig. 1C).

On the ipsilateral side of the carotid artery occlusion, temporal profiles of the mean surface CBF in the Core and the MCA region (but not the Penumbra region) differed significantly from the matching regions on the contralateral side, based on repeated-measures two-way ANOVA (Figs. 2A–C). The mean surface CBF in each of the three regions of each hemisphere was significantly decreased before the hypoxic exposure, compared with mean surface CBF in these regions before surgery. During the early reperfusion phase, the mean

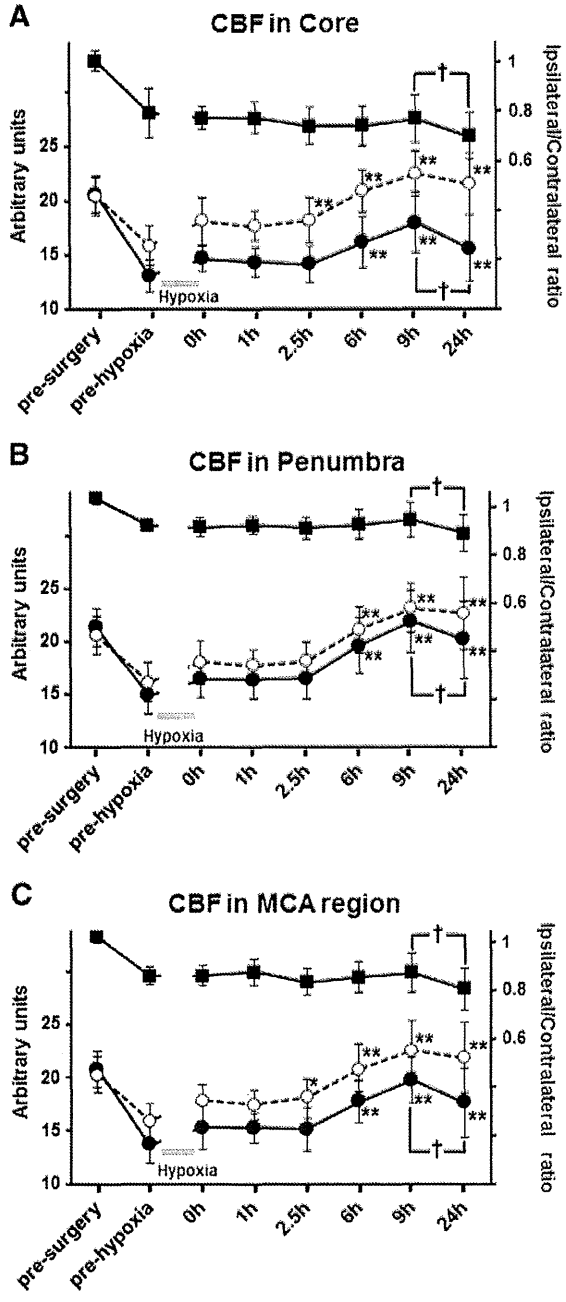

Fig. 2. The temporal profile of the mean CBF in mice in (A) the Core region; (B) the Penumbra region; and (C) the MCA region. The line with closed circles represents the ipsilateral CBF; the dashed line with open circles represents the contralateral CBF; the line with closed squares represents the ipsilateral/contralateral CBF ratio. The surface CBF level in each ROI in each hemisphere is significantly increased 6 h, 9 h, and 24 h after the end of hypoxia (i.e. the reperfusion phase), compared with the pre-hypoxia CBF level. *$p<0.05$; **$p<0.01$. The CBF in each ROI in the ipsilateral hemisphere (but not the contralateral hemisphere) is significantly decreased at 24 h, compared with the CBF at 9 h. † $p<0.05$

CBF in all three regions of both hemispheres increased slightly from the pre-hypoxic level; however, the increases were not statistically significant. During the late reperfusion phase (i.e., 6 h, 9 h, and 24 h after the end of hypoxia), the mean CBF in the three regions of both hemispheres increased significantly, compared with the mean CBF before the hypoxia. After 9 h of reperfusion, the mean CBF in the Penumbra region in the ipsilateral hemisphere and in all regions in the contralateral hemisphere were restored to their pre-surgery levels. At 24 h, the mean CBF in the three regions in the contralateral hemisphere remained the same; however, the mean CBF in all the three regions in the ipsilateral hemisphere was significantly decreased after 9 h. In the three regions, the ratio of the ipsilateral CBF to the contralateral CBF did not change significantly from before the hypoxic exposure to 9 h after the hypoxic exposure. From 9 h to 24 h, the ratio decreased significantly in the three regions.

Correlation between the CBF and later brain injury in mice

Brain injury was assessed histologically seven days after the HI insult. The CBF in each region at each time point was then compared with the histological brain injury in the mice. This was to determine whether the surface CBF (as measured by LSF during the reperfusion phase) can be used as an early indicator of later histological injury after an HI insult. The ratio of the ipsilateral CBF to the contralateral CBF was compared with the ratio of the ipsilateral hemispheric volume to the contralateral hemispheric volume (Fig. 3). Linear regression analysis demonstrated that the longer the time after the hypoxic exposure, the stronger was the correlation between the degree of reduced CBF and the degree of brain damage. The correlation was stronger in the Core than in the other two regions. Of all the regions and time points measured, the CBF in the Core at 24 h of reperfusion had the strongest correlation with brain injury ($R^2=0.89$).

We also used neuropathological injury scoring to examine the correlation between CBF and brain injury. Linear regression analysis demonstrated that the surface CBF in the Core at 24 h was significantly correlated with neuropathological scores in all four anatomical structures (the hippocampus, thalamus, cortex, and striatum) that were examined seven days after the HI insult (Fig. 4). Interestingly, the correlation was strongest for the injury score in the hippocampus ($R^2=0.58$) (data not shown), followed by the injury score in the thalamus. The correlation for the injury score in the cortex ($R^2=0.36$) was weaker than that of total score ($R^2=0.51$).

The effect of drug treatment on the correlation between CBF and later brain injury

The hemispheric volume was assessed seven days after the HI insult. Dexamethasone pretreatment, as expected, significantly reduced brain injury. However, this treatment did not improve CBF 24 h after the HI insult. The correlation between the CBF and brain injury in the dexamethasone-treated group was not significant ($P=0.08$) (Fig. 5). The loss of correlation between the CBF and brain injury after dexamethasone treatment indicates that its neuroprotective mechanisms are independent of CBF, at least at the time of measurement.

Temporal profile of CBF and its correlation with later brain injury in rats

Based on the results of repeated-measures two-way ANOVA, the temporal profile of the mean surface CBF in the Core on the ipsilateral side of the carotid artery occlusion significantly differed from the matching regions on the contralateral side in rats (Fig. 6A). The mean surface CBF in the Core on the ipsilateral hemisphere before the hypoxic exposure was significantly decreased from its pre-surgery level, while the mean surface CBF in the Core on the contralateral hemisphere remained the same. The mean CBF in the Core in both hemispheres significantly increased after 2.5 h of reperfusion, compared with the mean CBF before hypoxia. After 6 h of reperfusion, the mean CBF in the Core on the ipsilateral side was restored to its pre-surgery level, and the mean CBF in the matching region on the contralateral side increased beyond its pre-surgery level. The mean CBF in the Core of each hemisphere at 24 h was significantly decreased from its level at the previous time point measurement. The

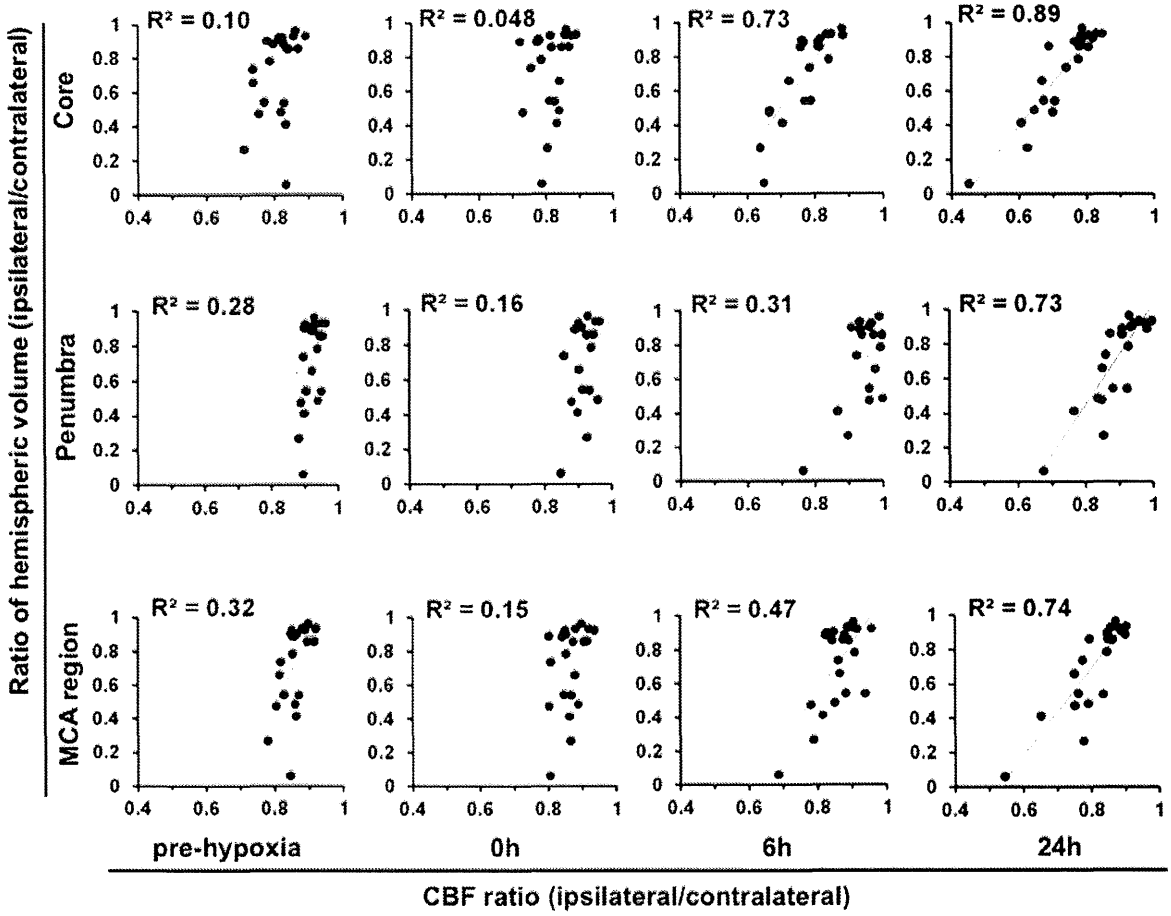

Fig. 3. The ratio of the ipsilateral CBF to the contralateral CBF at each time point was compared with the ratio of the ipsilateral hemispheric volume to the contralateral hemispheric volume (which was assessed seven days after the HI insult in the mice). The correlation between the degree of CBF reduction and the degree of brain damage is stronger in the Core than in the other two regions. The correlation was stronger at the later time points. Of all the time points and regions measured, the CBF in the Core at 24 h of reperfusion has the strongest correlation with later brain injury ($R^2 = 0.89$).

ratio of the ipsilateral CBF to the contralateral CBF increased from 2.5 h of reperfusion onward, compared with the pre-hypoxia ratio.

Among the three regions, the correlation between CBF and later brain injury (as assessed by the hemispheric volume) was the strongest in the Core in rats. This was also the case in mice. In the Core region, the CBF at 6 h of reperfusion had the strongest correlation with later brain injury ($R^2 = 0.35$) (Fig. 6B). When assessed by neuropathological injury scoring, a correlation was evident between the CBF and brain injury. Linear regression analysis demonstrated that the CBF in the Core at 6 h was significantly correlated with the total neuropathological scores, which were assessed seven days after the HI insult ($R^2 = 0.27$) (data not shown). These correlations were weaker in rats than in mice.

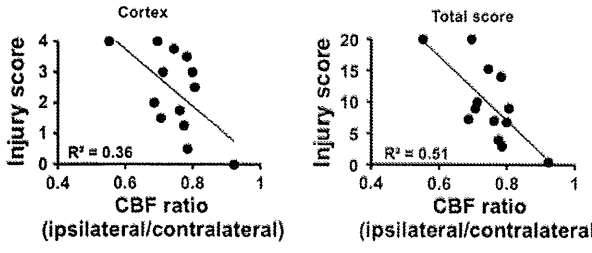

Fig. 4. The ratio of the ipsilateral CBF to contralateral CBF 24 h after the HI insult was compared with brain injury (as assessed by neuropathological scoring seven days after the HI exposure). Linear regression analysis demonstrates that the surface CBF in the Core at 24 h is significantly correlated with the neuropathological scores in the cortex and with the total score (which combines the scores of the cortex, hippocampus, striatum, and thalamus).

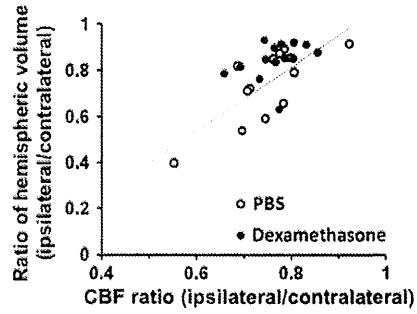

Fig. 5. The hemispheric volume (measured seven days after the HI insult) was used to assess the effect of the neuroprotective drug dexamethasone on the correlation between CBF (24 h after the HI insult) and brain injury. The data points show the correlation between CBF and brain injury in the PBS-pretreated mice and the dexamethasone-pretreated mice. The data points for the dexamethasone-pretreated mice tends to be shifted toward the top of the graph (indicating less brain injury), but is not shifted in the horizontal axis (indicating no change in the CBF).

