Cerebral activation of mitogen-activated protein kinases after circulatory arrest and low flow cardiopulmonary bypass

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Abstract

Objectives: Mitogen-activated protein kinases (MAPK) are important intermediates in the signal transduction pathways involved in neuronal dysfunction following cerebral ischemia-reperfusion injury. One subfamily, extracellular regulated kinase 1/2, has been heavily implicated in the pathogenesis of post-ischemic neuronal damage. However, the contribution of extracellular regulated kinase 1/2 to neuronal damage following deep hypothermic circulatory arrest and low flow cardiopulmonary bypass is unknown. We attempted to correlate the extent of neuronal damage present following deep hypothermic circulatory arrest and low flow cardiopulmonary bypass with phosphorylated extracellular regulated kinase 1/2 expression in the cerebral vascular endothelium.

Methods: Piglets underwent normal flow cardiopulmonary bypass ($n = 4$), deep hypothermic circulatory arrest ($n = 6$) and low flow cardiopulmonary bypass ($n = 5$). Brains were harvested following 24 h of post-cardiopulmonary bypass recovery. Cerebral cortical watershed zones, hippocampus, basal ganglia, thalamus, cerebellum, mesencephalon, pons and medulla were evaluated using hematoxylin and eosin staining. A section of ischemic cortex was evaluated by immunohistochemistry with rabbit polyclonal antibodies against phosphorylated extracellular regulated kinase 1/2.

Results: Compared to cardiopulmonary bypass controls, the deep hypothermic circulatory arrest and low flow cardiopulmonary bypass piglets exhibited diffuse ischemic changes with overlapping severity and distribution. Significant neuronal damage occurred in the frontal watershed zones and basal ganglia of the deep hypothermic circulatory arrest group ($P < 0.05$). No detectable phosphorylated extracellular regulated kinase 1/2 immunoreactivity was found in the cardiopulmonary bypass controls; however, ERK 1/2 immunoreactivity was present in the cerebral vascular endothelium of the deep hypothermic circulatory arrest and low flow cardiopulmonary bypass groups.

Conclusions: Our results indicate that phosphorylated extracellular regulated kinase 1/2 may play a prominent role in early cerebral ischemia-reperfusion injury and endothelial dysfunction. The pharmacologic inhibition of extracellular regulated kinase 1/2 represents a new and exciting opportunity for the modulation of cerebral tolerance to low flow cardiopulmonary bypass and deep hypothermic circulatory arrest.

Keywords: Brain; Cerebral ischemia; Reperfusion; Signal transduction

1. Introduction

Deep hypothermic circulatory arrest (DHCA) and low flow cardiopulmonary bypass (LFCPB) are important elements in complex congenital heart surgery. However, the cortical ischemia-reperfusion (I/R) injury associated with these perfusion strategies may result in both subtle and profound neurologic injury [1,2]. Additionally, focal cerebral ischemia results in cortical structural changes and an increased expression of immediate early and late response genes implicated in cerebral tissue injury, edema and apoptotic death. Such alterations may play a role in ischemia-induced cellular damage or the induction of protective mechanisms limiting cortical I/R injury.

Mitogen-activated protein kinases (MAPK) are highly conserved serine/threonine protein enzyme complexes which function as important intermediates in signal transduction pathways that connect a wide variety of cell surface receptors to key regulatory membrane, cytoplasmic...
and nuclear targets [1,3,4]. The MAPK cascade consists of three sequential intracellular protein kinase activation steps initiated by the activation of MAPK kinase kinase (MAP3K), which in turn activates MAPK kinase (MAP2K) and finally MAPK [1]. Each step of the MAPK cascade may be activated and/or inhibited by multiple upstream or downstream kinases, thus exponentially increasing the complexity and diversity of the cascade. Four distinct groups of MAPKs: extracellular regulated kinase 1 and 2 (ERK1/2), Jun amino-terminal kinases (JNK1/2/3), p38 proteins (p38alpha/beta/gamma) and ERK 5, are activated by specific MAPKK’s: MAPK/ERK kinase1/2 (MEK1/2) for ERK1/2, MKK3/6 for p38, MKK4/7 (JNKK1/2) for JNK and MEK5 for ERK5 [5]. The nuclear targets of MAPK signaling pathways are transcription factors, such as transcriptional factor activator protein-1 (AP-1) and nuclear factor-kappa B (NF-kB), which regulate the expression of various pro-inflammatory genes [1,6]. Multiple overlapping stimuli may activate the four major MAPK signal transduction pathways. The p38, JNK and ERK 5 kinases are activated in response to physiologic stress, lipopolysaccharide, ultraviolet radiation, inflammatory cytokines, and hyperosmolality [1,2,8]. ERK1/2 is activated by a variety of growth factors as well as the cellular stressors previously listed and may be important in cell survival and proliferation [5]. Inhibition of the ERK1/2 pathway has been shown to decrease cerebral I/R injury secondary to focal cerebral hypoxia [9].

