Brain oxygen and metabolism is dependent on the rate of low-flow cardiopulmonary bypass following circulatory arrest in newborn piglets

Peter Pastuszko a,*, Huiping Liub, Alberto Mendoza-Paredes b, Steven E. Schultz c, Scott D. Markowitz d, William J. Greeley d, David F. Wilson b, Anna Pastuszko b

a Department of Surgery, The University of Oklahoma, Oklahoma City, OK, United States
b Department of Biochemistry & Biophysics, The University of Pennsylvania School of Medicine, Philadelphia, PA, United States
c Department of Pediatrics, The University of Miami, Miami, FL, United States
d Department of Anesthesiology & Critical Care, The Children’s Hospital of Philadelphia, Philadelphia, PA, United States

Abstract

Objective: To determine the optimum rate of low-flow hypothermic cardiopulmonary bypass (LF), following circulatory arrest (DHCA) on brain oxygenation ($bO_2$), extracellular dopamine (DA), phosphorylation of select neuroregulatory proteins responsible for neuronal injury, and survival following ischemic brain injury: CREB, Erk1/2, Akt, Bcl-2, and Bax. Methods: The piglets were placed on cardiopulmonary bypass (CPB) and cooled to 18 °C. They were then subjected to 30 min of DHCA followed by 1 h of LF at 20, 50, or 80 ml/(kg/min), rewarmed, separated from CPB, and maintained for 2 h. The $bO_2$ was measured by quenching of phosphorescence; DA by microdialysis; phosphorylation of CREB, ERK1/2, Akt, Bcl-2, and Bax by Western blots. The results are means ± SD for seven experiments. Results: Pre-bypass $bO_2$ was 47.4 ± 4.2 mmHg and decreased to 1.9 ± 0.8 mmHg during DHCA. At the end of LF at 20, 50, and 80 ml/(kg/min), $bO_2$ was 11.8 ± 1.6, 26 ± 1.8, and 33.9 ± 2.6 mmHg, respectively. The DA increased 510-fold relative to control ($p < 0.001$) by 15 min of LF-20 with maximum increase occurring at 45 min. With LF-50, increase in DA was not statistically significant and no increase was observed when LF-80 was used. Bcl-2 immunoreactivity increased after LF-50 and LF-80 (140 ± 14.5%, $p < 0.05$ and 202 ± 34%, $p < 0.05$, respectively). Neither flow increased Bax immunoreactivity. The ratio of Bcl-2/Bax, pCREB, pAkt, pErk increased significantly with increasing the flow rate of LF. Conclusions: The protective effect of LF following DHCA on brain metabolism is dependent on the flow rate. Flow-dependent increase in pCREB, pErk1/2, pAkt, increase in Bcl-2/Bax, and decrease in DA indicated that to minimize DHCA-dependent neuronal injury, LF flow should be above 50 ml/(kg/min).

Keywords: Newborn; Brain injury; Cardiopulmonary bypass; Circulatory arrest; Oxygen

1. Introduction

Cardiopulmonary bypass (CPB) and prolonged deep hypothermic circulatory arrest (DHCA) may result in detrimental effects in young, particularly neonatal patients. The early, enthusiastic use of DHCA, particularly in neonates, has been tempered by the finding of significant neurological morbidity associated with its prolonged exposure observed in long-term follow up studies. Stroke, cognitive, and neuropsychological dysfunction, retinal microvascular pathology, impaired level of consciousness, seizures, and spinal cord injury, all can occur after cardiopulmonary bypass [1–4]. Some of the contributing factors of these injuries can be due to DHCA-dependent impaired recovery of cerebral blood flow and cerebral oxygen metabolism [5–9]. These findings have led surgeons to limit, or even eliminate, DHCA and shift to other perfusion techniques such as low-flow cardiopulmonary bypass (LF), intermittent perfusion, and selective cerebral perfusion [10–16]. Another promising strategy for mitigating adverse effects of prolonged DHCA is to use a shorter duration of DHCA followed by LF. However, as for the other techniques, whether this approach affords significant protection from neuronal injury is unknown. Furthermore, the optimum rate of LF has not been determined.