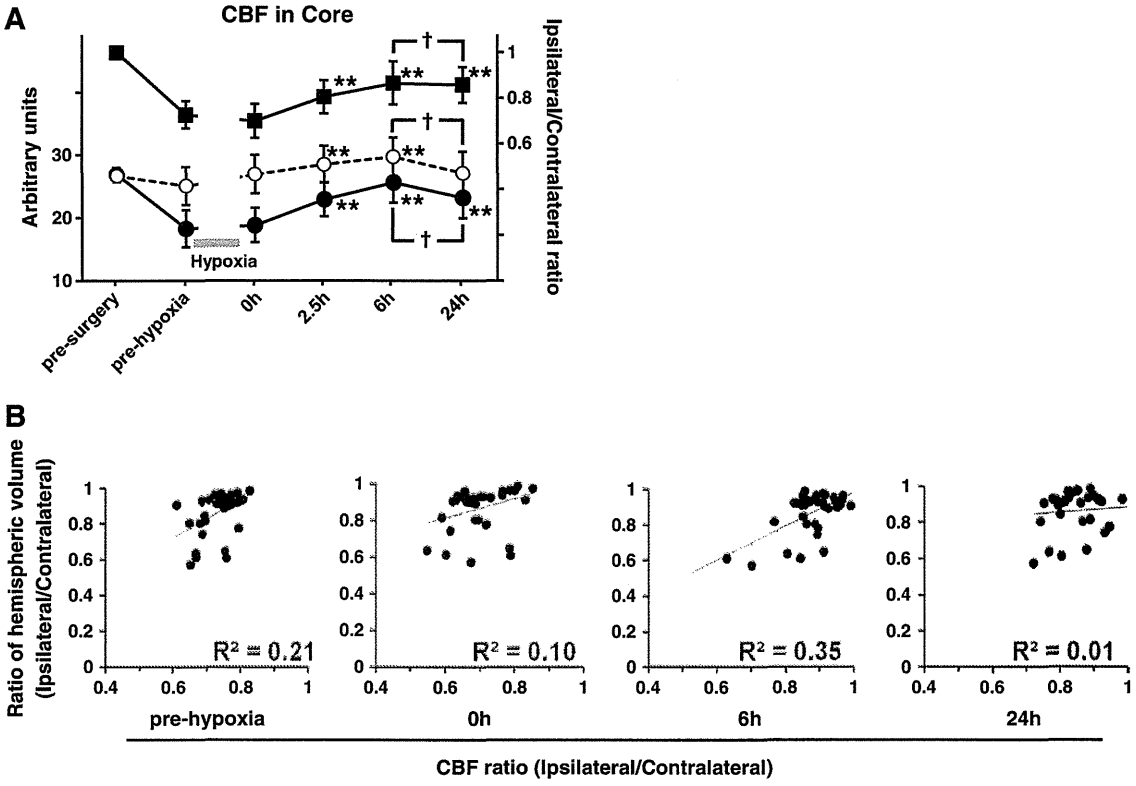

Fig. 6. (A) The temporal profile of CBF in the Core region in rat pups. The mean CBF in the ipsilateral hemisphere is significantly decreased after carotid artery ligation alone. After the hypoxic exposure, the mean CBF level is significantly increased at 2.5 h, 6 h, and 24 h of reperfusion, compared with its pre-hypoxia level. By contrast, the mean CBF in the contralateral hemisphere remains unchanged after carotid artery ligation alone. After the hypoxic exposure, the mean CBF level significantly increased at 2.5 h and 6 h, compared with the pre-hypoxia CBF level. $^{**}p<0.01$. The CBF level in each hemisphere is significantly decreased at 24 h, compared with its level at the 9 h. †$p<0.05$. (B) The ratio of the ipsilateral CBF to contralateral CBF in the Core at each time point was compared with the ratio of the ipsilateral hemispheric volume to the contralateral hemispheric volume that was assessed seven days after the HI injury in the rats. The correlation between CBF and brain injury is significant and strongest at 6 h of reperfusion.

Discussion

Temporal profiles of CBF

In the present study, we demonstrated the spatial and temporal profiles of cortical surface CBF in mouse and rat pups. After carotid artery ligation alone, the mean CBF was decreased in the ipsilateral hemisphere in the mouse and rat pups. Following the hypoxic insult (i.e., with the initiation of reperfusion), the mean CBF on the ipsilateral side gradually increased; it nearly approached its pre-surgery level during the late reperfusion phase (at 9 h in mice and at 6 h in rats). It then decreased by 24 h.

In a classical study that used carbon-14 autoradiography in immature rats, blood flow to individual structures in the ipsilateral cerebral hemisphere was not affected by unilateral arterial occlusion alone (Vannucci et al., 1988). During the HI insult, the regional CBF of the ipsilateral hemisphere decreased. In a subsequent study, the researchers found that the CBF was similar in both hemispheres after 30 min, 4 h, and 24 h of recovery, and that the CBF did not differ from age-matched controls (Mujsce et al., 1990).

Most non-invasive studies measure CBF only until the early reperfusion phase; however, one LDF study measured CBF changes occurring from before surgery until 24 h after an HI injury in immature rodents (Wainwright et al., 2007). The CBF in the ipsilateral hemisphere was significantly decreased after carotid artery ligation alone and at 2 h after the HI insult, compared with the pre-surgery level. The CBF level then slowly returned to its pre-surgery level. On the other hand, the CBF in the contralateral hemisphere increased during reperfusion, before normalizing 24 h after the HI insult. Using the magnetic resonance arterial spin-labeling technique, Qiao et al. (2004) measured CBF prior to hypoxia, during hypoxia, and 1 h and 24 h after hypoxia in P7 rats with an HI injury. Following carotid artery occlusion and prior to hypoxic exposure, the cortical CBF levels were reduced, compared with the levels in the sham animals. At 1 h of reperfusion (i.e., after the cessation of hypoxia), the CBF approached levels close to the pre-hypoxic exposure levels. At 24 h of reperfusion, the CBF in the ipsilateral cortex further recovered, although the flow was still below that of the sham animals. These two reports are mostly in accordance with our data.

The apparent conflicting results of our study with studies using autoradiography may be because of the different techniques and regions used to measure CBF. We measured the CBF on the cortical surface, while the other researchers used coronal sections. The CBF was measured with a diffusible indicator in the other studies. A diffusible indicator is more representative of plasma flow (Mujsce et al., 1990), whereas LSF and LDF reflect red blood cell flow in the brain surface. Even among studies using the same CBF measuring techniques, there are conflicting reports.

The intensity of an HI insult may also explain the discrepancy of the reported data on CBF during reperfusion (Karlsson et al., 1994; Todd et al., 1986). Mild to moderate ischemia is followed by an initial period of hyperperfusion; a severe insult is followed by hypoperfusion (Fellman and Raivio, 1997). In adult rats with ischemia, it has been shown that the degree of postischemic hypoperfusion increases with increasing duration of ischemia, while the onset of postischemic hypoperfusion is delayed with increasing duration of ischemia. The mechanisms of hypoperfusion have been variously

ascribed to endothelial injury and swelling, to granulocytic plugging of microvessels, and to intravascular clotting (Fellman and Raivio, 1997).

The temporal profiles of CBF after an HI insult were similar in mice and rats, although there were some differences. Apart from species differences, differences in the intensity of the HI insult may have influenced the results. The degree of brain injury was more severe in the mice than in the rats, even though we had tried to optimize the duration of the hypoxic exposure to obtain a similar degree of brain injury, to obtain a similar percentage of pups with no obvious brain lesion, and to obtain a similar mortality rate in each species. Another factor that may have influenced the differences is that the CB17 mouse is a strain in which there are few variations in the MCA branches (Taguchi et al., 2010).

Predicting later brain injury by CBF and reducing animal variability

Animal models of ischemic disease show a heterogeneous degree of brain injury. This lesion variability is highlighted by animals that do not develop any lesion, despite proper surgical procedures. These animals with an intact brain are seen in HI models and in stroke models that use transient occlusion of the MCA (Derugin et al., 2000; Wendland et al., 2008) or that use permanent occlusion of the MCA (Bonnin et al., 2011) and carotid artery (Comi et al., 2005). To offset this animal variability, we examined CBF during reperfusion to see if it could be utilized as a predictive factor for later brain injury. The correlation between CBF and later brain injury was significant in mice ($R^2=0.89$) and in rats ($R^2=0.35$). The strong correlation in mice in the current study is remarkable when compared with other measures in literature. It is even stronger than the coefficient of determination for the cortex ($R^2=0.83$), which was assessed by MRI (using the 9.4T system) and Nissl-stained histology (Ten et al., 2004). MRI and histological examinations were performed at the same time point (i.e., 10 weeks after the HI insult). (In our study, the CBF was analyzed hours after the HI insult and a histological examination was performed seven days after the HI insult. The correlation is nevertheless stronger in our study than in the MRI study.) The present study interestingly demonstrated that the cortical surface CBF, as measured by LSF, was well correlated with later injury in deep brain structures, such as the hippocampus and thalamus.

We believe that CBF, as measured by LSF, can be used in preclinical research with rodents in two different ways. First, the LSF technique optimizes the extent of brain injury by excluding a portion of pups with no lesion or excluding pups with a massive lesion. Second, the LSF technique enables the analysis of the effect of an intervention on temporal and spatial changes in CBF, so that the mechanisms of neuroprotection (in relation to the CBF) can be assessed.

We examined how effectively pups with no brain lesion or minimal brain lesion can be excluded by using CBF analysis. In the present study, there were seven mouse pups with no lesion or a minimal lesion, which was defined as an ipsilateral/contralateral hemispheric volume ratio greater than 0.90. With the ipsilateral/contralateral Core CBF ratio cut-off value set at 0.78 at 24 h of reperfusion, nine pups out of 22 pups were excluded. Using this criterion, all seven mouse pups with no lesion or a minimal lesion were accurately excluded. (Therefore, the sensitivity was 100%; and the specificity, 87%). There were seven rat pups with no lesion or a minimal lesion. In rats, a minimal lesion was defined as an ipsilateral/contralateral hemispheric volume ratio greater than 0.95. With the ipsilateral/contralateral Core CBF ratio cut-off value set at 0.90 at 6 h of reperfusion, the sensitivity was 57% and specificity was 65% for excluding pups with no lesion or a minimal lesion.

A limitation of this method is that excluding pups with no lesion may not be practical at an early time point. Most therapeutic interventions tested in experimental models are administered either before or immediately after an HI insult. In the mice in the current study, the correlation between later brain injury and the Core CBF after reperfusion remained strong at 2.5 h ($R^2=0.36$) and at 6 h ($R^2=0.73$). However, the correlation at 0 h ($R^2=0.05$) and at 1 h ($R^2=0.15$) was weaker than at later time points, and therefore was not useful for predicting brain injury. This was the same trend noted in rats.

Cell therapies such as umbilical cord blood mononuclear cells or mesenchymal stem cells derived from bone marrow have recently been shown to be effective treatments in ischemic models (Taguchi et al., 2004; van Velthoven et al., 2010). The cells are administered days after a brain injury. Our method of using CBF to predict brain injury would be particularly useful in studies in which interventions such as cell therapy are administered some time after the brain injury.

The graph with linear regression in Fig. 5 shows that a relationship is evident between neuroprotection and CBF. A flattened slope of linear regression indicates that neuroprotection is independent of CBF. By contrast, when the line is unchanged and the data points shift toward the right upper side of the line, neuroprotection would be mainly the result of increased CBF after an intervention. In this way, the mechanisms of the intervention, in relation to the CBF, can be speculated. In the present study, a graph of the correlation between CBF and brain injury shows that dexamethasone treatment shifted the plots to the top (which indicates decreased brain injury), but the treatment did not shift the plots horizontally (which indicates no change in the CBF). This finding is in line with a previous study that used carbon-14 autoradiography and showed that dexamethasone prevented cerebral infarction without affecting the CBF during hypoxia in neonatal rats (Tuor et al., 1993). LSF can be used to test speculations concerning the mechanisms of an intervention, regardless of the timing of the intervention.

Derugin et al. (2000) used diffusion-weighted MRI (DWI) in a neonatal rat model with transient MCA occlusion to offset animal variability. DWI performed 24 h after reoxygenation, predicted the histological development of a lesion seven days after an ischemic insult in nine of 10 rats studied. They were not able to predict a lesion when DWI was performed 2 h after the initiation of reoxygenation. DWI did not detect a lesion in three rats at 2 h or at 24 h after the HI insult; however, one of these three rats developed a brain injury (Wendland et al., 2008).

Bonnin et al. (2011) used color-coded pulsed Doppler ultrasound imaging to assess CBF in a neonatal rat model that used permanent MCA occlusion with transient left common carotid artery occlusion. They measured the mean blood-flow velocity in the internal carotid arteries and the basilar trunk at three time points: before arterial occlusion, during the left common carotid artery occlusion, and 15 min after reperfusion. The mean blood-flow velocity during the transient occlusion showed a predictive value for brain injury. To our knowledge, there are no other reports demonstrating useful predictive parameters for brain injury after an HI insult in immature rodents. We believe that our LSF method is a useful and easy-to-perform way to predict later brain injury. By contrast, MRI is a time-consuming technique to perform, and color-coded pulsed Doppler ultrasound imaging may require trained hands, especially when examining immature rodents.

Clinical studies in CBF after HIE

The data of temporal changes in CBF in asphyxiated neonates during the first day of life are scarce and contradictory. Most studies using MRI, single photon emission tomography, or positron emission tomography assess CBF several days or weeks after birth (Rosenbaum et al., 1997).