CPB induces a systemic inflammatory response including the activation of complement, the coagulation fibrinolytic cascade, upregulation of adhesion molecule receptors and an increase in endothelial permeability, all of which impair cerebrovascular function and result in neurological injury [10,11]. Central nervous system damage can be further exacerbated by the I/R injury associated with DHCA and LFCPB. Cerebral ischemia produces a variety of biochemical changes in cortical tissue and is associated with the early expression of activated or phosphorylated ERK1/2 (p-ERK 1/2) in the brain, suggesting that MAPKs may play an important role in the pathogenesis of cerebral I/R injury [12,13]. In this study, we examined the expression of p-ERK1/2 in the cerebral vascular endothelium using immunohistochemistry and quantified neuronal damage in the brains of piglets following normothermic CPB, DHCA and LFCPB in an effort to elucidate the contribution of the ERK 1/2 pathway to cerebral I/R injury following DHCA and LFCPB.

2. Materials and methods

2.1. Surgical preparation

Fifteen Duroc piglets with a mean body weight of 7.6 kg (range 6.0–10.3 kg) were divided into three groups: normal flow, normothermic CPB (CPB control, n = 4), DHCA (n = 6) and LFCPB (n = 5). All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources published by the National Institute of Health (NIH publication No. 85-23, revised in 1985).

Piglets were sedated with intramuscular ketamine (20 mg/kg) and acepromazine (0.03 mg/kg), transported to the laboratory, and attached to a pulse oximeter. Inhalational anesthesia with 1.5–2.5% Isoflurane was administered via a fitted, rubber-sealed mask. Once the animal reached a plane of surgical anesthesia as determined by toe-pinch response, jaw tone, heart rate, and response to stimulation, a rapid tracheostomy was performed with a 3.5–4.5 mm endotracheal tube. Piglets were ventilated with an FiO2 of 1.0, tidal volumes of 10–15 cc/kg and a rate of 18–24 respirations per minute (Modified Servo Anesthesia Ventilator 900C, Siemens Pediatric, Sweden) to achieve arterial oxygen tensions greater than 200 mmHg and carbon dioxide tensions of 35–45 mmHg. Anesthesia was maintained by continuous inhalation of 1.5–2.5% Isoflurane, except during CPB when an Isoflurane infusion was given through the bypass circuit. The right femoral artery and vein were cannulated for arterial pressure monitoring (Digital display, Gould, Valley View, OH), collection of blood samples for blood gas analyses (Blood Gas Analyzer, Synthesis 25, Instrumentation Laboratory, Lexington, MA), and administration of fluids. Prior to arterial cannulation, 300 IU/kg of heparin was administered via the femoral venous cannula. A rectal temperature probe and suprapubic-indwelling bladder catheter were inserted. Following administration of cephazolin (50 mg/kg IV) and a median sternotomy, bypass cannulas were placed into the ascending aorta (10–12 F Medtronic DLP), right atrium (16–20 F Medtronic DLP), and left atrium (10 F Medtronic DLP Vent Catheter). Temperature was maintained at 37 °C except for the period of induced hypothermia.

2.2. Conduct of CPB

The CPB circuit consisted of a roller pump (Cobe Cardiovascular Inc., Arvada, CO), cardiotomy reservoir, and a membrane oxygenator (Minimax plus® Medtronic Inc., Minneapolis, MN). The circuit was primed with fresh whole blood collected from an adult pig one day preoperatively, 2000 IU of heparin, 1 g of calcium chloride and 25 mEq of sodium bicarbonate. Hematocrit was maintained at 26–30% (mean 28%). The total prime volume was approximately 700 ml. CPB was initiated at 100–150 ml/kg per minute and adjusted to maintain a mean arterial pressure of 50–60 mmHg.

