Controlled arterial reflow after ischemia induces better outcomes in the juvenile rat brain

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Philippe Bonnin[1,2], Julien Pansiot[1,3], Elise Paven[1,2], Maxime Eloi[1,3], Sylvain Renolleau[4], Olivier Baud[1,3], Pierre-Louis Leger[1,3,5,*] and Christiane Charriaut-Marlangue[1,3]

Abstract

Our objective was to determine whether controlled reflow on one side and/or the other side after bilateral carotid occlusion release could reduce cell death in focal ischemic P14 rats. Arterial blood flow was measured using ultrasonography. Cell death, inflammation and nitrotyrosine were measured using immunofluorescence. When reflow was first induced in the contralateral side, we observed improved outcome markers compared with those when reflow was first induced in the ipsilateral side and/or simultaneous reflow was induced in both sides. Our data suggest that progressive rerouting of arterial flow through the circle of Willis toward the ischemic site reduced cell death.

Keywords

Ischemic stroke, macrocirculation, arterial re-flow, cell death

Introduction

Arterial ischemic stroke is increasingly recognized as a serious pediatric problem. The groups at risk of arterial ischemic stroke are newborns, especially full-term infants, and older children with sickle cell disease, or under specific conditions (surgery of congenital cardiopathy, extracorporeal life supports), although 1/3 of childhood strokes remain cryptogenic.[1]

In the adult brain, restoration of blood flow (BF) to the ischemic territory is characterized by significant hyperemia within the penumbra that occurs immediately after occlusion release.[2,3] In contrast, a progressive and incomplete reperfusion occurred in the cortical penumbra during early reflow in the neonatal P7 rat brain.[4] In the juvenile P14 rat brain, rapid and complete reperfusion, without hyperemia was detected in the penumbra.[5] In addition, juvenile rats responded to ischemia by producing prostaglandins in a COX-2- and mPGES1-dependent manner.[6]

Whereas ischemic postconditioning (a series of rapid intermittent interruptions of BF at reflow) was demonstrated to be a harmless procedure that attenuates cerebral BF (CBF) disturbances in adults, this paradigm did not ameliorate ischemic lesions in neonatal ischemic P7 rats, which suggests collateral support efficiency[7] and a lower capacity to activate NO production in the immature brain (for review see Leger et al.[8]).

In the present study, we evaluated whether controlling arterial reflow by gradual occlusion release alleviates damage using both imaging and molecular approaches.

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Material and methods

All experiments of this study complied with the ethical guidelines of the Robert Debre´ Hospital Research Council Review Board (A75-19-01), INSERM, and the ARRIVE guidelines (http://www.nc3rs.org/ARRIVE) and were approved by the local ethic committee (Paris7, France). All experiments were performed in a blinded and randomized manner.

Ischemia was induced in Wistar P14 rats (Janvier, Le genest St-Isle, France, 31.2 ± 3.12 g, $n = 75$) as previously reported.[5] Briefly, thermoregulated ($37.0 \pm 0.5^{\circ}$C) and anesthetized rats (induction 2%, maintenance 1.5% isoflurane in air) were exposed to ipsilateral left middle cerebral artery (MCA) electrocoagulation (MCAo) combined with a transient (60 min) double occlusion of the common carotid arteries (CCAo). Carotid blood flow restoration was assessed by release of either (1) both clips at the same time (control group), (2) first clip on the right CCA, then 5 min after on the left CCA (R-L group), and conversely, (3) first clip on the left CCA, then 5 min after on the right CCA (L-R group).

Randomized allocation of animals

For each litter, the 10 pups were labeled from 1 to 10. The numbered pups "1, 3 and 6" a priori constituted the R-L group, the numbered pups "2, 5 and 9" constituted the control group, and the numbered pups "4, 7 and 10" constituted the L-R group. Then, each randomized animal was subjected to ischemia and reperfusion. The numbered pup 8 could replace an animal that died during anesthesia.

Sample size calculation

Assuming a βeta risk of 0.2 and an αlpha risk of 0.05, it was estimated (using the BiostaTGV software (https://marne.u707.jussieu.fr/biostatgv)) that six animals in each group were needed to observe a significant difference in gene and protein expression, and US imaging as previously reported.[5,6] As an example (mean 1 = 13.5, mean 2 = 9.8, common SD = 2), four animals per group are necessary.

