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Systemic Inflammation following Hind-Limb Ischemia-Reperfusion Affects Brain in Neonatal Mice

M. Daniela Bianco-Batlles^a, Alexander Sosunov^b, Richard A. Polin^a, and Vadim S. Ten^a

^aDepartment of Pediatrics, Columbia University, New York, N.Y., USA

^bDepartment of Neurosurgery, Columbia University, New York, N.Y., USA

Abstract

Antenatal and postnatal infection and inflammation are associated with neurological injury in neonates. However, no direct role for systemic inflammation in mediating neurodamage has been shown. The study was aimed to determine whether systemic inflammation following ischemia-reperfusion (IR) of an organ remotely located from the brain results in cerebral injury. Neonatal mice were subjected to 2 h of hind-limb IR. At 48 h of reperfusion, brains were examined for activation of microglia and caspase-3. Lungs were assessed for pulmonary edema and granulocyte infiltration. The levels of circulating inflammatory mediators were measured at 24 h of reperfusion. In a separate cohort of mice, changes in the cerebral and hind-limb blood flow were measured. All data were compared to that in sham mice. Compared to shams the degree of pulmonary edema in IR mice was 33% ($p = 0.04$) greater. This was associated with significantly ($p = 0.0006$) greater granulocytic infiltration and a markedly increased level of circulating cytokines. The brains of these same mice exhibited significantly ($p = 0.02$) greater numbers of caspase-3-immunopositive cells and activation of microglia compared to sham mice. These data indicate that systemic inflammation following IR in the organ remote from the brain can induce neuroinflammation and cerebral proapoptotic changes.

Keywords

Systemic inflammation; Ischemia-reperfusion; Brain damage

Introduction

It is well established that maternal infection and fetal inflammation are strongly associated with white matter injury and cerebral palsy in preterm and term infants [1]. The fetal or systemic inflammatory response syndrome (SIRS) has been suggested in the pathogenesis of cerebral palsy. However, clinical and experimental data on the causative role of circulating cytokines in neonatal cerebral injury are conflicting. For instance, Nelson et al. [2] reported that none of the 11 circulating inflammatory mediators, including IL-1, IL-6, IL-8, TNF-1 α , measured in infants less than 32 weeks of gestation were predictive of cerebral palsy diagnosed at the age of 24 months. In contrast, the level of IL-6 measured in the umbilical cord blood and amniotic fluid has been reported as an independent predictor for the development of periventricular leukomalacia in preterm neonates [3]. Ellison et al. [4] reported that cerebrospinal fluid, but not plasma levels of IL-6, IL-10 and TNF-1 α , were associated with MRI-documented white matter injury in premature neonates. Maternal exposure to *Escherichia coli* LPS has been shown to induce astrogliosis, hypomyelination and significantly increased cellular death rate

in the periventricular striatum and deep gray matter in newborn rats [5]. However, Eklind et al. [6] using the same dose of LPS for neonatal rats reported that LPS-induced inflammation resulted in no or little damage to the intact (nonischemic) brain, but increased sensitivity to hypoxic-ischemic (HI) brain injury. Furthermore, it has been shown that cerebral morphological changes following intracervical maternal exposure to a low (100 µg/kg) dose of LPS were not associated with early and late sensorimotor deficit in neonatal rats [7]. If the association between fetal inflammatory response syndrome and the immature brain injury has a mechanistic link, then systemic inflammation of any origin might be expected to result in brain damage. Our study was undertaken to determine whether generalized inflammatory response following aseptic insult to an organ remotely located from the brain induces cerebral injury. To test this hypothesis we have chosen a model of hind-limb ischemia-reperfusion (IR). In adult rodents, this model produces damage in multiple organs by reperfusion-driven systemic inflammation [8]. However, there are no studies on cerebral damage in this model. We reasoned that if the immature brain is affected by remote-organ IR, this model could be used as experimental evidence for SIRS-mediated encephalopathy in neonates.

Material and Methods

Animal Model

At postnatal day 10–12 C57BL/6J (Jackson Lab) mice were anesthetized with intraperitoneal injection of xylosine and ketamine (0.01 mg/g xylosine, 0.1 mg/g ketamine) and underwent 120 min of ischemia of both hind limbs. IR was induced by application and release of rubber bands above the greater trochanter using the McGivney Hemorrhoidal Ligator (Miltex Instrument). The absence of blood perfusion in the tourniquetted limb was verified by the laser Doppler blood flowmetry. Sham mice were anesthetized, but did not undergo ischemia. During IR mice were kept in the infant incubator with ambient temperature of 32°C to mimic the temperature in the mouse nest. After releasing the rubber bands, mice were returned to their dams. In a separate cohort of mice changes in cerebral (CBF) and hind-limb blood flow in response to reperfusion were measured by laser Doppler as described [9]. At 48 h of reperfusion mice were sacrificed under deep isoflurane anesthesia. All experiments were conducted according to a protocol approved by the Columbia University Animal Care and Use Committee.