Piglets were subjected to one of the following conditions. The control group underwent normal flow (100–150 ml/kg per minute), normothermic (37 °C) CPB for 60 min without
aortic cross clamping. The DHCA and LFCPB groups were cooled to a rectal temperature of 20 °C. In the DHCA group, CPB was discontinued at the end of cooling phase and the piglet was maintained at 20 °C for the 60 min of DHCA. In the LFCPB group, the flow rate was reduced to one-quarter flow at the end of the cooling phase and maintained for 60 min. In both groups, myocardial protection was afforded by infusing high potassium blood cardioplegia (15 ml/kg, potassium concentration 20 mEq) and the application of iced saline slush during the interval of DHCA or LFCPB. Arterial blood gases were controlled by pH stat strategy. After 60 min, full flow CPB was re-initiated in both groups and the animals were re-warmed to a rectal temperature of 37 °C. Care was taken to avoid temperature differences between the perfusate and core temperature of more than 8 °C. Once normothermic the piglets were weaned from CPB, decannulated and protamine (1 mg/1000 IU heparin) was administered.

2.3. Post-operative management

The animals remained intubated and sedated and were recovered for 24 h. Physiologic data were recorded every hour. In order to avoid postoperative adverse effect, arterial blood gases, electrolytes, and hemodynamics were maintained within normal limits throughout the recovery period. Upon completion of the experiment, the cranial vault was opened and the whole brain was transected at the level of C1 and rapidly extracted in a state of stable hemodynamics with a continuous mean antegrade cerebral perfusion pressure of 50–60 mmHg.

2.4. Morphology of ischemic brain lesions

The brains were fixed in 10% neutral buffered formalin at room temperature. After 2 weeks, 11 sections were taken: (1) right frontal watershed area; (2) left frontal watershed area; (3) right parietal watershed area; (4) left parietal watershed area; (5) hippocampus; (6) basal ganglia; (7) anterior thalamus; (8) cerebellum; (9) mesencephalon; (10) pons; and (11) medulla oblongata. The tissue was processed overnight and subsequently embedded in paraffin. The brains were cut into 50–60 mmHg. Region-specific histopathologic damage, (ischemic neuronal damage, (scattered collections of ischemic neurons that collectively occupy one to three 100× magnification fields).

Ischemia was defined as nuclear pyknosis, eosinophilia and contraction of cytoplasm seen in neurons in a high-powered field (100×). Region-specific histopathologic scores represent the average among the slides taken from each region. An overall neuronal damage score was calculated for each animal by taking the sum of the eleven different region-specific neuronal damage scores.

2.5. Immunohistochemistry

After 2 weeks of fixation, a section of frontal watershed zone was examined by immunohistochemistry using polyclonal rabbit antibodies to p-ERK1/2 and total ERK1/2 from Cell Signaling (Beverly, MA). Immunohistochemistry was performed on a Ventana ES automated immunostainer (Ventana, Tucson, AZ). Optimal conditions were determined separately for each antibody. To avoid background artefactual unmasking of epitopes, no pretreatment was used. The antigen–antibody complexes were visualized using an avidin–biotin complex detection system (Vector Laboratories, Inc, Burlingame, CA) with 3,3′-diaminobenzidine as the chromogen. Sections were counterstained with hematoxylin. No significant immunoreactivity for each antibody was observed when substituting the primary antibodies with non-immune rabbit serum or by skipping the primary antibodies. The activation of ERK1/2 was assessed by the same blinded neuropathologist as either absent (no or rare expression of p-ERK1/2) or present (considerable positive expression of p-ERK1/2).