Inclusion/exclusion

All animals were included in this study (no mortality).

Animals were then subjected to 2D color-coded ultrasound imaging.[5] COXs and terminal synthesizing enzymes genes were evaluated at 1 h after reflow. Protein expression[6] (including cell death) were evaluated at 4 and/or 24 h after reflow. A detailed description of Materials and Methods can be found in the supplementary material.

Results

Blood flow redistribution according to occlusion release protocols

The three groups of animals displayed similar mBFV under basal conditions in the three great arteries of the circle of Willis. At the end of ischemia, when both CCAs were occluded, mBFV in the basilar trunk (BT) increased in a similar manner in the three groups (Figure 1(a); $p < 0.001$ compared to basal values; Supplemental Figure 1), suggesting collateral supply.[5] We also observed (for the three groups) residual mBFV in both intracranial carotid arteries (ICAs, Figure 1(b) and (c)) despite no visible flow in the CCA.

In the control group (simultaneous occlusion release in both CCAs), at 15 min after reflow, mBFV in the BT returned to basal values (11.1 ± 1.1 compared to 11.9 ± 1.7, respectively, NS), whereas reflow in both ICAs occurred 1 min after clip removal with a similar pattern in both the left and right ICAs, as previously reported.[5] Fifteen minutes after re-flow, mBFV in the right and left ICA were not significantly different from basal values (Figure 1(b) and (c); Supplemental Figure 1).

In the R-L group, reflow in the right CCA was accompanied by a return to basal mBFV in the right ICA; reflow in the left CCA was accompanied with a return to basal mBFV in the left ICA, whereas mBFV in the right ICA and BT decreased ($p < 0.01$ vs. control). Fifteen minutes after reflow, mBFV in the BT (7.9 ± 1.8 cm/s) and right ICA (9.7 ± 1.5 cm/s) remained reduced ($p < 0.01$) compared with the basal values (10.6 ± 2.0 and 13.3 ± 3.4 cm/s, respectively Figure 1(b) and (c); Supplemental Figure 1). Mean BFV in the left ICA (10.7 ± 3.4 cm/s) decreased compared with the basal (14.9 ± 2.9 cm/s) values ($p < 0.05$).

In the L-R group, reflow in the left CCA was accompanied by a return to basal mBFV in the left ICA; reflow in the right CCA was accompanied by a return to basal mBFV in the right and left ICA and the BT. Fifteen minutes after reflow in the three great arteries, mBFV were similar to basal mBFV. No significant variation in heart rates was detected between groups throughout the procedure.

COX-2 and mPGES1 gene and protein expression, microglial activation and cell death

One hour after ischemia-reperfusion, whereas an increase in the COX-2 ($p < 0.001$) and mPGES1 ($p < 0.001$) genes was observed in the ipsilateral (IL) cortex in the control group,[6] only a reduction in the mPGES1 ($p < 0.05$) gene was measured in the R-L group (Figure 2(a) and (b)).

Four hours after ischemia-reperfusion, the COX-2 and mPGES1 proteins in the IL cortex were reduced