Assessment of Injury in the Organs Remote to the IR Limbs

Lungs—The evidence of lung injury served as a positive control for SIRS-induced remote end-organ damage. Tissue preparation and immunohistological procedure were done as follows: after midsternotomy the right lung was excised. It was immediately weighed followed by desiccation at 90°C for 72 h to obtain a wet/dry weight (W/D) ratio. The W/D ratio has been reported as a measure of pulmonary edema in adult rats subjected to hind-limb IR [10]. The left lung was perfused with 4% paraformaldehyde, harvested and fixed in 4% paraformaldehyde in PBS (4°C). After paraffin mounting, three consecutive 5-µm sections from each (upper, middle and lower) lobes were obtained and processed for detection of granulocytes using granulocyte-specific antibody against Ly-6G/C [11]. Briefly, sections were incubated overnight with primary Ly-6G/C antibodies (BD Pharmingen) followed by incubation with secondary antibody. Using confocal microscopy, the number of extravascularly identifiable Ly-6G/C-positive cells was quantified manually in 10 nonadjacent fields (295 × 295 µm) per section in three sections per mouse.

Brain—Brains were fixed in 4% paraformaldehyde in PBS for 48 h (4°C). Forty-micrometer coronal sections were cut on vibratome and immunostained for activated caspase-3 (Cell Signaling Technology, Danvers, Mass., USA) and CD68, markers of activated microglia (AbD Serotec, Oxford, UK). Briefly, free-floating sections were pretreated with 0.3% hydrogen peroxide in 70% methanol, blocked in 10% donkey serum and incubated with primary

antibodies (overnight at 4°C). Sections were incubated with secondary biotinylated antibodies, washed in PBS and incubated in avidin-biotin complex linked to peroxidase (Vectastain Elite ABC kit, Vector Lab). The product of reaction was visualized with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) in 0.05 M Tris-HCl containing 0.01% H₂O₂. Using light microscopy cells immunopositive for activated caspase-3 and CD68 were identified as dark-brown cells in the cortex and hippocampus. Caspase-3-positive cells were manually counted in 5 nonadjacent identically magnified fields in each region of interest in three sections per mouse. The degree of CD68 immunopositivity in the same areas of interest was semiquantified as described [12] with minor modification. Briefly, images were captured under identical magnification. The ratio of the area (in square pixels) immunopositive for CD68 (dark brown) divided by total imaged field of interest was measured using Image Pro-Plus and expressed as staining index. Five nonadjacent fields in three separate sections (anterior -1 to 1.2 mm, middle -1.8 to 2 mm and posterior 2.5 mm in relation to the bregma) per mouse were examined. Data obtained from different cerebral regions (cortex and hippocampus) were summated and compared between IR mice and sham mice.

Cytokine Assay—In a separate cohort of mice at 24 h of reperfusion the level of circulating cytokines was measured using Mouse Inflammation Antibody ChemiArray I (Chemicon International) according to the manufacturer's instruction. Briefly, mice were subjected to hind-limb IR (as described above), a sham procedure, or received 80 µl of boiled *E. coli* DH5- α suspension as a crude source of LPS. At 24 h following the index event, blood was obtained by cardiac puncture, centrifuged and serum was processed for measurement of circulating cytokines. To obtain the volume sufficient for this study, the serum was pooled from 4 mice in each group (IR mice, sham mice and *E. coli*-exposed mice). Antibody array membranes were incubated with serum for 2 h, washed and incubated overnight with biotin-conjugated anticytokine primary antibody. Diluted HRP-conjugated streptavidin and detection buffer were applied. Different cytokine signals were identified using a chemiarray map, quantified by densitometry and expressed as a percentage of optical density (o.d.) relative to shams (100%).

Statistical Analysis

Data are expressed as mean $\pm$ SE. Unpaired t test analysis was used for comparative assessment of the extent of pulmonary edema, degree of pulmonary granulocyte infiltration, activation of cerebral microglia and activated caspase-3 expression in the brain. CBF and PBF changes during IR were analyzed using ANOVA for repeated measures. Simple regression analysis was used to assess correlation between degree of granulocyte infiltration in the lungs and the extent of microglial activation in the brain. Data were considered statistically significant if $p \leq 0.05$ between groups.

Results

The application of the rubber bands resulted in a complete cessation of the blood flow to the hind limbs. Once tourniquets were released, the blood flow in the affected limbs was restored immediately (fig. 1a, c). Immediately following tourniquet release, the CBF significantly increased at 5 s of reperfusion followed by a significant drop at 30 s of reperfusion compared to the limb-ischemic baseline (fig. 1 b). No significant changes compared to the pretourniquet release level were found during next 5 min of CBF recording (fig. 1 b).