The objective of this study was to examine the optimum CPB flow rate, when initiated after a 30 min period of DHCA, on brain tissue oxygenation, dopamine release, and the activity of select neuronal regulatory proteins. Dopamine release was chosen because this neurotransmitter is widely believed to play a crucial role in the physiology of brain...
function, and excess release of dopamine following circulatory arrest can play a major role in mediating neuronal damage. The activities of specific neuronal regulatory proteins that play a crucial role during neurodevelopment and are important determinants of the extent of neuronal cell survival and injury were studied. These regulatory proteins include cAMP response element binding protein (CREB), protein kinase B (Akt), extracellular signal-regulated kinase 1/2 (Erk1/2), Bcl-2, and Bax. We hypothesized that the higher CPB flow rates, after a brief duration of DHCa, would improve or mitigate any harmful effects of circulatory arrest, as determined by brain oxygenation, dopamine release, and regulatory protein activity.

2. Methods

2.1. Animal model

Newborn piglets, 3–5 days old (1.4–2.5 kg) were anesthetized with halothane, and a tracheotomy was performed. The piglets were then placed on a ventilator, and anesthesia was maintained with fentanyl, isoflurane 0.5%, and pancuronium. Femoral venous and arterial cannulas were placed for the collection of blood samples and for monitoring blood pressure. With the head of the animal in a stereotaxic holder, the scalp was removed and a cranial window approximately 8 mm in diameter was created over the right parietal hemisphere for measurement of cortical oxygenation. A small hole was drilled over the left parietal hemisphere for implantation of a microdialysis probe into the left striatum. After a 2-h stabilization period, cardiopulmonary bypass was initiated. Following bypass, the animals were recovered for 2 h and then euthanized with 4 M KCl.

All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and have been approved by the local Animal Care Committee.

2.2. CPB technique

The circuit was primed with Plasmalyte-A and then 25% albumin was added to the circuit. Donor whole blood was added to maintain a hematocrit of 25–30%. Heparin (1000 units), fentanyl (50 mcg), pancuronium (1 mg), CaCl₂ (500 mg), methylprednisolone (60 mg), cefazolin (100 mg), furosemide (2 mg), and NaHCO₃ (25 meq.) were then added to the pump prime. A membrane oxygenator (Lilliput) was used for CPB, a median sternotomy was performed. The piglets were then placed on a ventilator, and a tracheotomy was performed. The cranial window was prepared, bone wax was used to seal the vessels in the bone tissue, and the dura was left intact. A small amount of physiological saline was placed on the dura and then it was covered with a sheet of clear plastic film. The excitation and collection light guides (3 mm diameter fiber bundles with center-to-center distance of about 7 mm) were brought very close (< 1 mm) to the plastic film.

A near-infrared oxygen-sensitive phosphor (Oxyphor G2) was injected i.v. at approximately 1.5 mg/kg. The measurements were made using a multi-frequency phosphorescence lifetime instrument (PMOD 5000). The LED excitation lamp (635 nm), modulated by the sum of 37 sinusoidal waves with frequencies spaced between 100 Hz and 40 kHz, was carried into the tissue through a 3 mm light guide. The phosphorescence (~970 nm) emitted from the tissue was collected through a second light guide, placed against the tissue at approximately 8 mm (center-to-center) from the excitation light guide. This positioning of the light guides allowed effective sampling of brain tissue oxygenation down to approximately 6 mm under the neocortical surface. The phosphorescence signal was amplified, digitised, and analyzed to give distribution of phosphorescence lifetimes (oxygen histogram) in the volume of tissue sampled by the light. Since there were substantial differences among animals with respect to collected phosphorescence, etc., the oxygen histograms were normalized to have the same total amount of signal (integral) for oxygen pressures less than 140 mmHg. Using this method, oxygen measurements have been made over periods of several hours without observable injury to the tissue or evidence of measurement-induced alterations in the oxygen distributions in the tissue.

2.4. Measurements of oxygen pressure and oxygen distribution by the oxygen-dependent quenching of phosphorescence

Cortical oxygen pressure was measured using oxygen-dependent quenching of phosphorescence. The technical basis for determining the distribution of oxygen in the microcirculation of tissue from the distribution of phosphorescence lifetimes in the serum of blood has been described in detail [17,18]. The cranial window was prepared, bone wax was used to seal the vessels in the bone tissue, and the dura was left intact. A small amount of physiological saline was placed on the dura and then it was covered with a sheet of clear plastic film. The excitation and collection light guides (3 mm diameter fiber bundles with center-to-center distance of about 7 mm) were brought very close (< 1 mm) to the plastic film.