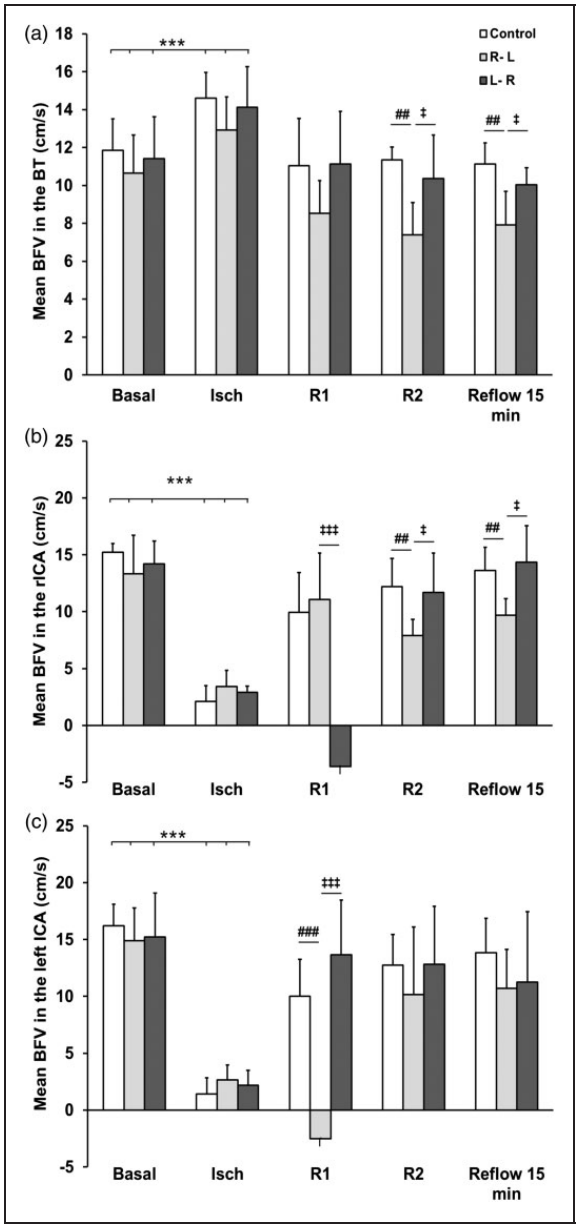

Figure 1. Blood flow redistribution in the three great arteries of the circle of Willis after the transient CCA occlusion release protocols. (a–c) Mean BFV in the basilar trunk (BT, a), the right ICA (rICA, b), and left ICA (c) in the control (white bars, simultaneous CCAs occlusion release, $n = 6$), R-L (gray bars, $n = 7$), and L-R (black bars, $n = 7$) groups under basal conditions, at end of ischemia (Isch), after the first CCA release (R1), after the second CCA release (R2 – 5 min later), and after 15 min of re-flow. $^{***}p < 0.001$ (vs. basal); $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ (control vs. R-L); $^{\ddagger}p < 0.05$, $^{\ddagger\ddagger\ddagger}p < 0.001$ (R-L vs. L-R).

($p < 0.001$, Figure 2(c) and (d), Supplemental Figure 2), as well as microglial density (determined with Iba-1 immunostaining, Figure 2(e), Supplemental Figure 3), in the R-L group compared with those in the control group. At 24 h after reperfusion, microglial density was reduced in the R-L group and increased in the L-R group (Figure 2(f), Supplemental Figure 3). The extent of protein nitrotyrosine formation (an index for reactive nitrogen species; Supplemental Figure 4) was higher in the control and L-R animals than that in R-L animals at 24 h after injury (Figure 2(g)). The density of cleaved-casp-3^{+}-cells (apoptotic marker) was increased in the L-R group ($p < 0.001$) compared with that in the control ($p < 0.001$) and R-L ($p < 0.001$) groups. Furthermore, the R-L animals displayed numerous cells with cytosolic cleaved-casp-3, whereas the L-R animals mostly displayed cells with nuclear cleaved-casp-3, suggesting completion of apoptosis in the latter group (Figure 2(g) and (h)) according to the pale lesion observed at 24 h after reflow (supplemental Figure 5).

Discussion

We here report that rerouting CBF by first inducing reflow in the CL side (R-L group) before reflow in the IL side may be beneficial by reducing inflammation, reactive nitrogen species and cell death.

To provide a better reperfusion in the ischemic penumbra, either thrombolysis or improvement of collateral supply has been proposed in the acute phase of ischemic stroke. Increased collateral recruitment can be obtained by the administration of drugs to increase vasodilation,[9,10] or by facilitating the synthesis of vasodilators[6] leading to the rerouting of BF through the numerous native anastomoses in the cerebral vascular network. We have also demonstrated that an increase in collateral supply induced by exogenous NO-donors is beneficial during ischemia but deleterious during reflow due to an elevation in oxidative stress.[10,11]

