Rolipram, a Phosphodiesterase Type IV Inhibitor, Exacerbates Periventricular White Matter Lesions in Rat Pups

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ABSTRACT: Periventricular white matter injury is the leading cause of cerebral palsy in premature infants for which no effective treatments are available. Our previous studies have demonstrated that pharmacological activation of the cAMP response element-binding protein (CREB) signaling pathway, before hypoxic-ischemia protected against neuronal injury in neonatal rats. We examined whether rolipram, a phosphodiesterase type IV inhibitor, treatment after hypoxic-ischemia is protective against white matter injury in neonatal rats. Rats were exposed to hypoxia-ischemia (HI) on P7 and then treated with daily injections of various doses of rolipram (P7–P11). Immunohistochemical staining for myelin basic protein, ED1, glial fibrillary acidic protein, CREB and O1 were examined on P11. We found that the periventricular white matter and deep cortical lesions were exacerbated by rolipram administration after HI injury. The lesions in the rolipram-treated group also showed increased astrogliosis and increased CREB phosphorylation in the activated astroglia and astrocytes. Furthermore, the rolipram-posttreated HI group had markedly depleted preoligodendrocytes in the ipsilateral hemisphere, which may be related to decreased preoligodendrocytes proliferation after rolipram treatment per se. These data suggest that rolipram treatment after hypoxic-ischemia is not protective; in contrast, rolipram may exacerbate hypoxic-ischemic white matter injury in neonatal rat brains. (Pediatr Res 64: 234–239, 2008)

Nearly 90% of low-birth weight infants now survive with the advances in neonatal intensive care. Of the survivors, however, 10–15% have cerebral palsy, and 25–50% have manifest cognitive and behavioral deficits (1). Periventricular white matter injury, or leukomalacia (PVL), is the major form of brain injury in preterm babies. The neuropathological findings are characterized by distinct cystic white matter lesions and, commonly, a more diffuse loss of white matter volume and secondary ventriculomegaly (2). Notably, despite the significant long-term morbidity, there is currently no specific treatment for these disorders.

Although, the exact cause of PVL in human infants is currently unknown, hypoxia-ischemia (HI) is believed to be a significant cause (3). Cell-type specific factors are also likely to underlie the mechanisms of PVL. Data from a recent study demonstrated that PVL in humans was related to oxidative damage that particularly targeted cells of the oligodendrocyte lineage; whereas neuronal and glial cells were markedly more resistant (4). The timing of appearance of preoligodendrocytes (preOLs) coincides with the high-risk period for PVL (5). Considerable data demonstrate that preOLs are highly susceptible to oxidative stress and hypoxic-ischemia injury (6–9). Compared with mature oligodendrocytes, preOLs express lower levels of antioxidant enzymes (6). These data suggest that acceleration of preOLs maturation might be a feasible strategy to reduce the severity of PVL.

cAMP plays an important role in inducing oligodendrocyte differentiation and myelin synthesis (10,11). Furthermore, the transcription factor, cAMP response element-binding protein (CREB) is a key mediator of stimulus-induced cAMP-mediated nuclear responses that underlie survival of neurons. CREB is phosphorylated on Ser133 (pCREB) and consequently binds to the cAMP response element of target genes. Recent data have revealed that virus-mediated expression of a constitutive active form of CREB can protect preOLs from excitotoxicity (12). Rolipram has been shown to elevate cAMP levels by inhibiting c-AMP specific phosphodiesterases type IV (13). Our previous studies demonstrated that pharmacological activation of the cAMP-CREB signaling pathway before hypoxic-ischemia with rolipram protected against neuronal injury in neonatal rats (14,15). It has also been shown that rolipram treatment after spinal cord injury promotes axonal regeneration, attenuates glial scar formation, and enhances remyelination and functional recovery in adult rats (16,17). Therefore, in this study, we investigated whether pharmacological activation of the cAMP-CREB signaling pathway with rolipram after hypoxic-ischemia is protective against white matter injury in neonatal rats.

MATERIALS AND METHODS

All procedures were approved by and in accordance with the guidelines of the Animal Care Review Committee at the Chang Gung Memorial Hospital. Ten to 12 pups per dam were housed with a 12/12-h light/dark schedule. We used postpartum day 7 (P7) male Sprague-Dawley rat pups when the cerebral white matter at that stage is primarily populated by preOLs, which increased the vulnerability of white matter to hypoxic-ischemia brain injury (18).