A near-infrared oxygen-sensitive phosphor (Oxyphor G2) was injected i.v. at approximately 1.5 mg/kg. The measurements were made using a multi-frequency phosphorescence lifetime instrument (PMOD 5000). The LED excitation lamp (635 nm), modulated by the sum of 37 sinusoidal waves with frequencies spaced between 100 Hz and 40 kHz, was carried into the tissue through a 3 mm light guide. The phosphorescence (λmax = 790 nm) emitted from the tissue was collected through a second light guide, placed against the tissue at approximately 8 mm (center-to-center) from the excitation light guide. This positioning of the light guides allowed effective sampling of brain tissue oxygenation down to approximately 6 mm under the neocortical surface. The phosphorescence signal was amplified, digitised, and analyzed to give distribution of phosphorescence lifetimes (oxygen histogram) in the volume of tissue sampled by the light. Since there were substantial differences among animals with respect to collected phosphorescence, etc., the oxygen histograms were normalized to have the same total amount of signal (integral) for oxygen pressures less than 140 mmHg. Using this method, oxygen measurements have been made over periods of several hours without observable injury to the tissue or evidence of measurement-induced alterations in the oxygen distributions in the tissue.

2.5. Measurement of striatal extracellular levels of dopamine by microdialysis

The extracellular level of dopamine in striatum was measured by in vivo microdialysis as described earlier [15]. Following a 2 h period of stabilization, the dialysis samples were collected at 15 min intervals during the bypass and post-bypass recovery and immediately analyzed for
dopamine level by high-pressure liquid chromatography with
electrochemical detector. Identification and quantitation of
dopamine were conducted by comparison with chromatograms
of standard solution of dopamine. The efficiency of the
microdialysis probe was determined in vitro at 18 and 37 °C.
The levels of dopamine in the dialysate are presented after
correction for relative recovery by the microdialysis probe.

2.6. Western blotting

The Western blots of CREB, ERK1/2, Bcl-2, Bax, and Akt
were performed as described earlier [19]. The specific
antibodies used for incubation with striatal membranes were
Erk1 (cross-reacting with Erk2), Bax (N-20), Bcl-2 (Santa Cruz
Biotechnology, Santa Cruz, CA, USA); phospho-Erk1/2,
phospho-Akt, Akt (Cell Signaling Technology, Beverly, MA,
USA); beta-actin (ABCAM, Cambridge, MA, USA); anti-
phosphorylated CREB antibodies (a-p-CREB; Upstate Bio-
technology, NY, USA; 1:200). Beta-actin antibody served as a
loading control.

2.7. Data analysis

Autoradiographic films were analyzed using Scion Image
software (NIH). Each blot contained two sets of samples, one
for an experimental group and one for the control group. The
data were normalized to the values obtained for the
untreated control group (assigned a value of 100). Statistical
analysis was performed using one-way analysis of variance
followed by Mann—Whitney test. A p < 0.05 was considered
statistically significant.

The cortical oxygen pressure and extracellular dopamine
values are expressed as means ± SD for seven experiments.
Statistical significance was determined using one-way
analysis of variance with repeated measures by Wilcoxon
signed-rank test. A p < 0.05 was considered statistically
significant.

3. Results

3.1. Dependence of cortical oxygen pressure on flow rate
of low-flow bypass following circulatory arrest in
newborn piglets

Pre-bypass cortical oxygen pressure was 47.4 ± 4.2 mmHg,
which decreased to 1.9 ± 0.8 mmHg during 30 min of DHCA.
During LF following DHCA, cortical oxygen increased with
time, and this increase was dependent on the rate of flow
(Fig. 1). At the end of a 60 min period of LF at flows of 20, 50,
and 80 ml/(kg/min), cortical oxygen pressures were 11.8 ±
1.6, 26 ± 1.8, and 33.9 ± 2.6 mmHg, respectively.

3.2. Effect of DHCA with LF on extracellular striatal level
of dopamine

The level of extracellular dopamine in striatum increased
by 510 times higher than the control by 15 min of LF-20 with
maximum increase occurring at 45 min (785-fold increase)
(Fig. 2). The time before the increase in extracellular
dopamine is similar to that for DHCA, described in our earlier

3.3. Levels of Bcl-2 and N-Bax in striatum following DHCA
and different rates of LF

As shown in Fig. 3, Bcl-2 immunoreactivity was decreased
after DHCA with LF-20 (71.0 ± 7.4% as compared to control,
p < 0.05) but was significantly increased after LF-50 and LF-
Neither LF-20, LF-50, nor LF-80 induced an increase in N-Bax immunoreactivity (115.2 ± 11.5%, 112.5 ± 12.3%, and 104.5 ± 9.3% as compared to control, respectively) (Fig. 4). The calculated ratio of Bcl-2/Bax increased with increase in the flow rate of LF following DHCA. At a flow rate of 20 ml/(kg/min), the ratio was 0.6, whereas when the flow rate was increased to 50 and 80 ml/(kg/min), the ratios increased to 1.3 and 2, respectively (Fig. 5).