At 24 h of reperfusion there was a considerable elevation in serum levels of IL-1, TNF-1 α and TIMK-1 (IL-1 = 151 o.d. = 132%, TNF-1 α = 109 o.d. = 143%, TIMK-1 = 326 o.d. = 196%) compared to their sham counterparts (IL-1 = 114 o.d., TNF-1 α = 76 o.d., TIMK-1 = 166 o.d.) (fig. 1 d). Similar elevation in levels of circulating cytokines was observed in mice pretreated with LPS (IL-1 = 154 o.d. = 135%, TNF-1 α = 159 o.d. = 209%, TIMK-1 = 392 o.d. = 236%).

At 48 h of reperfusion, IR mice exhibited a greater pulmonary W/D ratio ($p = 0.04$) and increased numbers of granulocytes infiltrating the lung compared to sham mice ($p = 0.0002$) (fig. 1e–h). Similarly, in IR mice the activation of cerebral microglia was significantly greater compared to sham mice ($p = 0.03$) (fig. 2a–c). This was associated with an increased total number of caspase-3-positive cells in the cortex and hippocampus of IR mice ($p = 0.02$) (fig. 2d). Caspase-3-immunopositive cells morphologically resembled neurons and glial cells (fig. 2e).

Regression analysis revealed significant ($p = 0.04$, $r = 0.47$) correlation between the degree of CD68 immunopositivity and amount of caspase-3-positive cells in the brain in sham ($n = 8$) and IR mice ($n = 13$). When we analyzed the association between inflammatory changes in the brain and lung of IR mice, there was a significant ($p = 0.01$, $r = 0.67$) correlation between the degree of microglial activation in the brain and the degree of granulocytic infiltration in the lung (fig. 2f).

Discussion

Our study indicates that the immature brain can be affected by systemic inflammation during reperfusion following ischemic insult to a remote organ. The use of a hind-limb (an organ remote from the brain) IR model which induced a robust microglial activation and proapoptotic changes in brain suggests that circulating cytokines mediate cerebral injury during reperfusion. Cytokines in SIRS have been proposed as the cause for encephalopathy in critically ill adult patients with sepsis [13]. In neonates, maternal infection and fetal inflammatory response have been associated with white-matter injury and cerebral palsy [14]. Although clinical observations indicate that systemic inflammatory response to fetal or maternal infection can affect the brain, it is possible that maternal bacterial/viral infection may also induce focal cerebral infection and subsequent encephalopathy. Thus, it is difficult to determine whether systemic inflammation alone can account for cerebral damage in human neonates.

In animals, exposure to LPS is the most common model to study the link between infection/inflammation and injury to the immature brain. It has been shown that ‘preexposure’ of neonatal rats to live *E. coli* followed by LPS injection resulted in significant long-term neurofunctional deficit [15]. It has been suggested that LPS-mediated brain injury is secondary to cerebral hypoperfusion/ischemia [16,17]. In human premature neonates cytokine-induced alterations in systemic hemodynamics may be a predisposing factor for development of chorioamnionitis-associated brain injury [18]. Of note, the same authors reported that neither chorioamnionitis nor elevated cytokine concentrations were associated with changes in blood flow velocities measured in a middle cerebral artery [18]. The model used in our study was associated with very brief fluctuations in CBF only for 30 s following the tourniquet release, which is very unlikely to cause ischemic changes and subsequent microglial activation. Therefore, the use of this model may be appropriate to study a pathogenic role for circulating cytokines in SIRS-induced cerebral injury. In particular, the use of this model can be considered more clinically relevant than the use of LPS to highlight a contributive effect of cytokines released from ischemic tissues during reperfusion to the cerebral injury in asphyxiated neonates. It should be noted that exacerbation of HI brain injury in neonatal rats was observed only when LPS pretreatment was given 4–6 and 72 h, but not 24 h prior to HI insult [6]. This indicates that LPS induces cerebral sensitization to HI injury exerted via both systemic (SIRS) and focal cerebral changes in the brain, as in 72-hour animals completely recovered from the systemic effects of LPS [6]. Of note, the same authors reported that LPS-induced sensitization to HI injury was partially abolished by glucose administration following LPS exposure. This indicates that the mechanism for LPS-induced exacerbation of HI brain damage, at least partially, can be explained by the LPS-mediated alteration in the glucose homeostasis [19].