Abbreviations: CREB, cAMP response element-binding protein; MBP, myelin basic protein; preOLs, pre-oligodendrocytes; PVL, periventricular leukomalacia

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Hypoxic-ischemia. The animals were anesthetized with 2.5% halothane (balance, room air), and the right common carotid artery was surgically exposed and permanently ligated with 5–0 paraformaldehyde. The brains were then perfused with saline followed by 4% paraformaldehyde. The brains were removed and immersed in the same fixative for 48 h. The brains were then dehydrated through a graded series of alcohols and finally embedded in paraffin. The coronal sections were dewaxed, hydrated through graded alcohols and placed in phosphate-buffered saline (PBS). Endogenous peroxidase was blocked for 30 min in 0.3% H$_2$O$_2$ in methanol. Antigen retrieval was performed by incubating the sections in sodium citrate at 95°C for 10 min. Nonspecific binding was blocked by incubating the sections in PBS buffer 5% goat serum for 1 h at room temperature. The sections were then incubated with various primary antibodies followed by a 60-min incubation at room temperature with secondary Ab (Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibodies used were myelin basic protein 1: 500 (MBP, Chemicon, Temecula, CA); glial fibrillary acidic protein 1:200 (GFAP, DAKO, Glostrup, Denmark); ED1 1:500 (Serotec, Raleigh, NC); and pCREB 1:200 (Upstate Biotechnology, Lake Placid, VA). The sections were developed in 3′,3′-diaminobenzidine (Sigma Chemical Co.) with the Vectastain ABC system (Vector Laboratories, Burlingame, CA). In every experiment, a control with the primary antibody omitted was used as a negative control.

Double-fluorescence immunocytochemistry. After blocking for 1 h, the slides were incubated overnight at 4°C with a mixture of two of the following primary antibodies: GFAP (1:200), ED1 (1:500), NeuN (1:200; Chemicon), and pCREB (1:200). The slides were washed three times with PBS and then incubated with Texas Red-conjugated anti-rabbit and fluorescein isothiocyanate-conjugated anti-mouse Ab (1:200; Jackson ImmunoResearch) for 1 h at room temperature. The fluorescence signals were detected using a Nikon E400 microscope at excitation-emission wavelengths of 596–615 nm (Texas Red) and 470–505 nm (fluorescein isothiocyanate).

Analysis of preOL proliferation. Rats were treated with daily rolipram (3 mg/kg, n = 9) or vehicle (n = 9) on P7–P11 as stated above, except that hypoxic-ischemic insult was not induced. Animals received concurrent injections of daily BrdU (100 mg/kg, Sigma Chemical Co.) during the treatment protocol on P10 and P11 and killed on P11. Coronal sections were labeled with O4 Ab (chemicon), fixed, and processed for BrdU labeling (14). BrdU (+) cells were counted in the corpus callosum and external capsule of sections stereotactically similar to the region evaluated for injury.

Analysis. Using IMAGE PRO software (Image Pro, Boston), under a microscope (Nikon, Japan), the areas of parietal cortex (area between the rhinal sulcus and cingulum) were measured. Additionally, the ratio of each area (ipsilateral to contralateral hemisphere) was calculated. The degree of neuronal damage was scored by hematoxylin and cosin staining: mild (few damaged neurons), moderate (moderate numbers of damaged neurons), and severe (extensive number of damaged neurons) (19). The degree of neuronal damage was assessed on three separate sections at the level of the striatum, the dorsal hippocampus, and the ventral hippocampus.

Statistics. Statistical significance (p < 0.05) was determined using one-way analysis of variance (SPSS Inc., Chicago, IL). Post hoc comparisons were made using the Mann-Whitney U test. Continuous data were expressed as mean ± standard error of the mean unless indicated otherwise.

RESULTS

Selective vulnerability of immature white matter to moderate hypoxic ischemia in rat pups. Four days after moderate hypoxic-ischemia in P7 rat pups, there was subtle atrophy in the cortex and striatum of the cerebral hemisphere ipsilateral to carotid artery ligation, although overall tissue integrity was preserved. Seventy-five percent of the rat pups had small histopathological changes manifesting as a conical loss of cells in the deep cortical layers of the ipsilateral parietal cortex. No overt pathologic changes in the thalamus, dorsal hippocampus, or striatum were observed in bilateral hemisphere. There was a nonsignificant trend of decreased right-to-left (R/L) ratios in the parietal cortex, striatum and thalamus in the hypoxic-ischemia group compared with the naive control. No differences in the dorsal hippocampus areas were found between the hypoxic-ischemia and control groups (data not shown).