2.6. Statistical analysis

The summary statistics for the continuous and categorical variables are presented as the mean ± the standard deviation (SD) (Tables 1 and 2). The pre- and post-operative data and intra-operative CPB profiles were compared using the Wilcoxon rank sum test for comparisons between two groups and Kruskal–Wallis ANOVA of the ranks for comparisons among multiple groups. The difference in overall neuronal damage among the three groups was evaluated using the Kruskal–Wallis ANOVA of the ranks. In an effort to control for overall type I error, the Wilcoxon rank sum test for inter-group comparisons was only performed if the Kruskal–Wallis ANOVA was positive. Furthermore, region-specific neuronal damage scores were only compared between those groups with significant differences based upon the inter-group comparison of overall neuronal damage by the Wilcoxon rank sum test. Statistical evaluation of the contribution of cerebral vascular ERK 1/2 expression to neuronal damage was performed by comparing the overall neuronal damage among the ERK 1/2 positive animals (n = 4) and ERK 1/2 negative animals (n = 11) across all three groups using the Wilcoxon rank sum test. In addition, the contribution of cerebral vascular
ERK 1/2 expression to neuronal damage within the DHCA group was assessed by comparing the overall neuronal damage scores in the ERK 1/2 positive DHCA animals (n = 3) and ERK 1/2 negative DHCA animals (n = 3) using the Wilcoxon rank sum test. A P-value less than 0.05 would be considered statistically significant.

3. Results

3.1. Comparison of experimental groups

A comparison of pre-operative and post-operative variables [body weight, pH, PaCO₂, PaO₂, hematocrit, Na, K, Ca, HCO₃⁻, mean arterial blood pressure, and FiO₂] revealed no significant differences among the groups. The mean total CPB time, excluding the sixty minutes of DHCA and LFCPB, was significantly longer in the DHCA group (86.5 min) than in the CPB control group (63.3 min) secondary to rewarming in the DHCA group. There were no other significant differences among the intra-operative variables (Table 1).

3.2. Evaluation of neuronal damage

The overall neuronal damage scores among the three groups (CPB, LFCPB and DHCA) were compared using the Kruskall-Wallis test (P-value = 0.027). Subsequent inter-group comparisons (DHCA vs. CPB, LFCPB vs. CPB, and DHCA vs. CPB, respectively) were performed using the Wilcoxon rank sum test and are summarized below.

3.3. Neuronal damage in DHCA

Overall and region-specific neuronal damage scores are displayed in Table 2. The CPB group possessed almost no detectable neuronal damage (Fig. 1A). In comparison to the CPB controls, the DHCA group demonstrated a significantly higher overall neuronal damage score (15.12 vs. 1.25, P = 0.04). Subsequent region- specific comparisons between the two groups revealed significantly higher neuronal damage scores in the right and left frontal and right parietal WSZs and the basal ganglia of the DHCA group (P < 0.05) (Table 2). Representative sections from the DHCA group demonstrated extensive ischemic neuronal necrosis with pyknosis of the nuclei, collapse of the cytoplasm and eosinophilia (Fig. 1B). Although no statistically significant difference in neuronal damage was seen between the DHCA and LFCPB groups (15.12 vs. 11.2, P = 0.65), there was an overall trend toward more extensive damage in the DHCA group (Fig. 2).

3.4. Neuronal damage in LFCPB

The Wilcoxon rank sum test failed to show a significant difference between the overall neuronal damage scores of the LFCPB and CPB groups (11.2 vs. 1.25, P = 0.095). For exploratory purposes region-specific comparisons of neuronal damage between the groups were carried out and revealed no significant differences. However, there was an obvious trend toward more severe neuronal damage within the LFCPB group (Fig. 2).

3.5. Activation of ERK1/2

Immunohistochemistry staining revealed positive expression of p-ERK 1/2 in the vascular endothelium of ischemic cerebral cortex in three of the six DHCA piglets and one of the five LFCPB piglets (Fig. 3A). In contrast, no expression of p-ERK 1/2 was found in the cerebral vascular
endothelium of animals from the CPB control group (Fig. 3B). In an attempt to correlate the extent of neuronal damage with the presence of cerebral vascular p-ERK 1/2 expression we first compared the overall neuronal damage scores from the ERK 1/2 positive \((n = 4)\) animals with those of the ERK 1/2 negative \((n = 11)\) animals throughout all groups (14.50 vs. 8.55, \(P = 0.23\)). While the ERK 1/2 positive group possessed a higher overall neuronal damage score, there was no significant difference between the overall or region specific neuronal damage scores among the two groups. Next, we analyzed the contribution of cerebral vascular p-ERK 1/2 expression to neuronal damage within the DHCA group only. The overall neuronal damage score from the ERK 1/2 positive DHCA animals \((n = 3)\) was not significantly different from that of the ERK 1/2 negative DHCA animals \((n = 3)\) (13.67 vs. 16.67, \(P = 0.68\)).