During ischemia, both hemispheric arterial networks experience a decrease in vascular resistance mediated by the loss of the myogenic tone (deep decrease in intravascular pressure),[12] and the accumulation of metabolic end-products as well as the initiation of anaerobic respiration (CO_2, glutamate, lactate, pyruvate, acidosis, Ca^{2+}).[13] The decrease in vascular resistance promotes the establishment of collateral recruitment after the changes in the arterial pressure gradient from the BT to the hemispheres (1) through the circle of Willis with perfusion of the two intracranial ICAs by the BT, through the posterior communicant arteries and the proximal segment of the posterior cerebral arteries (reverse-flow, the posterior cerebral artery is the first branch of the ICA in rodents)[14]; or (2) through the cortical arterial anastomoses that extend from the vascular supplies of the posterior and/or anterior cerebral arteries towards the vascular supply of the middle cerebral artery, particularly in the ischemic penumbra.[14]

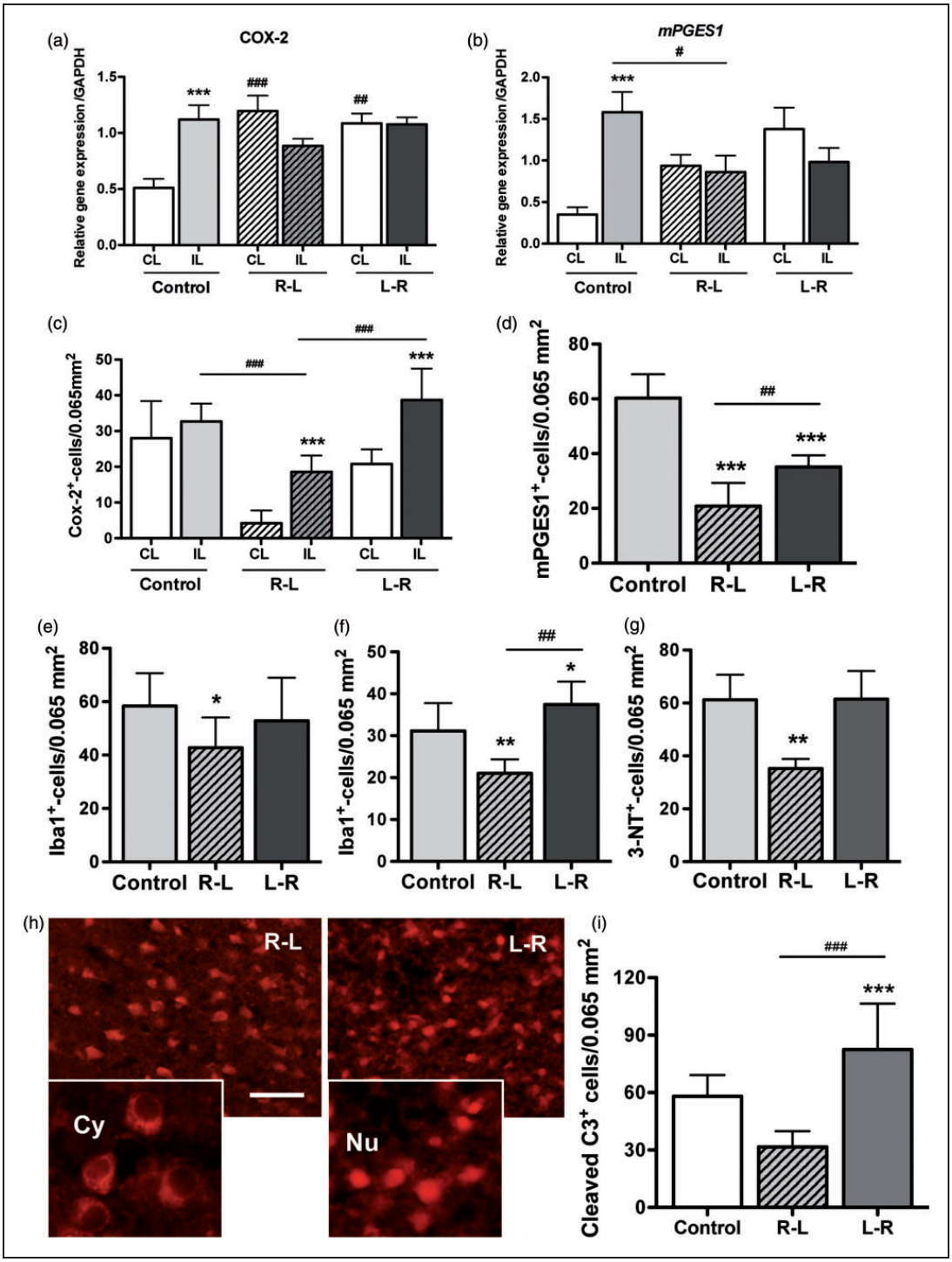

Figure 2. *COX-2* and *mPGES-1* gene and protein expression, nitrotyrosine formation and cell death. (a–d) Quantification for the COX-2 and mPGES1 genes (a–b) and proteins (c–d) in the three groups of animals ($n = 6$ per group) in the left (IL) and right (CL) cortex. (e–f) Quantification of microglial cells (immunostained with Iba-1) in the IL cortex at 4 (e, $n = 10$) and 24 (f, in remote peri-infarct, $n = 10$) hours after reflow. (g) Quantification of 3-nitrotyrosine (3-NT) formation at 24 h after reflow. (h–i) Number of cleaved-caspase-3^+-cells in the ipsilateral peri-infarct in the three groups of animals at 24 h after reflow. CL: contralateral; IL: ipsilateral; Cy: cytoplasm; Nu: nuclear. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ R-L group vs. L-R group.

During reflow, when the clamp on CCA one is released, arterial pressure and BF are instantaneously reestablished in a vasodilated vascular network. The vascular network requires a few seconds to control vascular tone and to restore adapted vascular resistance to the restored arterial pressure in order to control the local CBF. *In the R-L group*, arterial pressure and blood flow are reestablished in the right intracranial ICA first. The left ICA is then reperfused by a retrograde flow coming from the confluence of both anterior cerebral arteries (azygos artery in rodents), allowing the progressive pressure loading and the lavage of the metabolic end-products, particularly in the ischemic penumbra. Local vascular tone/resistance can therefore be restored progressively without a strong increase in the local CBF. Resupplying the arteries after low blood flow involves a decrease in metabolic end-products such as CO_2 and H^+, with changes deep acidosis to mild local acidosis that is known to be neuroprotective during post-conditioning.