There was substantially decreased MBP immunoreactivity in the ipsilateral periventricular white matter and external capsule in 80% of the rat pups with hypoxic-ischemia. Decreased MBP immunostaining in the ipsilateral internal capsule and striatum was also observed in 75% of these rat pups. Compared with the naïve group, a significant reduction in R/L ratio of MBP-positive areas was observed in the hypoxic-ischemia group (data not shown).

Postinsult treatment with rolipram exacerbates deep cortical and white matter injury in immature rats. Although, the overall tissue integrity was preserved (Fig. 1A and B), post-ischemia treatment with rolipram increased neuronal cell death in the deep cortex of the ipsilateral hemisphere (Fig. 1C and D). There were no significant differences in the R/L ratio of cortical areas between the naïve and the rats posttreated with rolipram or vehicle (Fig. 1G). Further scoring revealed that the vehicle-treated group had mild cortical neuronal damage, but most of the rolipram-treated groups had moderate to severe neuronal damage (Fig. 1H, p < 0.05).

We observed significant decreases in MBP immunostaining in the ipsilateral periventricular, external capsule and striatum areas after hypoxic ischemia injury in the rolipram-treated groups compared with those areas in the vehicle-treated group (Fig. 1E and F). There was a significant difference in right-to-left ratio of MBP immunoreactivity between the vehicle-treated and rolipram-treated groups (Fig. 1I, p < 0.05). Roli-

pramid-treated rats also had significantly a higher degree of ventriculomegaly in the ipsilateral cerebral hemisphere than the vehicle-treated rats (Fig. 1J, p < 0.001). Furthermore, there was a significant difference in ventriculomegaly between the rolipram-treated groups. The degree of ventriculomegaly was significantly higher in the Rol-3 group than in the Rol-1 and Rol-0.5 groups (p < 0.05).

Postinsult treatment with rolipram exacerbated reactive astroglisis. The vehicle-treated group had increased numbers of GFAP(+) cells within the deep cortical layers and the underlying white matter in the ipsilateral hemisphere and very few astrocytes in the contralateral hemisphere. The levels of GFAP expression in the corpus callosum, deep cortical layer, external capsule and internal capsule in the ipsilateral hemisphere were markedly higher and more diffuse in the rolipram-treated groups than in the vehicle-treated group. The astrocytes in the rolipram-treated groups had larger cell bodies and thicker processes than those in the vehicle-treated group (Fig. 2A and B). There was no significant difference in the GFAP
revealed that there were significant differences between the vehicle- and treated HI groups (dose-related effect on the severity of cortical lesions among the rolipram-naive and the rats posttreated with rolipram or vehicle (Holm-Sidak test, p < 0.05, one-way ANOVA test, n = 9/group). There was no significant difference in the MBP immunoreactivity ratio between the Rol-0.5, Rol-1 and Rol-3 groups (I). The degree of immunoreactivity between the Rol-0.5, Rol-1 and Rol-3 groups.

In the vehicle-treated group, there were some ED1(+) cells in the deep parietal cortex and corpus callosum in the ipsilateral hemisphere; in contrast, very few ED1(+) cells were seen in the contralateral hemisphere. Compared with vehicle treatment, rolipram treatment prominently increased the number of ED1(+) cells within the ipsilateral deep parietal cortex and underlying corpus callosum. These activated ED1(+) microglia was characterized by large round cell bodies with reduced, thick processes (Fig. 2C and D). There was no significant difference in the ED1 immunoreactivity between the Rol-0.5, Rol-1 and Rol-3 groups.

Postinsult treatment of rolipram caused diffuse CREB phosphorylation. There was a marked bilateral increase in pCREB immunoreactivity in the cortex in the rolipram-treated group, which contrasted with the weak pCREB immunoreactivity in the vehicle-treated group (Fig. 3A and B). Double immunofluorescence analysis in the rolipram-treated group showed that pCREB was expressed in the activated microglia and astrocytes, as well as neurons in the ipsilateral hemisphere (Fig. 3C–K).
Rolipram treatment decreased the proliferation of preOLs.