In some studies, decreased CBF during reperfusion indicated a poor prognosis. One study used near-infrared spectroscopy (NIRS)

to assess changes in cerebral hemodynamics (van Bel et al., 1993). In neonates with an adverse outcome at one year of age, the cerebral blood volume had been decreased during the first 12 h of life (compared with the baseline value), suggesting a decrease in cerebral perfusion. Kirimi et al. (2002) used ultrasonography to assess the hemodynamic parameters of the MCA during the first 12 h of life in term neonates with HIE. The peak systolic velocity and the end diastolic velocity were significantly lower (and the resistive index was significantly higher) in neonates with a poor prognosis than in neonates with a good prognosis. By contrast, the cerebral blood volume was stable or increased during the first 12 h of life in infants with a normal one-year outcome.

In other studies, increased CBF during reperfusion indicated a poor prognosis. In asphyxiated term neonates, the global CBF during the first day of life was assessed by using the intravenous xenon 133 technique (Pryds et al., 1990). The mean CBF in infants with a poor prognosis was significantly higher than the mean CBF in infants with a good prognosis or in the control infants. Using Doppler ultrasonography, Ilves et al. (1998) assessed changes in CBF velocity in the major cranial arteries. Asphyxiated infants with a moderate stage of HIE had a significantly low CBF velocity (i.e., hypoperfusion) in the MCA at the age of 12 h. From the age of 24 h onward, there were no differences in the mean CBF velocity in these infants, compared with the CBF velocity in normal infants. Infants with a severe stage of HIE had a significantly high CBF velocity (i.e., hyperperfusion) in the MCA from the age of 12 h onwards. All of the infants that had a severe stage of HIE and a markedly high mean CBF velocity at the age of 12 h either died or developed multicystic degeneration of the brain.

Conclusions

Our major finding is that the degree of the CBF during the late reperfusion phase is strongly associated with the extent of later morphological brain damage. Using LSF to analyze CBF reduces animal variability, thereby reducing the number of animals that need to be used. Conflicting results in reports suggest that hemodynamic responses to an HI insult are sensitive to the severity and the duration of the insult; the time points and regions measured; and the strains and species of animals used. Because of this sensitivity, an easy and repeatable technique is crucial in assessing the CBF in animals with an HI insult. We believe that our LSF method is useful for studies using immature rodents with an HI insult and stroke. We also believe that this method would make detailed analyses possible.

Disclosure/conflict of interest

None

Sources of funding

This work was supported by a Grant-in-Aid for Scientific Research (JSPS KAKENHI 22890254) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Acknowledgments

We thank Manami Sone for excellent technical assistance with histological preparations, Akiko Kada for statistical assistance.

References

Ayata, C., Dunn, A.K., Gursoy, O.Y., Huang, Z., Boas, D.A., Moskowitz, M.A., 2004. Laser speckle flowmetry for the study of cerebrovascular physiology in normal and ischemic mouse cortex. J. Cereb. Blood Flow Metab. 24, 744–755.

Bonnin, P., Leger, P.L., Deroide, N., Fau, S., Baud, O., Pocard, M., et al., 2011. Impact of intracranial blood-flow redistribution on stroke size during ischemia-reperfusion in 7-day-old rats. J. Neurosci. Methods 198, 103–109.

Comi, A.M., Johnston, M.V., Wilson, M.A., 2005. Strain variability, injury distribution, and seizure onset in a mouse model of stroke in the immature brain. Dev. Neurosci. 27, 127–133.

Derugin, N., Wendland, M., Muramatsu, K., Roberts, T.P., Gregory, G., Ferriero, D.M., et al., 2000. Evolution of brain injury after transient middle cerebral artery occlusion in neonatal rats. Stroke 31, 1752–1761.

Dunn, A.K., Bolay, H., Moskowitz, M.A., Boas, D.A., 2001. Dynamic imaging of cerebral blood flow using laser speckle. J. Cereb. Blood Flow Metab. 21, 195–201.

Fabian, R.H., Perez-Polo, J.R., Kent, T.A., 2008. Perivascular nitric oxide and superoxide in neonatal cerebral hypoxia–ischemia. Am. J. Physiol. Heart Circ. Physiol. 295, H1809–H1814.

Fellman, V., Raivio, K.O., 1997. Reperfusion injury as the mechanism of brain damage after perinatal asphyxia. Pediatr. Res. 41, 599–606.

Forrester, K.R., Stewart, C., Tulip, J., Leonard, C., Bray, R.C., 2002. Comparison of laser speckle and laser doppler perfusion imaging: measurement in human skin and rabbit articular tissue. Med. Biol. Eng. Comput. 40, 687–697.

Fujita, Y., Ihara, M., Ushiki, T., Hirai, H., Kizaka-Kondoh, S., Hiraoka, M., et al., 2010. Early protective effect of bone marrow mononuclear cells against ischemic white matter damage through augmentation of cerebral blood flow. Stroke 41, 2938–2943.

Ilves, P., Talvik, R., Talvik, T., 1998. Changes in Doppler ultrasonography in asphyxiated term infants with hypoxic–ischaemic encephalopathy. Acta Paediatr. 87, 680–684.

Ioroi, T., Yonetani, M., Nakamura, H., 1998. Effects of hypoxia and reoxygenation on nitric oxide production and cerebral blood flow in developing rat striatum. Pediatr. Res. 43, 733–737.

Johnston, M.V., Ferriero, D.M., Vannucci, S.J., Hagberg, H., 2005. Models of cerebral palsy: which ones are best? J. Child Neurol. 20, 984–987.

Karlsson, B.R., Grogaard, B., Gerdin, B., Steen, P.A., 1994. The severity of postischemic hypoperfusion increases with duration of cerebral ischemia in rats. Acta Anaesthesiol. Scand. 38, 248–253.

Kirimi, E., Tuncer, O., Atas, B., Sakarya, M.E., Ceylan, A., 2002. Clinical value of color doppler ultrasonography measurements of full-term newborns with perinatal asphyxia and hypoxic ischemic encephalopathy in the first 12 hours of life and long-term prognosis. Tohoku J. Exp. Med. 197, 27–33.

Lindström, K., Lagerroos, P., Gillberg, C., Fernell, E., 2006. Teenage outcome after being born at term with moderate neonatal encephalopathy. Pediatr. Neurol. 35, 268–274.

Liu, X.H., Kwon, D., Schielke, G.P., Yang, G.Y., Silverstein, F.S., Barks, J.D., 1999. Mice deficient in interleukin-1 converting enzyme are resistant to neonatal hypoxic–ischemic brain damage. J. Cereb. Blood Flow Metab. 19, 1099–1108.

Matsiukevich, D., Randis, T.M., Utkina-Sosunova, I., Polin, R.A., Ten, V.S., 2010. The state of systemic circulation, collapsed or preserved defines the need for hyperoxic or normoxic resuscitation in neonatal mice with hypoxia–ischemia. Resuscitation 81, 224–229.

Mujsce, D.J., Christensen, M.A., Vannucci, R.C., 1990. Cerebral blood flow and edema in perinatal hypoxic–ischemic brain damage. Pediatr. Res. 27, 450–453.

Pryds, O., Greisen, G., Lou, H., Friis-Hansen, B., 1990. Vasoparalysis associated with brain damage in asphyxiated term infants. J. Pediatr. 117, 119–125.

Qiao, M., Latta, P., Foniok, T., Buist, R., Meng, S., Tomanek, B., et al., 2004. Cerebral blood flow response to a hypoxic–ischemic insult differs in neonatal and juvenile rats. MAGMA 17, 117–124.

Rice III, J.E., Vannucci, R.C., Brierley, J.B., 1981. The influence of immaturity on hypoxic–ischemic brain damage in the rat. Ann. Neurol. 9, 131–141.

Ringel, M., Bryan, R.M., Vannucci, R.C., 1991. Regional cerebral blood flow during hypoxia–ischemia in the immature rat: comparison of iodoantipyrine and iodoamphetamine as radioactive tracers. Brain Res. Dev. Brain Res. 59, 231–235.

Riyamongkol, P., Zhao, W., Liu, Y., Belayev, L., Busto, R., Ginsberg, M.D., 2002. Automated registration of laser Doppler perfusion images by an adaptive correlation approach: application to focal cerebral ischemia in the rat. J. Neurosci. Methods 122, 79–90.

Rosenbaum, J.L., Almli, C.R., Yundt, K.D., Altman, D.I., Powers, W.J., 1997. Higher neonatal cerebral blood flow correlates with worse childhood neurologic outcome. Neurology 49, 1035–1041.

Sheldon, R.A., Sedik, C., Ferriero, D.M., 1998. Strain-related brain injury in neonatal mice subjected to hypoxia–ischemia. Brain Res. 810, 114–122.

Stern, M.D., Lappe, D.L., Bowen, P.D., Chimosky, J.E., Holloway Jr., G.A., Keiser, H.R., et al., 1977. Continuous measurement of tissue blood flow by laser-Doppler spectroscopy. Am. J. Physiol. 232, H441–H448.

Strong, A.J., Bezzina, E.L., Anderson, P.J., Boutelle, M.G., Hopwood, S.E., Dunn, A.K., 2006. Evaluation of laser speckle flowmetry for imaging cortical perfusion in experimental stroke studies: quantitation of perfusion and detection of peri-infarct depolarisations. J. Cereb. Blood Flow Metab. 26, 645–653.

Taguchi, A., Soma, T., Tanaka, H., Kanda, T., Nishimura, H., Yoshikawa, H., et al., 2004. Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. J. Clin. Invest. 114, 330–338.

Taguchi, A., Kasahara, Y., Nakagomi, T., Stern, D.M., Fukunaga, M., Ishikawa, M., et al., 2010. A reproducible and simple model of permanent cerebral ischemia in CB-17 and SCID mice. J. Exp. Stroke Transl. Med. 3, 28–33.

Taniguchi, H., Mohri, I., Okabe-Arahori, H., Aritake, K., Wada, K., Kanekiyo, T., et al., 2007. Prostaglandin D2 protects neonatal mouse brain from hypoxic ischemic injury. J. Neurosci. 27, 4303–4312.

Ten, V.S., Wu, E.X., Tang, H., Bradley-Moore, M., Fedarau, M.V., Ratner, V.I., et al., 2004. Late measures of brain injury after neonatal hypoxia–ischemia in mice. Stroke 35, 2183–2188.

Todd, N.V., Picozzi, P., Crockard, H.A., Russell, R.R., 1986. Reperfusion after cerebral ischemia: influence of duration of ischemia. Stroke 17, 460–466.

Tsuji, M., Wilson, M.A., Lange, M.S., Johnston, M.V., 2004. Minocycline worsens hypoxic–ischemic brain injury in a neonatal mouse model. Exp. Neurol. 189, 58–65.

Tuor, U.I., 1995. Dexamethasone and the prevention of neonatal hypoxic–ischemic brain damage. Ann. N. Y. Acad. Sci. 765, 179–195.
Tuor, U.I., Simone, C.S., Barks, J.D., Post, M., 1993. Dexamethasone prevents cerebral infarction without affecting cerebral blood flow in neonatal rats. Stroke 24, 452–457.
van Bel, F., Dorrepaal, C.A., Benders, M.J., Zeeuwe, P.E., van de Bor, M., Berger, H.M., 1993. Changes in cerebral hemodynamics and oxygenation in the first 24 hours after birth asphyxia. Pediatrics 92, 365–372.
van Handel, M., Swaab, H., de Vries, L.S., Jongmans, M.J., 2007. Long-term cognitive and behavioral consequences of neonatal encephalopathy following perinatal asphyxia: a review. Eur. J. Pediatr. 166, 645–654.
van Velthoven, C.T., Kavelaars, A., van Bel, F., Heijnen, C.J., 2010. Nasal administration of stem cells: a promising novel route to treat neonatal ischemic brain damage. Pediatr. Res. 68, 419–422.
Vannucci, R.C., Lyons, D.T., Vasta, F., 1988. Regional cerebral blood flow during hypoxia–ischemia in immature rats. Stroke 19, 245–250.
Wainwright, M.S., Grundhoefer, D., Sharma, S., Black, S.M., 2007. A nitric oxide donor reduces brain injury and enhances recovery of cerebral blood flow after hypoxia–ischemia in the newborn rat. Neurosci. Lett. 415, 124–129.
Wendland, M.F., Faustino, J., West, T., Manabat, C., Holtzman, D.M., Vexler, Z.S., 2008. Early diffusion-weighted MRI as a predictor of caspase-3 activation after hypoxic–ischemic insult in neonatal rodents. Stroke 39, 1862–1868.

Stroke
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Cilostazol Reduces the Risk of Hemorrhagic Infarction After Administration of Tissue-Type Plasminogen Activator in a Murine Stroke Model

Yukiko Kasahara, Takayuki Nakagomi, Tomohiro Matsuyama, David Stern and Akihiko Taguchi

Stroke. 2012;43:499-506; originally published online October 27, 2011;
doi: 10.1161/STROKEAHA.111.635417
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0039-2499. Online ISSN: 1524-4628

Cilostazol Reduces the Risk of Hemorrhagic Infarction After Administration of Tissue-Type Plasminogen Activator in a Murine Stroke Model

Yukiko Kasahara; Takayuki Nakagomi, MD; Tomohiro Matsuyama, MD; David Stern, MD; Akihiko Taguchi, MD

Background and Purpose—Prior use of antiplatelet agents improves stroke outcome in patients undergoing thrombolytic therapy as shown by reduced arterial reocclusion, although the risk of cerebral hemorrhage can be increased.

Methods—The effect of cilostazol, an antiplatelet drug that improves endothelial function through upregulation of intracellular cAMP, on cerebral hemorrhage after thrombolytic therapy was investigated using a highly reproducible transient ischemia model.