[15] Moreover, a mild hypoxic exposure after severe hypoxia in adult rats constitutes a postconditioning mode that attenuates post-hypoxic neuronal injury and reduces brain edema with improvement in recovery after severe hypoxia.[16] This postconditioning mode enhances the expression of HIF-1-alpha and erythropoietin in surviving rats after severe hypoxia.[16] Here, 5 min after the right CCA clamp release and the consecutive release of the left CCA clamp, reflow is established in the ipsilateral vascular network with a previously restored vascular tone, avoiding any supplemental increase in local oxidative stress and inflammation. Altogether, these events may constitute ''hypoxic post-conditioning'', which has been shown to be neuroprotective when the duration of hypoxia is less than 10 min.[15] *In the L-R group*, the arterial pressure and blood flow are instantaneously restored in the intracranial left ICA vascular network, particularly in the cortical anastomoses and thus in the ischemic penumbra. Upon reperfusion, the sudden resupply of oxygen and nutriments re-energizes mitochondrial aerobic respiration, resulting in a burst in reactive oxygen species production,[17] which leads to elevated local oxidative stress and the consecutive extension of the ischemic lesion. Identical events occur in case of concomitant release of CCAs clamps. In addition, the development of high CBF velocities in severely asphyxiated infants has been reported to predict the development of severe hypoxic-ischemic encephalopathy and poor prognosis.[18]

Compared with the control and L-R groups, our data show that the animals in the R-L group exhibit less prostaglandins synthesis, microglial activation, 3-nitrotyrosine formations, and cell death. In conclusion, the duration of arterial occlusion is of course mainly responsible of the lesion size; nevertheless, after an embolic stroke, controlled mechanical ablation of the thrombus to ensure the progressive restoration of arterial pressure and blood supply could be beneficial and avoid the ''blood tsunami'' that leads to local oxidative stress and nitrogen species synthesis. Our work shows that a slowly progressive cerebral arterial-recanalization following stroke may induce better outcomes. However, additional studies in rodents are needed to define the optimal rate of reperfusion.

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Declaration of conflicting interests

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Author's contributions

CCM and PLL designed the work; PB, JP, EP, ME, SR performed experiments; PB, PLL, OB and CCM analyzed data and wrote the manuscript.

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