In the vehicle-treated HI group, there was a substantial decrease in O1 immunoreactivity in the periventricular white matter and external capsule in the ipsilateral hemisphere after hypoxic-ischemia, as compared with the contralateral hemisphere. There was markedly less O1 immunostaining in the ipsilateral periventricular areas in the rolipram-treated HI group compared with the vehicle-treated HI group (Fig. 4A and B).

To examine the effect of rolipram per se on normal white matter development, rat pups were treated with 3 mg/Kg rolipram or vehicle, concurrent with injection of BrdU, but without HI injury. There were no significant differences in the O4(+) cells between the rolipram- and vehicle-treated rats (Fig. 4D). The number of O4(+)-BrdU(+) cells in the periventricular areas, however, was markedly lower in the rolipram-treated group than in the vehicle-treated group (Fig. 4C and E).

DISCUSSION

The present study shows that unilateral carotid artery ligation in combination with moderate hypoxia results in selective white matter injury with relatively cortical sparing. Although, the level of CREB phosphorylation increased, rolipram administered after hypoxic-ischemic injury exacerbated the degree of periventricular white matter injury and led to an increase of deep cortical lesions. There was also increased astrogliosis in the ipsilateral periventricular areas and deep cortical layer in the rolipram-treated hypoxic-ischemic group, with increased CREB phosphorylation in these activated microglia and astrocytes. Furthermore, the rolipram-treated hypoxic-ischemic groups had markedly depleted preOLs in the ipsilateral hemisphere, which may be related to decreased preOLs proliferation after rolipram treatment per se. These data suggest that rolipram treatment after hypoxic-ischemia is not protective; in contrast, it may exacerbate neuronal and white matter injury in the neonatal rat brains.

It has been well documented that the cAMP-CREB signaling pathway plays an important neuroprotective role in both immature and mature brains (15,20). Rolipram pretreatment in rat pups has been shown to protect against hypoxic-ischemic neuronal injury via CREB activation (15). It has also been shown that virus-mediated overexpression of CREB protected preOLs from excitotoxicity in vitro and in vivo (12). However, in our study, rolipram posttreatment increased the periventricular white matter and deep cortical neuronal injury. We observed diffuse CREB phosphorylation in activated microglia and astrocytes, in addition to neurons, in the ipsilateral hemisphere after rolipram posttreatment. Previous studies have shown transient neuronal but persistent astroglial CREB activation after focal brain injury, suggesting a crucial role of CREB in perilesional reactive astrogliosis (21). Recent studies suggested that the induction of proinflammatory genes, such as interleukin-6 and tumor necrosis factor-α is mediated via activation of NF-kB and CREB in neonatal glial cells (22). Therefore, differential modulation of target genes by transcription factors, which is region, cell type and time specific, may explain these divergent results for CREB activation (23). Furthermore, rolipram can increase cAMP, which in turn regulates many important intracellular metabolic processes besides its effect on CREB. Many cAMP-dependent and
cAMP binding effectors have been identified, and several alternative pathways have been discovered (24). The cellular compartment in which cAMP is released is also of prime importance as it determines which downstream effectors are activated (25). Differential modulation of these targets may explain the divergent effects observed in the various pharmacological agents used in rat models (26).

The deleterious effects of rolipram posttreatment on hypoxic-ischemic white matter injury in neonatal rat brain may be related to the inhibitory effects of rolipram per se on preOLs proliferation. We found that the numbers of preOLs incorporating BrdU in the white matter were significantly lower in rats treated with rolipram than in those treated with vehicle. Oligodendrocytes undergo a series of sequential and well-defined changes in their phenotypic characteristics, gradually evolving from proliferative immature precursors into postmitotic mature cells (27). cAMP-elevating agents can promote preOLs lineage progression and differentiation but may inhibit their proliferation (17,28,29). Although mature oligodendrocytes are more resistant to hypoxic-ischemic injury than preOLs (6–9), rolipram treatment may in fact deplete the number of proliferating preOLs after hypoxic-ischemic injury, as demonstrated in our study.

Much evidence points to a pathologic contribution of microglia toward periventricular white matter damage (30,31). Therefore, anti-inflammatory therapy, such as antenatal betamethasone treatment (32), represents a potential treatment of this disorder. Rolipram has been shown to prevent immune cell activation and proinflammatory cytokine production (17). This does not appear to be the case in the present study, because the degree of astrogliosis was significantly higher in the rolipram-treated group than in the vehicle-treated group. Nevertheless, anti-inflammatory therapy, such as rolipram treatment, may worsen outcomes in the developing brain.

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