3.4. Levels of phosphorylated CREB in striatal tissue measured after 2 h recovery following DHCA and LF

Western blots of proteins isolated from striatal tissues of piglets from control group and the groups subjected to DHCA + LF-20, LF-50, and LF-80, probed with anti-pCREB antibodies, are presented in Fig. 6. The phospho-CREB immunoreactivity in striata from these three experimental groups of animals was increased when compared to the sham-operated group of animals (4.56 ± 1.28%, p < 0.01; 5.67 ± 2.197%, p < 0.01 and 7.15 ± 2.046%, p < 0.005, respectively). The degree of increase was dependent on the flow rate during low-flow bypass and was highest in LF-80 group.

3.5. Levels of Akt in striatum following DHCA and different rates of LF

As can be seen in Fig. 7, the phospho-Akt (pAkt) immunoreactivity increased in all experimental groups, and percent of increase depended on the rate of low flow used following DHCA. LF-20, LF-50, and LF-80 induced a significant increase in pAkt immunoreactivity (169 ± 52%, p < 0.05; 207 ± 45%, p < 0.05; 234 ± 55%, p < 0.05) as compared to control. The differences in the increase in pAkt between LF-20 and LF-50 were not statistically
significant but were statistically significant in the increase between LF-20 and LF-80.

3.6. Levels of phosphorylated Erk1/2* in striatal tissue measured after 2 h recovery following DHCA and different rates of LF

The changes in the phospho-Erk (pErk) immunoreactivity in all experimental groups of animals are shown in Fig. 8. The pErk1/2 immunoreactivity increased in all experimental groups, but the highest increase was observed in LF-80 group. Following LF-80, the increases in pErk1 and pErk2 immunoreactivities, as compared to control, were 2870 ± 140%, p < 0.005 and 7970 ± 960%, p < 0.005, respectively. There was significant increase in pErk1 and pErk2 immunoreactivities between LF-80 group and LF-20 and LF-50 groups.

4. Discussion

The ideal CPB perfusion strategy has not been elucidated. Combination of DHCA and LF may be the most effective approach to repairing the more complex defects, specifically hypoplastic left heart syndrome and other hypoplastic aortic arch lesions. This method might allow for a technically satisfactory result and, at the same time, minimize the risks of neurological injury.

The present study determined the response of newborn brain metabolism to the rate of LF following 30 min of DHCA using α-stat strategy. Our earlier experiments had established that when using α-stat management the extracellular level of striatal dopamine significantly increased following 30 min of DHCA. The present study extends those observations, asking if this increase in extracellular dopamine can be decreased or abolished by LF, and if so, what flow rate is required. Even if it is accepted that in newborns and children, in contrast to adults, pH-stat blood gas management during CPB provides better protection from DHCA-dependent injury to the brain than does α-stat management, the published data are not conclusive [20–23]. The specific time periods used in this study are selected as representative of those used in clinical practice.

Our data show a significant dependence of brain oxygenation following DHCA on the rate of low-flow CPB. Cortical oxygen was measured by using the oxygen-dependent quenching of the phosphorescence. This method measures the oxygen pressure in the microcirculation of the cortex and some underlying white matter, down to about 6 mm from the surface. The presented values are the approximate mean for the sampled volume of tissue. A perfusion flow of 20 ml/(kg/min) does not protect against the release of extracellular dopamine in the striatum observed after 30 min of DHCA and reperfusion at this low rate. Increasing CPB reperfusion flow to 50 ml/(kg/min) significantly suppressed the release of extracellular dopamine compared to a flow of 20 ml/(kg/min), and the increase was no longer significantly different from control. A large standard deviation was present in the data for this group of animals, indicating a varying response of the animals to a flow of 50 ml/(kg/min) after 30 min of DHCA. From the group of seven piglets, four animals showed substantial increase in the dopamine levels, whereas in three animals, extracellular dopamine was not different from the control level. Further increase in the flow to 80 ml/(kg/min) completely abolished the release of dopamine.