Results—Treatment with cilostazol for 7 days before ischemia significantly suppressed the risk and severity of cerebral hemorrhage after injection of tissue-type plasminogen activator, although treatment with aspirin had no such protective effect compared with nontreated mice. Immunohistological analysis revealed that treatment with cilostazol suppressed disruption of the microvasculature in the ischemic area associated with reduced matrix metalloproteinase-9 activity.

Conclusions—Our results suggest that patients treated with cilostazol before onset of stroke could have a lower risk of cerebral hemorrhage after thrombolytic therapy and might also have a longer therapeutic time window for thrombolysis. Furthermore, the risk of cerebral hemorrhage can be significantly altered by prestroke therapies, and analysis of the effects of multiple drugs on tissue-type plasminogen activator-induced cerebral hemorrhage in animal models is essential for the extending safe and effective thrombolytic therapy to a wider group of patients. **(*Stroke*. 2012;43:499-506.)**

Key Words: antiplatelet drugs ■ brain ischemia ■ ICH ■ murine model ■ thrombolysis

Intravenous thrombolysis with tissue-type plasminogen activator (tPA) has been shown to improve functional outcomes of patients with stroke when given within 3 hours from the onset of stroke.[1,2] However, treatment with tPA significantly increases the risk of bleeding events, including hemorrhagic infarction.[3,4] Although a number of clinical studies indicate that prior use of antiplatelet agents increases the risk of cerebral hemorrhage after thrombolytic therapy,[5–7] the use of antiplatelet agents had been shown to improve stroke outcome compared with patients without such therapy.[8,9] The beneficial effects of antiplatelet drugs may be attributed to improved microcirculatory function and diminished reocclusion after tPA treatment, the latter observed in 20% to 34% of patients after initially successful recanalization.[10,11] Reocclusion after tPA treatment is induced, at least in part, by the activation of the coagulation cascade by tPA.[12,13] These findings indicate that the use of antiplatelet drugs is potentially a double-edged sword in the context of tPA treatment; that is, use of antiplatelet agents is likely to be beneficial for stroke outcome despite increased risk of hemorrhagic infarction. Consistent with these findings, a randomized controlled trial in the Netherlands has shown that thrombolysis in combination with antiplatelet drugs prevented reocclusion and improved the clinical outcome.[14]

Recently, cilostazol, an antiplatelet drug that inhibits the activity of cAMP phosphodiesterase Type 3, has been shown to be superior to aspirin for secondary prevention of stroke with fewer hemorrhagic events.[15] Compared with other antiplatelet drugs, cilostazol is known to have milder hemorrhagic side effects[16] and prevent the increase in bleeding time.[17] In this study, we focused on cilostazol and investigated its effect on hemorrhagic infarction after treatment with tPA using a murine ischemia–reperfusion model.

Materials and Methods

All procedures were performed under the auspices of an approved protocol of the National Cerebral and Cardiovascular Center Animal Care and Use Committee.

Received August 9, 2011; final revision received September 12, 2011; accepted September 29, 2011.
Miguel Perez-Pinzon, PhD, was the Guest Editor for this paper.
From the Department of Cerebrovascular Disease (Y.K., A.T.), National Cerebral and Cardiovascular Center, Osaka, Japan; the Institute for Advanced Medical Sciences (T.N., T.M.), Hyogo College of Medicine, Hyogo, Japan; and the Executive Dean's Office (D.S.), University of Tennessee, Knoxville, TN.
Correspondence to Akihiko Taguchi, MD, Department of Cerebrovascular Disease, National Cerebral and Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita, Osaka, Japan, 565-8565. E-mail taguchi@ri.ncvc.go.jp

Stroke **is available at http://stroke.ahajournals.org** **DOI: 10.1161/STROKEAHA.111.635417**

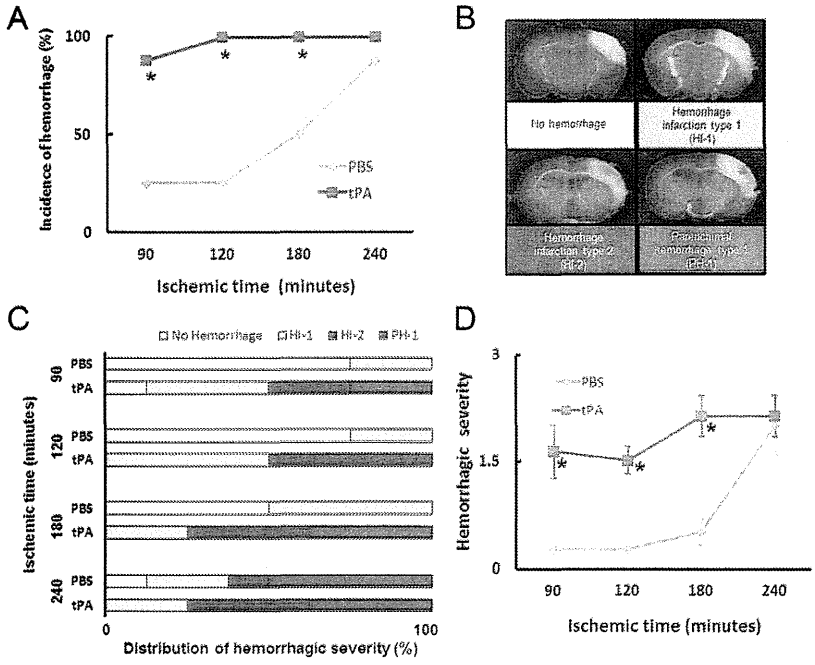

Figure 1. Administration of tPA and hemorrhagic infarction after transient ischemia. **A**, Administration of tPA at 90, 120, and 180 minutes after transient ischemia significantly increased the risk of cerebral hemorrhage compared with controls treated with PBS. **B**, Severity of cerebral hemorrhage was scored according to its type and extension by blinded investigator. Representative photographs of each hemorrhagic severity are shown. **C–D**, Distribution of each hemorrhagic severity is shown in **C**. Quantitative analysis revealed increased severity of cerebral hemorrhage with injection of tPA at 90, 120, and 180 minutes after ischemia compared with PBS injection (**D**). * $P<0.05$ vs PBS control (**A, D**). N=8, in each group. tPA indicates tissue-type plasminogen activator; PBS, phosphate-buffered saline.

Induction of Focal Cerebral Ischemia

To evaluate the effect of cilostazol on tPA-induced hemorrhagic infarction, we developed a highly reproducible murine transient cerebral ischemia model based on modification of our previous method.[18] In brief, the left middle cerebral artery (MCA) was isolated in male 7-week-old CB17/Icr mice (Clea, Tokyo, Japan) under halothane inhalation (3%) anesthesia and transient focal cerebral ischemia was induced under direct vision by transiently occluding the distal portion of the left MCA with a monofilament nylon suture (7-0 in size; Tyco) for 90, 120, 180, or 240 minutes. During surgical procedures, rectal temperature was monitored and controlled at 37.0±0.2°C by a feedback-regulated heating pad. Cerebral blood flow in the MCA area was monitored as described.[19] All mice showed a >75% decrease in cerebral blood flow rapidly after occlusion and restored cerebral blood flow (>0%) soon after reperfusion compared with before transient ligation of the MCA. tPA (10 mg/kg body weight in 0.1 mL saline; Mitsubishi Tanabe Pharmaceutical Co, Tokyo, Japan) was infused through the tail vein just before reperfusion. In sham-operated controls, the same procedure was used and intravenous saline (same volume) was injected in place of tPA.

Drug Administration

Mice were fed cilostazol (0.3% in the diet; Otsuka, Tokushima, Japan), aspirin (0.1% in the diet; Eizai, Tokyo, Japan), or a normal diet for 7 days before induction of ischemia. Doses of cilostazol and aspirin were determined according to previous reports.[20–22] tPA (10 mg/kg) was administered through the tail vein just before reperfusion. The dose of tPA was determined according to previous reports.[23,24]

Assessment of Hemorrhage and Infarction

Hemorrhagic infarction was evaluated at 24 hours after induction of ischemia as described previously.[25,26] Briefly, coronal forebrain sections (1 mm thick) were stained with 1% 2,3,5-triphenyltetrazolium (Sigma-Aldrich, St. Louis, MO) for 20 minutes at 37°C and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS; pH 7.4). Infarct volume was measured using a microscopic digital camera system (Olympus, Tokyo, Japan) as described previously.[27] Briefly, the 2,3,5-triphenyltetrazolium-positive area of each hemisphere was estimated using National Institutes of Health Image software (Version 1.62), and volume of the surviving/viable tissue was calculated by integrating the overall coronally oriented area. Percent stroke volume was evaluated by [(contralateral hemisphere volume)−(infarcted hemisphere volume)]/[(contralateral hemisphere volume)×2]×100%.

The severity of cerebral hemorrhage was quantified by investigators who were not informed regarding the experimental protocol and identity of samples under study, as described previously[28,29]: nonhemorrhage (Score 0); hemorrhagic infarction Type 1 (HI-1), defined as heterogeneous small petechiae, generally along the boundary of the infarct (Score 1); hemorrhagic infarction Type 2 (HI-2), with more confluent petechiae within the infarcted area (Score 2); parenchymal hemorrhage Type 1 (PH-1), characterized by hematoma covering <30% of the injured parenchyma (Score 3); and parenchymal hemorrhage Type 2 (PH-2) with dense hematoma in >30% of the infarct (Score 4). Examples of each score are demonstrated in Figure 1B (no mouse showed PH-2 in our experiment).

Immunohistochemistry

Twenty-four hours after reperfusion, mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde. Forebrain coronal sections (20 μm) were prepared using a vibroslicer (Leica, Wetzlar, Germany) and immunostained with antibodies to platelet endothelial cell adhesion molecule 1 (PECAM-1; BD Pharmingen, San Jose, CA; dilution 1:500), lectin (Invitrogen, Carlsbad, CA; 1:50), and matrix metalloproteinase (MMP)-9 (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:100) using standard immunohistochemical procedures. Anti-PECAM-1 and lectin were visualized by the 3,3′-diaminobenzidine method. Alexa488 or Alexa555 antibody was used as the secondary antibody for anti-MMP-9 and PECAM-1. Vascular density was evaluated using anti-PECAM-1 antibody as described previously.[27]

Briefly, the number of PECAM-1-positive vascular structures in the anterior cerebral artery area (border of cerebral ischemia; approximately 0.5 mm from the border of infarction), MCA area (stroke area), and contralateral cortex at the exact center of the forebrain section was counted by investigators who were not informed regarding the experimental protocol and identity of samples under study (3 random fields in each section were scored and the area of each field was 0.12 mm^2). Sections stained with antilectin

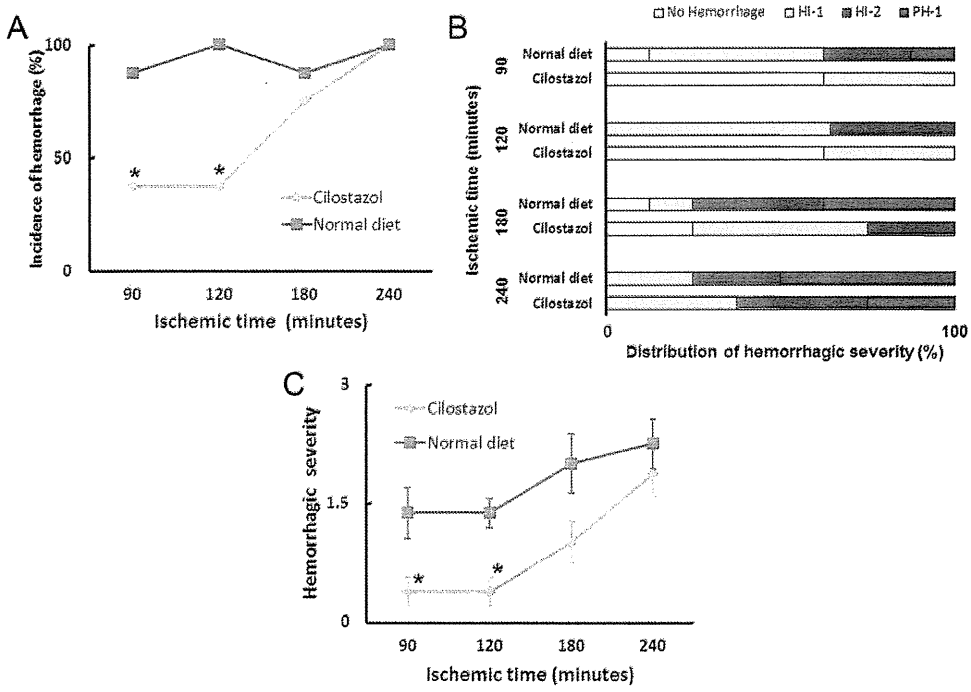

Figure 2. Administration of cilostazol reduced the risk of cerebral hemorrhage after tPA treatment. **A**, Mice fed a cilostazol-containing diet for 7 days before ischemia showed significantly reduced risk of cerebral hemorrhage after 90 and 120 minutes ischemia with tPA injection compared with mice fed a normal diet. **B–C**, The distribution of each hemorrhagic severity is shown in **B**. Quantitative analysis revealed reduced severity of cerebral hemorrhage in mice fed cilostazol after 90 and 120 minutes of ischemia with tPA injection compared with mice fed a normal diet (**C**). *$P<0.05$ vs normal diet control (**A, C**). N=8, in each group. tPA indicates tissue-type plasminogen activator.

were counterstained with Mayer hematoxylin solution (Wako, Osaka, Japan).