4. Discussion

MAPKs are a family of serine–threonine protein kinases, involved in cell growth, differentiation, transformation and apoptosis. They are activated in response to a variety of extracellular stimuli including LPS, hypoxia and inflammatory cytokine release [5]. These protein kinases play a pivotal role in the transmission of signals from cell surface receptors to the transcriptional machinery in the nucleus [7]. Activation of MAPKs in response to ischemia...
and reperfusion has been shown to occur in the kidney [15], liver [11] and heart [16,17] in animal models and in human myocardium during CPB [18]. More importantly, MAPKs have been shown to be important intermediates in the signal transduction pathways implicated in neuronal and endothelial dysfunction secondary to cerebral I/R injury [13,19].

In vivo studies have revealed that the ERK1/2 pathway of the MAPK signal transduction cascade is activated in the brain following ischemia and plays a crucial role in ischemic brain injury [9,20]. Namura et al. demonstrated that a significant activation of ERK1/2 occurred with reperfusion following temporary bilateral carotid artery occlusion in gerbils [9]. Additionally, they showed that pretreatment with U0126 (a potent MEK 1/2 specific inhibitor), not only significantly decreased ERK 1/2 activation, but attenuated hippocampal injury following temporary bilateral carotid artery occlusion and permanent middle cerebral artery occlusion [9]. The early expression of p-ERK1/2 following cerebral ischemia and the associated hippocampal injury demonstrate that the ERK1/2 pathway plays an important role in cerebral I/R injury. Furthermore, the ability to inhibit ERK1/2 activation and prevent hippocampal injury with U0126 suggests that ERK1/2 represents a potential molecular target whose inhibition may attenuate the effects of cerebral I/R injury.

Our study is the first to investigate the activation of ERK1/2 in the brain following normothermic CPB, hypothermic LFCPB and DHCA. We sought to utilize simple, clinically applicable methods of LFCPB and DHCA to establish their effects vs. that of normothermic, normal flow CPB on neuronal damage 24 h post-operatively. Additionally, we wished to correlate the expression of p-ERK1/2 with the degree of neuronal damage present. Therefore, we began by performing an extensive assessment of neuronal damage following normal flow CPB, hypothermic LFCPB and DHCA. Each anatomic area of the brain was scored for severity of ischemic neuronal damage by a single, blinded neuropathologist. The normothermic, normal flow CPB group displayed no measureable degree of neuronal damage. The LFCPB and DHCA groups exhibited marked early ischemic changes in multiple regions with overlapping severity and distribution. The overall neuronal damage score and the region-specific neuronal damage scores for the right and left frontal WSZ, right parietal WSZ, and the basal ganglia of the DHCA group were found to be significantly higher than those of the CPB group. While the neuronal damage within the LFCPB group was not found to be significantly higher than the CPB group, there was a clear trend towards more severe damage within this group vs. the CPB group and we believe these differences would easily achieve statistical significance with a larger sample size. In summary, within the first component of our study we have successfully established models of LFCPB and DHCA capable of demonstrating the harmful effects of these complex perfusion strategies on neuronal damage vs. the more benign effect of normothermic, normal flow CPB 24 h post-operatively in a piglet model.