Dopamine is widely believed to play a critical role in the pathophysiology of brain function, and the release of dopamine following circulatory arrest during LF-20 can play a major role in mediating neuronal damage, particularly in the striatum. Possible mechanisms of dopamine toxicity have
been described in detail in early publications [24,25]. Dopamine could potentiate neuronal damage through over-stimulation of the glutamnergic receptors, an increase the production of free radicals, and formation 6-hydroxydopa-
mine, very potent dopaminergic neurotoxin.

The suppression of extracellular dopamine release in the striatum is a good indicator of neuronal integrity. The CPB flow resumed following 30 min DHCA must be above 50 ml/(min/kg) to provide suppression of dopamine release and substantial protection to the brain. Our data obtained on protein phosphorylation and gene expression for selected proteins discussed below support this conclusion.

The activities of intracellular regulatory proteins such as Bcl-2, Bax, CREB, Akt, and Erk are important determinants of the extent of neuronal cell survival and injury. Key regulatory factors in apoptotic events are the proteins of Bcl-2 family, which can either promote cell survival (Bcl-2) or promote cell death (Bax). Our study shows that at flow rates of 50 and 80 ml/(kg/min), Bcl-2 expression was significantly higher than control, whereas at a flow of 20 ml/(kg/min), Bcl-2 expression was below the control values. There were no significant differences in expression of Bax among the experimental groups. The relative amounts and ratios of these pro- and anti-apoptotic proteins influence the susceptibility of cells to apoptosis. The calculated ratio of Bcl-2 to Bax was higher following 80 ml/(kg/min) flow than following a flow of 50 ml/(kg/min), suggesting that this flow can be the best in providing protection to the brain from DHCA-dependent injury.

The CREB (cAMP response element binding protein) is a regulatory protein activated by diverse signal transduction pathways influenced by cAMP and calcium, and is thought to be involved in the neuronal signal processing. Our data show that, in all three experimental groups, CREB phosphorylation significantly increased compared to control. The degree of increase was dependent on the CPB flow rate, with the greatest increase in the LF-80 group. This progressive increase in CREB phosphorylation may indicate, similar to the results of Bcl-2 and Bax, that using higher CPB flow rate will more likely be best for protecting the brain from ischemic damage.

Akt protein plays an essential role in neuronal survival and is best known for its ability to inhibit cell death pathways by directly phosphorylating and inactivating proteins involved in apoptosis. Akt targets several key proteins that help stressed cells to survive, including apoptosis regulators and transcription factors. Similar to our CREB findings, the phosphorylation of Akt increased progressively with increase in the rate of LF.

Erk1/2 is a member of the MAPK family that has been proposed to play an important role in the pathogenesis of cerebral ischemic injury and also in neuroprotection. Several lines of evidence suggest that sustained Erk1/2 activation may indicate serious neuronal injury, possibly leading to cell death. On the contrary, it has been suggested that prolonged Erk activation accompanied by an increase in CREB can contribute to cell survival and plasticity. While CREB phosphorylation can be induced by other kinases, activation of Erk1/2 is necessary for inducing expression of CREB target genes, which include Bcl-2, an anti-apoptotic protein. Thus, it appears that the relationship of Erk and CREB phosphor-
ylation may ultimately determine whether cells die or survive within a given region. Our data indicate there is a major increase in phosphorylation of Erk1/2 in the LF-80 group, and this flow also results in the greatest increase in CREB phosphorylation and expression. We conclude that LF-80, given the combination of the largest increases in Erk1/2 and CREB phosphorylation in the striatum, is likely to provide the best conditions for neuronal survival.

In summary, we have shown that a flow of 80 ml/(kg/min) following 30 min of DHCA, and not flows of less than 50 ml/(kg/min), abolished the large increase in extracellular dopamine observed with longer periods of DHCA. This CPB flow rate also caused largest increases in phosphorylation of CREB, Erk1/2, and Akt, and increased the Bcl-2/Bax ratio, which are favorable conditions for neuronal survival.

A limitation of our study was that measurement of phosphorylation and regulatory proteins were done only at one time during the recovery period. Future experiments are already in progress to define the time course of the changes more completely and the degree to which the observed changes, as measured at 2 h, correlate with cell survival and neuronal injury. However, on the basis of the present results, we conclude that, in a newborn animal model, for continued neuronal protection following DHCA, where CPB perfusion is required for additional time for surgical repair, the flow should be above 50 ml/(kg/min), probably between 60 and 80 ml/(kg/min).

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