Gelatin Zymography

The level of MMP-9 in the ischemic brain was evaluated by gelatin zymography, as described previously.[23] Briefly, at 24 hours after reperfusion with injection of tPA, brain tissue from the ipsilateral ischemic and contralateral nonischemic hemispheres was removed and homogenized in lysis buffer (CelLytic MT; Sigma). After centrifugation at 500 g for 10 minutes, supernatant was collected and protein concentrations were measured by the Bradford assay (Bio-Rad). Protein samples (35 $\mu g/\mu L$) were mixed with 2× zymogram sample buffer (TEFCO, Tokyo, Japan) and loaded onto 10% Zymogram-PAGE mini (TEFCO). After electrophoresis, the gel was stained with Coomassie blue R-250 according to the manufacturer's protocol (ZYMOGRAM buffer kit; TEFCO). Gel images were captured using a digital camera (Olympus, Tokyo, Japan) with reversed brightness, and the intensity of each band was quantified with National Institutes of Health Image.

Data Analysis

Statistical comparisons among groups were determined using the Kruskal-Wallis test to compare with controls. Data are expressed as mean±SE.

Results

Administration of tPA Increases the Risk of Cerebral Hemorrhage After Transient Ischemia

To confirm the increased risk of cerebral hemorrhage after administration of tPA, transient ischemia was induced and tPA or PBS was injected just before reperfusion. The incidence and degree of hemorrhagic infarction were evaluated at 24 hours after reperfusion. As shown in Figure 1A, the incidence of hemorrhagic infarction was significantly increased with tPA injection after 90, 120, and 180 minutes of transient ischemia, although no significant increase was observed after 240 minutes ischemia. To investigate the severity of hemorrhagic infarction, degrees of hemorrhage were scored according to 5 subtypes, as described previously.[28,29] In our experimental groups, no mice showed PH-2 (Grade 4). Representative photographs of hemorrhage subtypes are shown in Figure 1B. It is notable that none of the mice that received PBS before reperfusion showed parenchymal hematoma (Grade ≥2) after 90, 120, and 180 minutes transient ischemia (Figure 1C). In contrast, mice receiving tPA showed PH even after 90 minutes ischemia. In both treatment groups, more than half of the mice showed PH after 240 minutes ischemia. Quantitative analysis revealed a significant increase in hemorrhagic score in mice treated with tPA at 90, 120, and 180 minutes after transient ischemia compared with PBS-treated groups (Figure 1D). The volume of infarcted tissue at 24 hours, consequent to 90 minutes ischemia to induce stroke, was evaluated. There was no significant difference in percent stroke volume between treatment with tPA and PBS (13.8%±1.1% and 13.7%±0.8%, respectively; $P=0.96$).

Cilostazol Reduced the Risk of tPA-Induced Cerebral Hemorrhage

To evaluate the risk of cilostazol on tPA-induced cerebral hemorrhage, mice were fed cilostazol for 7 days and transient ischemia was induced followed by injection of tPA. Contrary

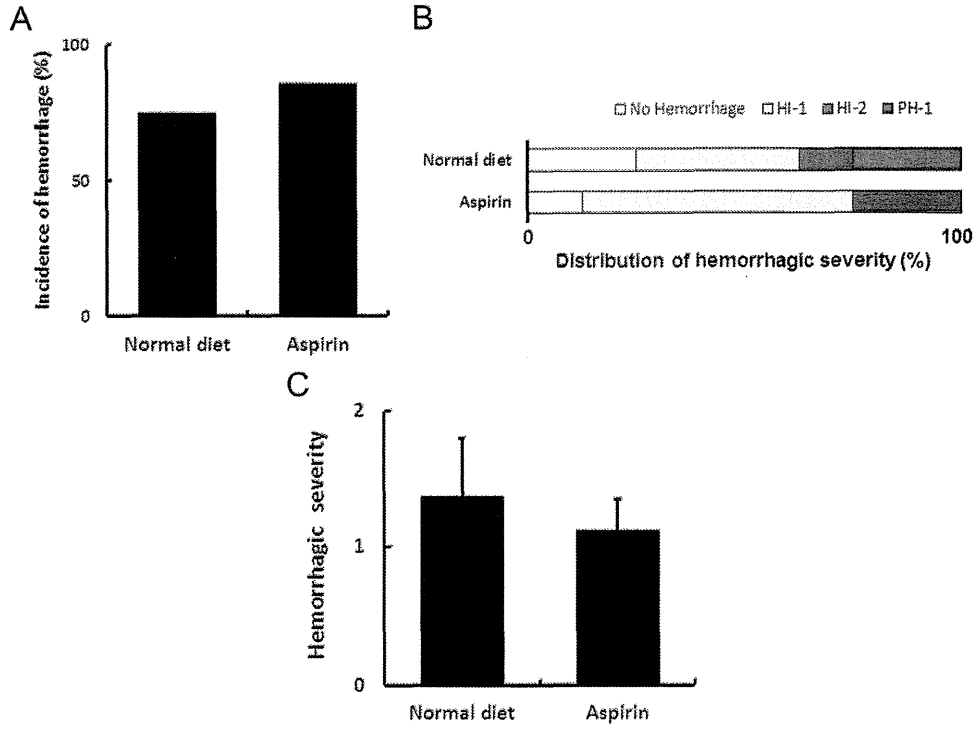

Figure 3. Aspirin administration did not alter the risk of cerebral hemorrhage after tPA treatment. **A**, Mice fed an aspirin-containing diet for 7 days before ischemia did not show a significant change in the risk of cerebral hemorrhage after 90 minutes of ischemia and tPA injection compared with mice fed a normal diet. **B–C**, Distribution of each hemorrhagic severity is shown in **B**. Quantitative analysis revealed no significant difference in the severity of cerebral hemorrhage between mice fed aspirin and the normal diet (**C**). N=8, in each group. tPA indicates tissue-type plasminogen activator.

to our initial expectation, the incidence of cerebral hemorrhage was significantly reduced on administration of cilostazol in mice after 90 and 120 minutes of transient ischemia, although no significant difference was observed after 180 and 240 minutes (Figure 2A). Figure 2B shows the distribution of severity in each group. It is notable that all of the mice fed cilostazol before injection of tPA showed no or mild hemorrhage (score 0 or 1) after 90 or 120 minutes ischemia. Quantitative analysis using the hemorrhagic score revealed a significant reduction of severity in mice pretreated with cilostazol at 90 and 120 minutes after transient ischemia compared with the nontreated group (Figure 2C). However, no statistical difference in the severity was observed between groups after 180 or 240 minutes of transient ischemia.

Aspirin Did Not Reduce the Risk of tPA-Induced Cerebral Hemorrhage

Aspirin is known to increase the risk of cerebral hemorrhage.[5,9,30] To investigate its effect on tPA-induced cerebral hemorrhage, mice were treated with aspirin for 1 week and transient ischemia was induced (90 minutes). The results displayed no significant reduction or increase in the incidence of cerebral hemorrhage on treatment with aspirin compared with normal controls (Figure 3A). Analysis of severity using the hemorrhagic score also revealed no significant change between the aspirin-treated and normal diet groups (Figure 3B–C). Although the incidence of hemorrhage was dependent on the time to reperfusion, these results indicate that aspirin has a nonsignificant effect on reduction of tPA-induced cerebral hemorrhage after 90 minutes of transient ischemia, whereas cilostazol had significant protective effects.

Cilostazol Prevented the Degradation of Cerebrovasculature After Transient Ischemia and Administration of tPA

To investigate mechanisms underlying the protective effect of cilostazol pretreatment on cerebral hemorrhage, morphological changes in cerebromicrovasculature were investigated at 24 hours after induction of transient ischemia (90 minutes). Immunohistological analysis revealed a decrease in PECAM-1-positive microvasculature at the border of cerebral ischemia after transient ischemia with tPA injection compared with the contralateral cortex (Figure 4A, contralateral; Figure 4B; ipsilateral). In contrast, pretreatment with cilostazol prevented the reduction in PECAM-1-positive microvasculature (Figure 4C). These impressions were confirmed by quantitative analysis of PECAM-1-positive vascular density (Figure 4D). PECAM-1 is known to be important for survival, migration, and functional organization of endothelial cells,[31] and our data indicate a beneficial effect of cilostazol on the preservation of these endothelial functions at the border of the stroke. In contrast, pretreatment with aspirin had no effect on the preservation of microvasculature (Figure 4E–F).

Next, we investigated possible degradation of cerebrovasculature in the stroke area with antilectin antibody, a marker of vascular morphology.[27] At 24 hours after transient ischemia (90 minutes) with tPA injection, a marked dissociation of microvasculature was observed in the ischemic brain in mice

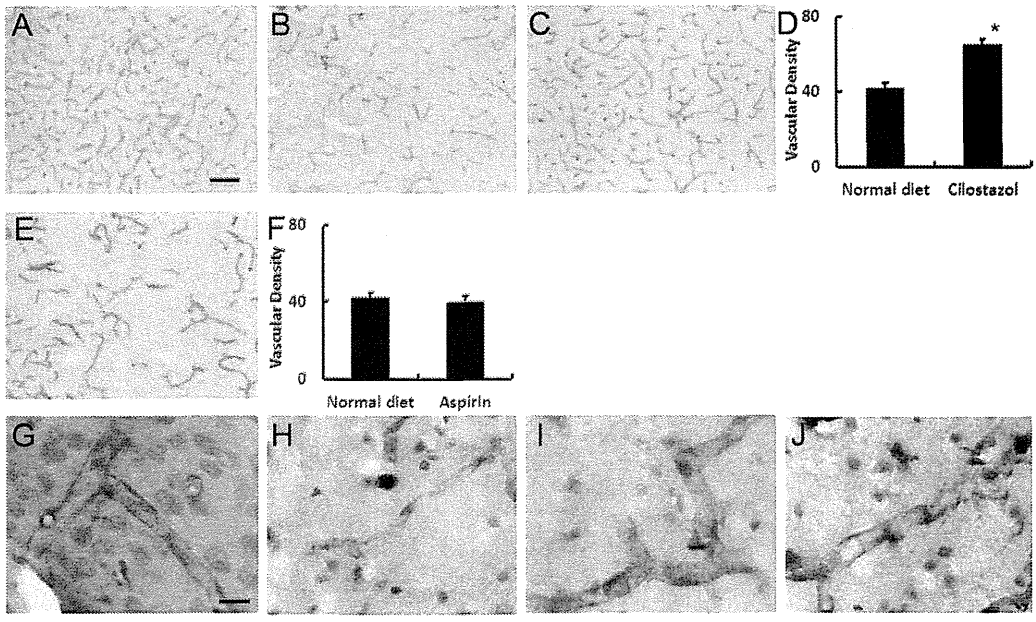

Figure 4. Pretreatment with cilostazol prevented cerebrovascular disruption after transient ischemia with tPA injection. **A–D**, Representative micrographs of cerebral cortex from the stroke-affected area in mice fed a normal diet (**A**, contralateral; **B**, ispilateral) or cilostazol (**B**) at 24 hours after ischemia. Quantitative analysis confirmed significant preservation of microvasculature by pretreatment with cilostazol compared with a normal diet alone (**D**). **E–F**, Representative micrographs of the stroke-affected cortex in mice pretreated with aspirin (**E**). No significant change in vascular density was observed between normal diet and aspirin-treated groups (**F**). **G–J**, Immunohistochemical staining with lectin at 24 hours after induction of ischemia. In contrast to the contralateral cortex (**G**), disruption of cerebral vascular structures was observed in stroke-affected cortex in mice fed a normal diet (**H**). Treatment with cilostazol had a protective effect on the cerebral vasculature (**I**), although no such effect was observed by treatment with aspirin (**J**). *$P<0.05$ vs normal diet control (**D**). N=4, in each group. Scale bars, 100 μm (**A**) and 20 μm (**G**). tPA indicates tissue-type plasminogen activator.

fed a normal diet (Figure 4G, contralateral; Figure 4H, ipsilateral). In contrast, preservation of vascular structure in the stroke area was observed in mice pretreated with cilostazol (Figure 4I). Similar to the results obtained with anti-PECAM-1 antibody, the pretreatment with aspirin had no protective effect on degradation of cerebrovasculature at the poststroke area (Figure 4J).

Cilostazol Prevented Activation of MMP-9 in the Poststroke Cortex

Activation of MMP-9 is well known to cause the deterioration of tight junctions and basement membranes.[32–34] To investigate activation of MMP-9 in the vasculature in the poststroke cortex, brain sections were costained with anti-PECAM-1 and anti-MMP-9 antibodies. Although no MMP-9-positive vascular structures were observed in the contralateral cortex (Figure 5A–C), MMP-9-positive vasculature was observed in the poststroke cortex in control mice (Figure 5D–F). In contrast, no MMP-9-positive vasculature was observed in mice pretreated with cilostazol (Figure 5G–I). In contrast, pretreatment with aspirin did not prevent activation of MMP-9 in the poststroke cortex (Figure 5J–L). To confirm these results, protein samples were extracted from each brain and MMP-9 activity was investigated by zymography. Consistent with results obtained by immunohistologic analysis, suppressed expression of MMP-9 activity was observed with pretreatment with cilostazol compared with pretreatment with aspirin (Figure 5M–N).

Discussion

In this study, we have demonstrated that treatment with cilostazol for 7 days before induction of cerebral ischemia significantly reduced the hemorrhagic risk accompanying tPA injection and was associated with suppressed MMP-9 activity in stroke vasculature (and its endothelium).

Thrombolysis with tPA after stroke is associated with an increased risk of hemorrhagic transformation.[33,35] In addition to endothelial cell injury caused by reperfusion after transient ischemia, tPA is known to induce disruption of the blood–brain barrier.[23,36,37] Consistent with these reports, administration of tPA after 90, 120, or 180 minutes of transient ischemia significantly increased the risk of cerebral hemorrhage, compared with PBS-injected mice, in our experimental model. Because of the homogeneity of cerebral vascular structure/organization between animals in CB-17 mice, the ischemia induced in this strain by transient occlusion of the MCA under direct visualization produced a highly reproducible ischemic area.[38] Although thrombolytic effects of tPA cannot be addressed in this model, these findings indicate the model in CB-17 mice is suitable to evaluate the effect of drugs on hemorrhagic transformation caused by tPA injection with high reproducibility.