Hind-limb IR results in a significant increase in the level of circulating cytokines (IL-6, TNF- α) in rodents [8,20]. In our study we found a robust elevation of circulating levels of TNF-1 α , IL-1 β and tissue inhibitor of metalloproteinase-1 (TIMP-1) following hind-limb IR. Elevated serum levels of TNF-1 α and IL-1 β were associated with brain injury and cerebral palsy in neonates [21]. Interestingly, it has been suggested that focal (hippocampal) induction of TIMP-1 following global cerebral IR in rats [22] may represent a natural neuroprotective response, presumably directed against neurotoxic effects of matrix metalloproteinase-9 [23]. It is unclear whether a markedly increased signal for circulating TIMP-1 following hind-limb IR contributes to or attenuates cerebral apoptosis. We realize that the use of a single value to study differences in circulating cytokine levels between groups of mice is a considerable methodological limitation in our study. However, the systemic cytokine-induced nature of injury after hind-limb IR in our experimental design is supported by simultaneous involvement of two different organs, brain and lungs. A strong correlation between the severity of inflammatory changes in lungs and brain coupled with significantly increased proapoptotic changes in brain suggests a causative role for circulating mediators in cerebral damage. A significant rise of circulating cytokine levels following hind-limb IR in animals and humans has been reported [8,24]. Simultaneous proinflammatory response to hind-limb IR in two independent organs (lungs and brain) reported here can serve as evidence that SIRS can affect multiple organs, including brain. Multiple organ failure is a long-known diagnosis which clinically defines pathophysiology of SIRS. Thus, we believe that our data support the concept that generalized inflammation affects developing cerebral tissue by circulating mediators.

We did not investigate the molecular mechanisms affecting the brain in our model. However, a robust activation of microglia detected following remote IR coupled with a significant positive correlation with the number of caspase-3-activated cells in brain indicates that the activation of microglia may be partially responsible for proapoptotic changes in our model. Activated microglia produce reactive oxygen species [25,26], nitric oxide [27] and TNF-1 α [28]. Mallard et al. [29] reported that intravenous injection of LPS induces periventricular brain injury in fetal sheep, which was associated with significant microglial activation. Recently, Jin et al. [30] demonstrated that attenuation of microglial activation was associated with a significant improvement of neurocognitive performance in rats subjected to intracerebral LPS infusion. We realize that our study does not answer an important question: do morphological changes in brain following IR of the remote organ result in a long-term neurological deficit? Given that hind-limb IR is associated with a robust microglial activation in hippocampus and neocortex our future study will be focused on long-term neurological outcome in our model.

In our experiments we did not study markers for permeability of the blood-brain barrier or white matter damage. The main focus of this work was to prove the concept that systemic inflammation induced by the remote IR is capable of affecting an intact brain. Experimental data indicate that intrauterine inflammation results in damage to both, gray and white matter [5]. In this study we have shown that IR in an organ remote to the brain induces gray matter (hippocampal and cortical) proapoptotic changes in neurons and glial cells. It is still to be determined whether white matter is affected in this IR model. We have stained cerebral specimens for myelin basic protein, the marker for integrity of myelination. No difference was found in the level of cerebral myelination between IR mice and sham counterparts (data not shown). It is possible that in our model more sensitive methods are needed to detect damage to white matter or that an injurious insult must be produced at an earlier stage of cerebral development.

In conclusion, this is a first report that SIRS which originated in response to IR in an organ(s) distantly located from the intact brain should be considered as a potential risk factor for brain injury of an inflammatory nature.

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Fig. 1. Blood flow changes in hind limb (a) and brain (b, c) in response to tourniquet release in IR mice (n = 9). Arrow indicates the time of the tourniquet release. * $p = 0.04$, ** $p = 0.005$ compared to the pretourniquet release level. d Optical density of circulating cytokine signals. Groups of mice are indicated. Pulmonary W/D ratio in sham (n = 8) and IR (n = 10) mice (e) and granulocyte count in lungs in sham (n = 8) and IR (n = 13) mice (f) at 48 h of reperfusion after 2 h of hind-limb ischemia. * $p < 0.05$ compared to shams. Representative images of granulocytes (pink) in pulmonary tissue obtained from sham (g) and IR (h) mice. Scale bar = 50 μ m.

Fig. 2.

CD68 immunostaining (dark brown) in the dentate gyrus and CA1 region of the hippocampus, part of internal capsule (IC) and cortex (C) in sham (a) and in IR (b) mice. Scale bar = 200 μ m. c CD68 immunoreactivity index in cortex and hippocampus in sham mice (n = 8) and IR mice (n = 13). A total number of caspase-3-positive cells/field in cortex and hippocampus in sham mice (n = 8) and IR mice (n = 13) (d) and morphological appearance of caspase-3-positive cells (e). Scale bar = 50 μ m. * $p < 0.05$ compared to sham mice. f A simple regression analysis for correlation between the degree of granulocytic infiltration in lungs and CD68 immunoreactivity index in brain in IR mice at 48 h of reperfusion ($r = 0.67$, $p = 0.01$).