In the second component of our study, sections of cerebral cortex from each of these groups were subjected to analysis using immunohistochemistry. Although qualitative analysis of MAPK expression in the vascular endothelia of ischemic cerebral cortex using immunohistochemistry did not allow us to discriminate between the absolute amount of p-ERK 1/2 present in each group, the DHCA group clearly showed a higher percentage of p-ERK1/2 expression than the LFCPB or normothermic CPB groups. In an attempt to more clearly define the relationship of cerebral vascular p-ERK 1/2 expression to neuronal damage within our study we performed two separate statistical comparisons. Initially, to ascertain whether cerebral vascular p-ERK 1/2 expression leads to more severe neuronal damage within our model regardless of the perfusion strategy employed we compared neuronal damage in ERK 1/2 positive animals (n = 4) vs. ERK 1/2 negative animals (n = 11) across all three groups. Although the ERK 1/2 positive animals possessed higher neuronal damage scores there were no significant differences in the overall and region-specific scores. In our second comparison we attempted to isolate the effect of cerebral vascular ERK 1/2 expression even further by assessing its effect within the DHCA group only. Among the DHCA group there were three ERK 1/2 positive animals and three ERK 1/2 negative animals. A comparison of the overall and region-specific scores revealed no significant differences. While we were unable to find a direct statistical correlation between cerebral vascular p-ERK 1/2 expression and neuronal damage within our model, our results represent the first report of the activation of the ERK1/2 pathway by cerebral I/R injury after LFCPB and DHCA. Additionally, these results provide the first evidence suggesting that the ERK1/2 pathway may be involved in the pathogenesis of the post-ischemic neuronal injury and cerebral endothelial dysfunction seen following LFCPB and DHCA.

An accurate analysis of our results requires an understanding of three important methodological aspects of our model of LFCPB and DHCA. Initially, the small number of animals in our study made arriving at conclusions with statistical certainty difficult. While we would have liked to accumulate a larger sample size within each group, we were constrained by the complex nature of the individual experiments, the large number of personnel involved and the number of specimens that our neuropathologist could accommodate. Additionally, in this preliminary study we wished only to discover whether our model would reliably detect the differences in neuronal damage between the perfusion strategies employed and whether we could accurately detect cerebral vascular p-ERK 1/2 expression. Now that we have verified the ability of our model to accomplish these goals future studies with larger number of animals are justified. Secondly, the timing of cerebral extraction was a critical element of the study. Previous work has demonstrated that neuronal death induced by
cerebral I/R injury requires a minimum of 24 h before an accurate neuropathological assessment of the injured tissue can be made [14]. As a result, we choose to extract brains at this time to ensure that an accurate assessment of neuronal death could be performed. However, prior studies in small animal models have reported that maximal ERK1/2 activation following cerebral I/R injury occurs 2 h after reperfusion [13]. Therefore, while the ideal time point to extract brains for immunohistochemistry would have been prior to 24 h, we decided to remove brains at this time so that we could accurately assess the ability of our model to distinguish between the severity of the cerebral insult caused by normothermic CPB, LFCPB and DHCA. The delayed extraction time employed coupled with the qualitative method of ERK 1/2 expression analysis used (immunohistochemistry) could potentially have resulted in several false negatives that might have been picked up if the brains had been extracted at an earlier time point. Finally, although the perfusion-fixation technique of cerebral extraction was not used, our method of rapid extraction (<5 min) under a state of stable hemodynamics with continuous antegrade cerebral perfusion was found to be reliable and safe. Analysis of the brains from the CPB control group revealed no significant neuronal injury or expression of p-ERK1/2, which demonstrates that our extraction technique has not introduced experimental error into our model.

Our ability to establish models capable of differentially assessing the degree of neuronal damage and cerebral vascular p-ERK1/2 expression present following LFCPB and DHCA and the recognition of the ERK 1/2 pathway as a potential molecular target for attenuating the cerebral I/R injury associated with these perfusion strategies has opened up avenues for future research. Initially, the correlation that we have proposed between the presence of cerebral vascular p-ERK 1/2 expression and the extent of ischemic neuronal damage seen 24 h following normothermic CPB, LFCPB and DHCA should be verified by specimens taken 2 h post-bypass at the peak of ERK 1/2 activation [13]. Studies aimed at qualitatively and quantitatively assessing the degree of cerebral vascular p-ERK 1/2 expression at 2 h would provide more concrete evidence of the involvement of the ERK 1/2 pathway in the development of neuronal damage after LFCPB and DHCA. Additionally, the use of selective inhibitors of the ERK 1/2 pathway, such as U0126, in clinically relevant models of LFCPB and DHCA would definitively establish the role of the ERK 1/2 pathway in the development of neuronal damage following LFCPB and DHCA. Such studies are exciting and important as they represent a novel method for the pharmacologic modulation of cerebral tolerance to LFCPB and DHCA and might lead to the development of a comprehensive pharmacologic inhibitor capable of reducing cellular injury and neurologic morbidity following LFCPB and DHCA.

References