Intracerebral hemorrhage is associated with worse clinical outcomes in the context of stroke.[39,40] Prior use of antiplatelet drugs remains a concern in terms of increasing the risk of hemorrhage after tPA treatment.[8,9] However, patients who received aspirin for prevention of stroke showed better

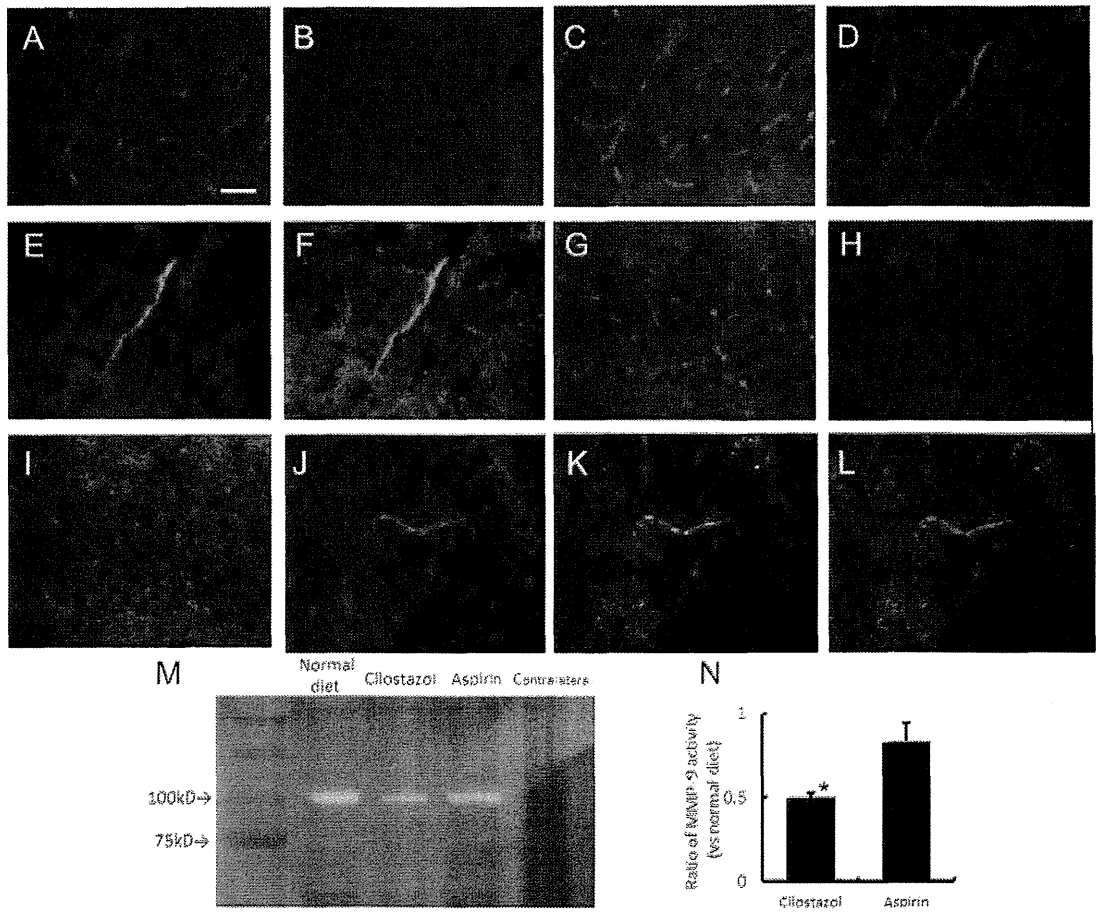

Figure 5. Pretreatment with cilostazol suppressed activation of MMP-9 in vasculature in the poststroke cortex. **A–L**, Representative micrographs of the contralateral (**A–C**) and ipsilateral cortex (**D–L**) at 24 hours after ischemia with tPA treatment (PECAM-1 [**A, D, G**, red]; MMP-9 [**B, E, K**, green]; and merged image [**C, F, L**, yellow]). Although no expression of MMP-9 was observed in the microvasculature of the contralateral cortex (**A–C**), expression of MMP-9 was observed in the ipsilateral cortex in mice pretreated with a normal diet (**D–F**). In contrast, reduced expression of MMP-9 was observed in the stroke-affected cortex in mice pretreated with cilostazol (**G–I**). Pretreatment with aspirin did not change the expression of MMP-9 in the microvasculature in the stroke-affected cortex compared with the normal diet (**J–L**). **M–N**, Representative photograph of zymogram. Suppressed activity of MMP-9 (105 kDa) was observed with pretreatment with cilostazol, although no change was observed with aspirin (**M**). Reverse images were obtained and the ratio of activity between cilostazol or aspirin vs the normal diet was quantified. Significant reduction of MMP-9 activity was observed in mice pretreated with cilostazol compared with mice pretreated with aspirin (**N**). $^*P<0.05$ vs aspirin. N=3, in each group. Scale bar, 80 μm (**A**). MMP indicates matrix metalloproteinase; tPA, tissue-type plasminogen activator; PECAM-1, platelet endothelial cell adhesion molecule 1.

clinical outcomes after treatment with tPA, although some studies reported increased risk of cerebral hemorrhage in patients with aspirin compared with patients who did not receive it.[5,9,41,42] This discrepancy can be attributed to reocclusion of the artery after initial successful recanalization by tPA,[10,11] which can be suppressed by antiplatelet drugs, thereby improving outcome.[14] Cilostazol is an antiplatelet drug with additional effects, including improvement in function of vascular endothelium.[43] It is known to be superior to aspirin in terms of reduction of the risk of cerebral hemorrhage.[16] Consistent with these previous reports, pretreatment with cilostazol for 7 days before ischemia and subsequent tPA administration significantly suppressed the occurrence/extent of cerebral hemorrhage. In contrast, pretreatment with aspirin had no effect on the risk of bleeding compared with nontreated control mice. These findings suggest that patients treated with cilostazol for prevention of ischemic diseases would have a lower risk of hemorrhagic transformation after thrombolytic therapy compared with nontreated or aspirin-treated patients. Cilostazol-treated patients might be expected to have a reduced risk of reoccluding the recanalized cerebral artery compared with nontreated patients.

To extend the therapeutic time window for effective thrombolytic therapy, the risk of cerebral hemorrhage must be evaluated in individual cases. Our current study demonstrates that the risk of cerebral hemorrhage can be significantly modified by treatments administered before the onset of stroke. However, the effects of other commonly used drugs for patients with a high risk of stroke such as calcium channel blockers, angiotensin receptor blockers, and statins are still controversial.[44,45] We believe that analysis of the effects of multiple drugs on tPA-induced cerebral hemorrhage in animal models is essential for extending safe and effective thrombolytic therapy to a wider group of patients, especially for those beyond the current 3-hour window for treatment.

Activation of MMP-9 in injured endothelial cells has been suggested as a mechanism for tPA-induced cerebral hemorrhage[23,37,46] in addition to direct injury due to ischemia-reperfusion. MMP-9 activation enhances the permeability and decreases structural integrity of the blood–brain barrier in postischemic brain.[32,47] Our studies have shown that pretreatment with cilostazol markedly reduced the expression of MMP-9 in endothelial cells after injection of tPA and suppressed degradation of cerebral vasculature in the ischemic brain. Our findings are consistent with a previous study demonstrating that cilostazol decreased MMP-9 expression in balloon-injured vasculature.[48] Cilostazol is known to raise the intracellular cAMP concentration in endothelial cells. In this context, cAMP promotes functional integrity of tight junctions between endothelial cells in the blood–brain barrier.[49,50] The vasculoprotective effect of cilostazol was also shown in other studies in which cilostazol suppressed endothelial hyperpermeability by inhibiting redistribution of the actin-based cytoskeleton[51] and protected endothelial cells against lipopolysaccharide-induced apoptosis by the activation of MAP kinase.[52] These findings indicate that the beneficial effect of cilostazol on cerebral hemorrhage might be achieved, at least in part, through suppression of endothelial injury after thrombolysis with tPA injection. Consistent with these findings, cilostazol-treated mice displayed retention of vascular density in ischemic cerebral cortex after tPA treatment, whereas aspirin did not prevent reduction in the number of cerebral microvessels. In the current study, we used 1 dose of cilostazol. Because both antiplatelet and vasculoprotective activity of cilostazol are known to be dose-dependent,[52–54] further study will be necessary to determine the optimal dose of cilostazol to suppress cerebral hemorrhage after tPA treatment.

In conclusion, our results suggest that treatment of patients with cilostazol for prevention of stroke may have significant merit with regard to suppressing the risk of hemorrhagic transformation after thrombolytic therapy as well as reducing the risk of cerebral hemorrhage[15] compared with treatment with aspirin. Furthermore, our data suggest that the therapeutic time window of thrombolytic therapy using tPA might be extended in patients treated with cilostazol. Furthermore, antithrombotic treatment might be safely started with cilostazol soon after injection of tPA to reduce the incidence of reocclusion of the artery after initial successful recanalization.

Acknowledgments

Food mixed with aspirin or cilostazol was kindly provided by Otsuka Pharmaceutical, Tokyo, Japan.

Sources of Funding

This work was supported by the Japan Cardiovascular Research Foundation.

Disclosures

None.

References

1. Hacke W, Donnan G, Fieschi C, Kaste M, von Kummer R, Broderick JP, et al. Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rtPA stroke trials. *Lancet*. 2004; 363:768–774.
2. Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, et al. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N Engl J Med*. 2008;359:1317–1329.
3. Meschia JF, Brott TG. New insights on thrombolytic treatment of acute ischemic stroke. *Curr Neurol Neurosci Rep*. 2001;1:19–25.
4. Trouillas P, von Kummer R. Classification and pathogenesis of cerebral hemorrhages after thrombolysis in ischemic stroke. *Stroke*. 2006;37: 556–561.
5. Larrue V, von Kummer RR, Muller A, Bluhmki E. Risk factors for severe hemorrhagic transformation in ischemic stroke patients treated with recombinant tissue plasminogen activator: a secondary analysis of the European–Australasian Acute Stroke Study (ECASS II). *Stroke*. 2001;32: 438–441.
6. Schmulling S, Rudolf J, Strotmann-Tack T, Grond M, Schneweis S, Sobesky J, et al. Acetylsalicylic acid pretreatment, concomitant heparin therapy and the risk of early intracranial hemorrhage following systemic thrombolysis for acute ischemic stroke. *Cerebrovasc Dis*. 2003;16: 183–190.
7. Diedler J, Ahmed N, Sykora M, Uyttenboogaart M, Overgaard K, Luijckx GJ, et al. Safety of intravenous thrombolysis for acute ischemic stroke in patients receiving antiplatelet therapy at stroke onset. *Stroke*. 2010;41: 288–294.
8. Grotta JC, Welch KM, Fagan SC, Lu M, Frankel MR, Brott T, et al. Clinical deterioration following improvement in the NINDS rtPA stroke trial. *Stroke*. 2001;32:661–668.
9. Uyttenboogaart M, Koch MW, Koopman K, Vroomen PC, De Keyser J, Luijckx GJ. Safety of antiplatelet therapy prior to intravenous thrombolysis in acute ischemic stroke. *Arch Neurol*. 2008;65:607–611.
10. Rubiera M, Alvarez-Sabin J, Ribo M, Montaner J, Santamarina E, Arenillas JF, et al. Predictors of early arterial reocclusion after tissue plasminogen activator-induced recanalization in acute ischemic stroke. *Stroke*. 2005;36:1452–1456.
11. Alexandrov AV, Grotta JC. Arterial reocclusion in stroke patients treated with intravenous tissue plasminogen activator. *Neurology*. 2002;59: 862–867.
12. Fassbender K, Dempfle CE, Mielke O, Schwartz A, Daffertshofer M, Eschenfelder C, et al. Changes in coagulation and fibrinolysis markers in acute ischemic stroke treated with recombinant tissue plasminogen activator. *Stroke*. 1999;30:2101–2104.
13. Tanne D, Macko RF, Lin Y, Tilley BC, Levine SR. Hemostatic activation and outcome after recombinant tissue plasminogen activator therapy for acute ischemic stroke. *Stroke*. 2006;37:1798–1804.
14. Zinkstok SM, Vermeulen M, Stam J, de Haan RJ, Roos YB. A randomised controlled trial of antiplatelet therapy in combination with rtPA thrombolysis in ischemic stroke: rationale and design of the ARTIS-trial. *Trials*. 2010;11:51.
15. Shinohara Y, Katayama Y, Uchiyama S, Yamaguchi T, Handa S, Matsuoka K, et al. Cilostazol for prevention of secondary stroke (CSPS 2): an aspirin-controlled, double-blind, randomised non-inferiority trial. *Lancet Neurol*. 2010;9:959–968.
16. Kamal AK, Naqvi I, Husain MR, Khealani BA. Cilostazol versus aspirin for secondary prevention of vascular events after stroke of arterial origin. *Cochrane Database Syst Rev*. 2011;1:CD008076.
17. Wilhite DB, Comerota AJ, Schmieder FA, Throm RC, Gaughan JP, Rao AK. Managing pad with multiple platelet inhibitors: the effect of combination therapy on bleeding time. *J Vasc Surg*. 2003;38:710–713.
18. Kasahara Y, Taguchi A, Uno H, Nakano A, Nakagomi T, Hirose H, et al. Telmisartan suppresses cerebral injury in a murine model of transient focal ischemia. *Brain Res*. 2010;1340:70–80.
19. Matsushita K, Matsuyama T, Nishimura H, Takaoka T, Kuwabara K, Tsukamoto Y, et al. Marked, sustained expression of a novel 150-kDa oxygen-regulated stress protein, in severely ischemic mouse neurons. *Brain Res Mol Brain Res*. 1998;60:98–106.
20. Oyama N, Yagita Y, Kawamura M, Sugiyama Y, Terasaki Y, Omura-Matsuoka E, et al. Cilostazol, not aspirin, reduces ischemic brain injury via endothelial protection in spontaneously hypertensive rats. *Stroke*. 2011;42:2571–2577.
21. Takase H, Hashimoto A, Okutsu R, Hirose Y, Ito H, Imaizumi T, et al. Anti-atherosclerotic effect of cilostazol in apolipoprotein-E knockout mice. *Arzneimittelforschung*. 2007;57:185–191.
22. Ito H, Hashimoto A, Matsumoto Y, Yao H, Miyakoda G. Cilostazol, a phosphodiesterase inhibitor, attenuates photothrombotic focal ischemic

brain injury in hypertensive rats. *J Cereb Blood Flow Metab.* 2010;30:343–351.
23. Sumii T, Lo EH. Involvement of matrix metalloproteinase in thrombolysis-associated hemorrhagic transformation after embolic focal ischemia in rats. *Stroke.* 2002;33:831–836.
24. Yamashita T, Kamiya T, Deguchi K, Inaba T, Zhang H, Shang J, et al. Dissociation and protection of the neurovascular unit after thrombolysis and reperfusion in ischemic rat brain. *J Cereb Blood Flow Metab.* 2009;29:715–725.
25. Hata R, Mies G, Wiessner C, Fritze K, Hesselbarth D, Brinker G, et al. A reproducible model of middle cerebral artery occlusion in mice: hemodynamic, biochemical, and magnetic resonance imaging. *J Cereb Blood Flow Metab.* 1998;18:367–375.
26. Saavedra JM, Benicky J, Zhou J. Mechanisms of the anti-ischemic effect of angiotensin II AT(1) receptor antagonists in the brain. *Cell Mol Neurobiol.* 2006;26:1099–1111.
27. Taguchi A, Zhu P, Cao F, Kikuchi-Taura A, Kasahara Y, Stern DM, et al. Reduced ischemic brain injury by partial rejuvenation of bone marrow cells in aged rats. *J Cereb Blood Flow Metab.* 2011;31:855–867.
28. Aronowski J, Strong R, Shirzadi A, Grotta JC. Ethanol plus caffeine (caffeinol) for treatment of ischemic stroke: preclinical experience. *Stroke.* 2003;34:1246–1251.
29. Berger C, Fiorelli M, Steiner T, Schabitz WR, Bozzao L, Bluhmki E, et al. Hemorrhagic transformation of ischemic brain tissue: asymptomatic or symptomatic? *Stroke.* 2001;32:1330–1335.
30. Clark WM, Madden KP, Lyden PD, Zivin JA. Cerebral hemorrhagic risk of aspirin or heparin therapy with thrombolytic treatment in rabbits. *Stroke.* 1991;22:872–876.
31. Park S, DiMaio TA, Scheef EA, Sorenson CM, Sheibani N. PECAM-1 regulates proangiogenic properties of endothelial cells through modulation of cell–cell and cell–matrix interactions. *Am J Physiol Cell Physiol.* 2010;299:C1468–1484.
32. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA. Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *J Cereb Blood Flow Metab.* 2007;27:697–709.
33. Yepes M, Sandkvist M, Moore EG, Bugge TH, Strickland DK, Lawrence DA. Tissue-type plasminogen activator induces opening of the blood–brain barrier via the LDL receptor-related protein. *J Clin Invest.* 2003;112:1533–1540.
34. Duran-Vilaregut J, Del Valle J, Manich G, Camins A, Pallas M, Vilaplana J, et al. Role of MMP-9 in striatal blood–brain barrier disruption in a 3-nitropropionic acid model of Huntington's disease. *Neuropathol Appl Neurobiol.* 2010;37:525–537.
35. Vivien D, Buisson A. Serine protease inhibitors: novel therapeutic targets for stroke? *J Cereb Blood Flow Metab.* 2000;20:755–764.
36. Kahles T, Foerch C, Sitzer M, Schroeter M, Steinmetz H, Rami A, et al. Tissue plasminogen activator mediated blood–brain barrier damage in transient focal cerebral ischemia in rats: relevance of interactions between thrombotic material and thrombolytic agent. *Vascul Pharmacol.* 2005;43:254–259.
37. Tsuji K, Aoki T, Tejima E, Arai K, Lee SR, Atochin DN, et al. Tissue plasminogen activator promotes matrix metalloproteinase-9 upregulation after focal cerebral ischemia. *Stroke.* 2005;36:1954–1959.
38. Taguchi A, Kasahara Y, Nakagomi T, Stern DM, Fukunaga M, Ishikawa M, et al. A reproducible and simple model of permanent cerebral ischemia in CB-17 and SCID mice. *J Exp Stroke Transl Med.* 2010;3:28–33.
39. Valiente RA, de Miranda-Alves MA, Silva GS, Gomes DL, Brucki SM, Rocha MS, et al. Clinical features associated with early hospital arrival after acute intracerebral hemorrhage: challenges for new trials. *Cerebrovasc Dis.* 2008;26:404–408.
40. Hanggi D, Steiger HJ. Spontaneous intracerebral haemorrhage in adults: a literature overview. *Acta Neurochir (Wien).* 2008;150:371–379; discussion 379.
41. Wahlgren N, Ahmed N, Eriksson N, Aichner F, Bluhmki E, Davalos A, et al. Multivariable analysis of outcome predictors and adjustment of main outcome results to baseline data profile in randomized controlled trials: Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST). *Stroke.* 2008;39:3316–3322.
42. Wahlgren N, Ahmed N, Davalos A, Ford GA, Grond M, Hacke W, et al. Thrombolysis with alteplase for acute ischaemic stroke in the Safe Implementation of Thrombolysis in Stroke-Monitoring Study (SITS-MOST): an observational study. *Lancet.* 2007;369:275–282.
43. Goto S. Cilostazol: potential mechanism of action for antithrombotic effects accompanied by a low rate of bleeding. *Atheroscler Suppl.* 2005;6:3–11.
44. Lapchak PA, Han MK. The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor simvastatin reduces thrombolytic-induced intracerebral hemorrhage in embolized rabbits. *Brain Res.* 2009;1303:144–150.
45. Uyttenboogaart M, Koch MW, Koopman K, Vroomen PC, Luijckx GJ, De Keyser J. Lipid profile, statin use, and outcome after intravenous thrombolysis for acute ischaemic stroke. *J Neurol.* 2008;255:875–880.
46. Yagi K, Kitazato KT, Uno M, Tada Y, Kinouchi T, Shimada K, et al. Edaravone, a free radical scavenger, inhibits MMP-9-related brain hemorrhage in rats treated with tissue plasminogen activator. *Stroke.* 2009;40:626–631.
47. Bauer AT, Burgers HF, Rabie T, Marti HH. Matrix metalloproteinase-9 mediates hypoxia-induced vascular leakage in the brain via tight junction rearrangement. *J Cereb Blood Flow Metab.* 2010;30:837–848.
48. Tsai CS, Lin FY, Chen YH, Yang TL, Wang HJ, Huang GS, et al. Cilostazol attenuates MCP-1 and MMP-9 expression in vivo in LPS-administrated balloon-injured rabbit aorta and in vitro in LPS-treated monocytic THP-1 cells. *J Cell Biochem.* 2008;103:54–66.
49. Ishizaki T, Chiba H, Kojima T, Fujibe M, Soma T, Miyajima H, et al. Cyclic AMP induces phosphorylation of claudin-5 immunoprecipitates and expression of claudin-5 gene in blood–brain-barrier endothelial cells via protein kinase A-dependent and -independent pathways. *Exp Cell Res.* 2003;290:275–288.
50. Yan SF, Ogawa S, Stern DM, Pinsky DJ. Hypoxia-induced modulation of endothelial cell properties: regulation of barrier function and expression of interleukin-6. *Kidney Int.* 1997;51:419–425.
51. Torii H, Kubota H, Ishihara H, Suzuki M. Cilostazol inhibits the redistribution of the actin cytoskeleton and junctional proteins on the blood–brain barrier under hypoxia/reoxygenation. *Pharmacol Res.* 2007;55:104–110.
52. Lim JH, Woo JS, Shin YW. Cilostazol protects endothelial cells against lipopolysaccharide-induced apoptosis through ERK1/2- and P38 MAPK-dependent pathways. *Korean J Intern Med.* 2009;24:113–122.
53. Igawa T, Tani T, Chijiwa T, Shiragiku T, Shimidzu S, Kawamura K, Kato S, Unemi F, Kimura Y. Potentiation of anti-platelet aggregating activity of cilostazol with vascular endothelial cells. *Thromb Res.* 1990;57:617–623.
54. Aoki C, Hattori Y, Tomizawa A, Jojima T, Kasai K. Anti-inflammatory role of cilostazol in vascular smooth muscle cells in vitro and in vivo. *J Atheroscler Thromb.* 2010;17:503–509.

Experimental Neurology 233 (2012) 214–220

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Progesterone and allopregnanolone exacerbate hypoxic-ischemic brain injury in immature rats

Masahiro Tsuji [a,*], Akihiko Taguchi [a], Makiko Ohshima [a], Yukiko Kasahara [a], Tomoaki Ikeda [a,b]

[a] Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center Research Institute, 5-7-1, Fujishiro-dai, Suita, Osaka, 565–8565, Japan
[b] Department of Perinatology, National Cerebral and Cardiovascular Center Research Institute, 5-7-1, Fujishiro-dai, Suita, Osaka, 565–8565, Japan

ARTICLE INFO

Article history:
Received 7 April 2011
Revised 28 September 2011
Accepted 4 October 2011
Available online 14 October 2011

Keywords:
Progesterone
Allopregnanolone
Hypoxic–ischemic brain injury
GABA
Immature rat

ABSTRACT

Progesterone and its metabolite, allopregnanolone, are neurosteroids that are present at high concentrations in fetal brains that decrease right after birth. Allopregnanolone is a potent positive modulator of γ-aminobutyric acid A ($GABA_A$) receptor function. We examined the effect of exogenous administration of these steroids on hypoxic–ischemic encephalopathy in immature rats. Progesterone (10 mg/kg), allopregnanolone (10 mg/kg), or vehicle alone was intraperitoneally administered immediately before and then subcutaneously 6 h and 24 h after hypoxia–ischemia to postnatal day 7 (P7), day 14 (P14), and day 21 (P21) rats. The effects of the treatments were evaluated using histological analyses (hemispheric volumes and semi-quantitative scoring for neuropathologic injury). Both progesterone and allopregnanolone significantly exacerbated brain injury in P7 and P14 rats, but not in P21 rats. This detrimental effect was similar across the examined brain regions (the cortex, striatum, hippocampus, and thalamus) and showed no sex differences. Co-administration of the $GABA_A$ receptor antagonist, bicuculline, partially mitigated the exacerbating effect of allopregnanolone. Based on the similarity of the effects of these neurosteroids, we speculate that progesterone accentuates neuronal injury mainly via the activity of allopregnanolone. The present study indicates that the detrimental effects of allopregnanolone were, at least in part, mediated via GABAergic neuroexcitability. This is in line with the notion that GABA is excitatory for immature neurons, while it is inhibitory for mature neurons.

Introduction

Fetuses physiologically experience hypoxic conditions because they have a relatively low oxyhemoglobin saturation (65%) in their cerebral circulation (du Plessis, 2009). Hence, we assumed that fetuses might have innate mechanisms for coping with hypoxia and possibly protect themselves from hypoxia–ischemia (HI) better than children and adults. We hypothesized that certain compounds present at higher concentrations in the brain during the fetal period compared with other periods of life might have neuroprotective properties against hypoxia. Neonatal HI encephalopathy is caused by respiratory and/or circulatory insufficiency, and many survivors have long-term cognitive dysfunctions, as well as cerebral palsy (Lindstrom et al., 2006).

Progesterone (PROG) and its metabolite, allopregnanolone (ALLO, 3α-hydroxy-5α-pregnan-20-one, 3α,5α-tetrahydroprogesterone), are neuroactive steroid hormones that are also known as neurosteroids because they are synthesized *de novo* in the nervous system (Belelli and Lambert, 2005). PROG and ALLO are present at high concentrations in the brains of fetal rats and sheep (Grobin et al., 2003, Nguyen et al., 2003). These two neurosteroids are both supplied from the maternal circulation and synthesized in the fetal brain. Serum PROG and ALLO levels in pregnant women continue to increase during pregnancy, with the highest levels at term, i.e., 10 to 100 times higher than during preconception (Luisi et al., 2000). The levels of ALLO in umbilical cord blood are almost the same as those in maternal blood (Hill et al., 2000), and these steroids easily penetrate the brain (Wang et al., 2010). The PROG and ALLO concentrations in the fetal brain decrease right after birth, mainly due to the loss of the maternal blood supply (Grobin et al., 2003, Nguyen et al., 2003). Given that the fetal brain is exposed to high levels of PROG and ALLO, we hypothesized that these neurosteroids might have some neuroprotective properties against hypoxia and that an exogenous supply of these steroids might alleviate HI-induced brain injury in immature subjects. Erythropoietin, for example, which is prominent in the fetal brain, has shown to be neuroprotective in rodents with HI injury when administered exogenously after birth, and is

Abbreviations: HI, hypoxic–ischemic, hypoxia–ischemia; PROG, Progesterone; ALLO, allopregnanolone; P, postnatal day; $GABA_A$, γ-aminobutyric acid A; ANOVA, analysis of variance.

* Corresponding author. Fax: +81 6 6835 5496.
E-mail addresses: mtsuji@ri.ncvc.go.jp, mtsujimd@ybb.ne.jp (M. Tsuji), taguchi@ri.ncvc.go.jp (A. Taguchi), oshimam@ri.ncvc.go.jp (M. Ohshima), kasahara@ri.ncvc.go.jp (Y. Kasahara), tikeda@hsp.ncvc.go.jp (T. Ikeda).

0014-4886/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2011.10.004

currently being tested for infants with HIE and for extremely-low birth weight infants (Juul, 2000; McPherson and Juul, 2010).

Despite the appeal of the hypothesis that compounds present at high concentrations in the fetal brain could have neuroprotective properties, there is a critical concern regarding the use of PROG and ALLO in immature animals and humans. ALLO acts as a potent positive modulator of γ-aminobutyric acid A ($GABA_A$) receptors (Belelli and Lambert, 2005). GABA depolarizes immature neurons and is excitatory, while it hyperpolarizes mature neurons and is inhibitory (Ben-Ari et al., 2007). Therefore, PROG and ALLO treatment could potentially exacerbate neonatal HI encephalopathy through a neuroexcitatory mechanism involving $GABA_A$ receptors.

To our knowledge, no study has examined the effect of an exogenous supply of PROG or ALLO on immature animals with brain injury. The purpose of this study was to examine the effects of PROG and ALLO on immature rats with HI-induced brain injury.

Materials and methods

Hypoxia–ischemia

Seven-day-old (P7; experimental paradigm), 14-day-old (P14), and 21-day-old (P21) Wistar rat (Japan SLC, Hamamatsu, Japan) pups were prepared for surgery. All experiments were performed in accordance with protocols approved by the Experimental Animal Care and Use Committee of the National Cerebral and Cardiovascular Center. Rats were subjected to a modified Rice–Vannucci procedure to produce HI injury. The Rice–Vannucci model combines permanent ligation of the unilateral carotid artery with exposure to hypoxia for several hours in 7-day-old rat pups and has been widely used for numerous studies on the pathogenesis of HI injury (Rice et al., 1981; Johnston et al., 2005). The brain of newborn rats cannot be damaged by either anoxia alone or unilateral carotid artery ligation alone (Rice et al., 1981). Briefly, under isoflurane anesthesia (4.0% for induction and 1.5 to 2.0% for maintenance), the left carotid artery was permanently occluded. After a 1–2 h recovery period, the P7, P14, and P21 rats were subjected to hypoxia (8% oxygen and 92% nitrogen, at 33.0 °C) for 120, 80, and 50 min, respectively. The duration of the hypoxic exposure was optimized to obtain a similar degree of brain injury in each group as assessed by hemispheric volume and neuropathological scores. After 1 h recovery in a temperature-controlled incubator, rats were returned to the dams until sacrifice.

Drug administration

PROG (Sigma-Aldrich, St. Louis, MO) and ALLO (Calbiochem/EMD Biosciences, San Diego, CA) were dissolved in 22.5% (2-hydroxypropyl)-β-cyclodextrin. Bicuculline (Sigma-Aldrich, St. Louis, MO) was dissolved in hydrochloric acid and then titrated to pH 5.2 by adding sodium hydroxide and phosphate-buffered saline (PBS). A total of five different experimental groups were used: regular dose paradigm in P7, P14, and P21 rats, low dose paradigm in P7 rats, and bicuculline paradigm in P7 rats. Ten to fourteen littermates, both males and females, were randomly assigned to one of three or four different treatment groups. As sex differences were designed to be assessed in Experiment 1 (P7), double the number of littermates was assigned to each treatment group, so that each sex group consisted of approximately 10 pups. As four different treatment groups were assessed in Experiment 3 (P7), 14–20 pups were used.

Experiment 1 (P7): To produce physiological prenatal levels of the two steroids in P7 rats (the level of brain maturation in P7 rats is generally considered comparable to that of P0 human neonates (Dobbing and Sands, 1979), although other authors have suggested that P12–13 rats fulfill this criterion (Romijn et al., 1991) (Clancy et al., 2007)), PROG and ALLO were each administered at a dose of 10 mg/kg body weight (5 mg/ml) immediately before the start of the hypoxic exposure. To simulate the clinical situation of treating newborn babies in the P7 rats, the steroids were administered 6 and 24 h after the start of the hypoxic exposure. The first injections (immediately before hypoxia) were given intraperitoneally to ensure rapid absorption, and the subsequent injections were given subcutaneously for more gradual absorption. The vehicle was administered in the same manner. This protocol is based on the one reported for neuroprotective effects in adult rats with stroke (Jiang et al., 1996, Sayeed et al., 2006), with minor modifications.

Experiment 2 (P7): In this protocol, ALLO was administered at a dose of either 3 mg/kg or 1 mg/kg. Other than the dosage, the protocol was same as that used in experiment 1. The vehicle was also administered in the same manner.

Experiment 3 (P7): Littermates were randomly assigned to one of four groups: vehicle (PBS) + vehicle (β-cyclodextrin), bicuculline + vehicle (β-cyclodextrin), vehicle (PBS) + ALLO, or bicuculline + ALLO. ALLO and the vehicle (β-cyclodextrin) were both administered in the same manner as that described in experiment 1. The $GABA_A$ receptor antagonist, bicuculline (2 mg/kg), and its vehicle (PBS adjusted to pH 5.2) were each administered intraperitoneally just before and subcutaneously 6 h after each ALLO injection, for a total of 5 injections (Fig. 1A). This protocol is based on one used previously to study the effects of $GABA_A$ blockade in immature rats (Galanopoulou, 2008).

Experiments 4 (P14) and 5 (P21): The same protocol used in experiment 1 was used for P14 and P21 rats.

Quantitative histological analysis

Seven days after the HI insult, the rats were deeply anesthetized with an overdose of pentobarbital and perfused with saline followed by 4% formaldehyde via the left ventricle. After perfusion, the brains were removed and sectioned coronally into 2-mm slices using a rat brain slicer (Neuroscience Inc., Tokyo, Japan). The area (mm^2) of the contralateral and ipsilateral hemispheres in each brain section was measured using NIH Image software (ImageJ, 1.43r). The hemispheric volume of each brain was estimated by summing the hemispheric area of the brain slices and multiplying by the section interval thickness. The injury was evaluated in hematoxylin-eosin-stained sections from four brain regions (cortex, striatum, hippocampus, and thalamus). The system we previously developed for evaluating neuropathologic injury (Tsuji et al., 2004) was used in the present study. Neuropathologic injury in the cerebral cortex was scored from 0 to 4 (0: no injury, 4: extensive confluent infarction). Neuropathologic injury in the hippocampus, striatum, and thalamus was scored from 0 to 6. The total score (0–22) was the sum of these ratings. Both hemispheric volume measurement and neuropathological scoring were assessed blindly.

Statistics

The effects of the neurosteroid treatment on the cerebral hemispheric volumes were assessed using a two-way analysis of variance (ANOVA) followed by Bonferroni's test. The injury scores were not distributed normally, so differences in injury scores were assessed using a Kruskal–Wallis test, followed by Dunn's multiple comparison. Sex differences in the injury scores were assessed using Mann–Whitney *U* test with Bonferroni's correction for multiple comparisons. The death rate of the animals was analyzed using Fisher's exact test with Bonferroni's correction for multiple comparisons. The differences in body weight and in rectal temperature were analyzed using a one-way ANOVA, followed by Bonferroni's test. Differences were considered significant at $P<0.05$. The results are presented as the mean ± standard error of the mean (SEM).

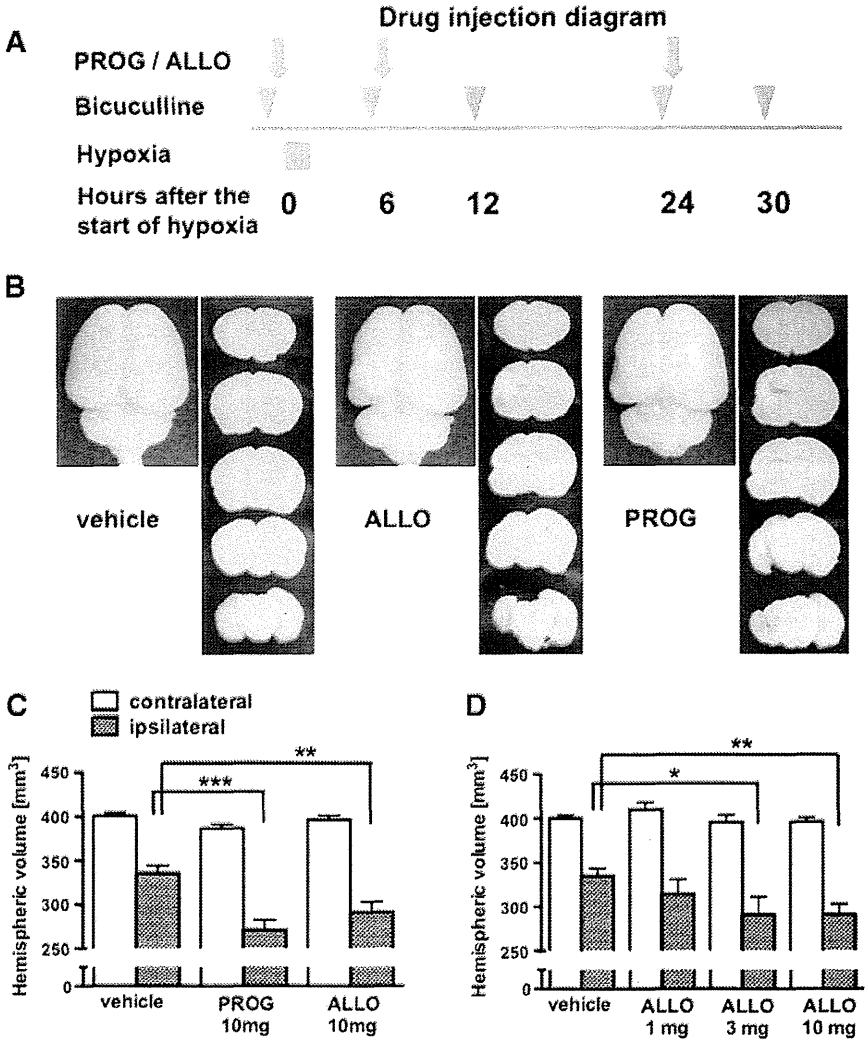

Fig. 1. [Exps. 1–3] Diagram of drug injections (A). Data from three experiments (Exps. 1–3) were pooled and analyzed together. Effects of progesterone (PROG; 10 mg/kg) and allopregnanolone (ALLO; 10 mg/kg) administration in postnatal day 7 (P7) rats. Representative photographs of rat brains at 7 days after hypoxia–ischemia (HI) (B). PROG and ALLO reduced the ipsilateral hemispheric volume (C). A lower dose of ALLO (1 mg/kg) did not reduce the hemispheric volume (D). ** $P<0.01$, *** $P<0.001$. (vehicle $n=42$; PROG $n=22$; ALLO, 10 mg $n=28$; ALLO, 3 mg $n=10$; ALLO, 1 mg $n=10$).

Results

PROG and ALLO exacerbate brain injury in P7 rats

First, the effect of PROG (10 mg/kg×3) and ALLO (10 mg/kg×3) administration on P7 rats with HI-induced brain injury was examined [Exp. 1]. With respect to hemispheric volumes, two-way ANOVA revealed a hemispheric difference and a treatment group difference. Ipsilateral hemispheric volume was significantly reduced in the PROG-treated group (271 ± 11 mm^3) and the ALLO-treated group (293 ± 15 mm^3) compared with the vehicle-treated group (345 ± 14 mm^3) (Fig. 1B). Second, the effect of the dose of ALLO administration on P7 rats with HI-induced brain injury was examined [Exp. 2]. Two-way ANOVA did not reveal a dose difference. Because the vehicle-control groups in three experiments with P7 rats [Exps. 1–3] did not differ from each other (two-way ANOVA) with respect to hemispheric volumes, these data were pooled together into a single control group. The exacerbating effects of PROG and ALLO were the same as the original analysis (Fig. 1C), and the effect of the dose of ALLO became significant. The administration of 10 mg/kg×3 ALLO or 3 mg/kg×3 ALLO, but not 1 mg/kg×3 ALLO, significantly reduced the ipsilateral hemispheric volume compared with that of the vehicle-treated group (Fig. 1D).

Based on the neuropathological scores, PROG (10 mg/kg×3) significantly exacerbated injury in all four regions examined, the cortex, striatum, hippocampus, and thalamus (Fig. 2A). Although ALLO (10 mg/kg×3) increased the injury scores, its effect was not statistically significant in any of the four regions. Given that the mortality rate was significantly higher in the ALLO-treated, but not the PROG-treated group, than in the vehicle group (Table 1), ALLO may be detrimental in HI-induced brain injury. The number of pups that died or were severely injured with a neuropathological score greater than 10 was significantly higher in both the PROG- and ALLO-treated groups compared with that in the vehicle-treated group ($P<0.05$, Fisher's exact test with Bonferroni's correction) (Fig. 2B).

There were no sex differences in the effects of PROG or ALLO on either evaluation of brain damage, i.e., hemispheric volume (data not shown) or the neuropathological injury score (Fig. 2C).

$GABA_A$ receptor antagonism abolishes the exacerbating effect

To better understand the mechanism behind the exacerbation caused by ALLO, bicuculline, a $GABA_A$ receptor antagonist, was co-administered with ALLO to the rats [Exp. 3]. After pooling data from the three P7 experiments [Exps. 1–3], two-way ANOVA revealed a