Long-term assessment of NFκB expression in the brain and neurologic outcome following deep hypothermic circulatory arrest in rats

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Abstract

Objectives: Inflammatory response is discussed as a contributor to neurologic deficits following cardiac surgery using deep hypothermic circulatory arrest (DHCA). Nuclear Factor Kappa B (NFκB) presents a central transcription factor whose expression pattern and subsequent role very much depend on the type and manner of cerebral injury. This study was designed to assess the time course of cerebral NFκB expression in relation to neurologic performance over 28 days following 45min of DHCA in rats.

Methods: With Institutional Review Board approval, 30 rats were subjected to cardiopulmonary bypass (CPB) with 45min of DHCA (rectal temperature 15-18°C) and randomly assigned to 1, 3, 7, 14 and 28 days of postoperative survival. Untreated animals served as control (n=6). Cerebral NFκB expression was analyzed immunohistochemically, cyclooxygenase-2 (COX-2) and inhibitor of kappa B-alpha (IκBα) using Western Blot and the number of eosinophilic neurons with hematoxylin and eosin (HE) staining. Neurologic outcome was assessed pre- and postoperatively.

Results: Neuronal expression of NFκB in the hippocampus peaked at day one, remaining elevated in the motor cortex until day 28. Rats showed neurologic deficits on postoperative day one. Cerebral COX-2 was increased during the first postoperative week and IκBα peaked on day 14. Histologic damage in the motor cortex and hippocampus persisted until day 28. No systemic inflammation was detectable postoperatively.

Conclusions: Postoperative day one presents with the highest NFκB-expression in the ischemia-sensitive hippocampus, accompanied by neurologic dysfunction and histologic damage following 45min of DHCA in rats.

Keywords
cerebral inflammation; deep hypothermic circulatory arrest; cardiopulmonary bypass; neurologic deficits; cerebral ischemia; Nuclear Factor Kappa B

Introduction

Inflammatory reaction is discussed as one of the obvious suspects contributing to neurologic injuries following cardiac surgery utilizing deep hypothermic circulatory arrest (DHCA).1-3 This reaction is mainly mediated by nuclear factor kappa (NFκB), one of the most important transcription factors activating many genes encoding cytokines, adhesion molecules and inducible enzymes such as cyclooxygenase-2 (COX-2).4 This influential role within the inflammatory reaction marks NFκB as a potential therapeutic target. This idea is supported by non-clinical studies that found an inhibition of NFκB to be neuroprotective and even suggested the down-regulation of NFκB activity as one of the underlying mechanisms of the anti-inflammatory effect of hypothermia.5,6 However, within the setting of DHCA, no description of the time course of cerebral NFκB expression exists to date. Neither was the time point of maximum NFκB availability which would present the suitable time point to investigate the effects of anti-inflammatory drugs or strategies yet determined.

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investigate cerebral NFκB expression, we used a rodent recovery model of cardiopulmonary bypass (CPB) and DHCA, with free access to brain tissue. The same setup will allow future studies additionally to elucidate the effect of such anti-inflammatory interventions on neurologic outcome, e.g. the maximum deficit or the persistence of dysfunction. Therefore, the current experiment was designed to investigate the time course of cerebral NFκB-expression and neurologic performance over 28 days following 45 minutes of DHCA in rats. As secondary endpoints, cerebral inhibitor of kappaB (IkBα) and COX-2 expression, systemic inflammatory parameters interleukin-6 (IL-6) and tumor necrosis factor (TNFα), as well as histologic outcome, were determined.

Methods

Surgical Preparation

The following experimental protocols were approved by the institutional animal care committee. Male Sprague Dawley rats from Charles River Laboratories (Sulzfeld, Germany) were housed under standard laboratory conditions (12 h light / 12 h dark, lights on at 0:30 am, 22°C, 60% humidity and free access to water and standard rat chow) two weeks prior to the experiments for acclimatization to the changed day-night rhythm. Thirty-six animals were randomly assigned to one of six groups, with 1, 3, 7, 14 or 28 days of survival after 45 minutes of DHCA or untreated control.

The animals (330 – 380g) were anesthetized, intubated and mechanically ventilated at a FiO2 = 40% with aPaCO2 at 32 – 40 mmHg. All instruments were disinfected and the catheters gas sterilized before use. The surgical sites were infiltrated with 2% xylocaine and surgery was carried out in an aseptic manner. During surgical preparation, anesthesia was maintained with 2 – 2.5% isoflurane and boluses of 5µg fentanyl. One hundred and fifty international units of heparin were administered to avoid clotting. During DHCA, anesthesia was discontinued. During rewarming, the MAP was kept above 50 mmHg, by norepinephrin, as soon as a rectal temperature of 30°C and a blood flow of 150 ml/min/kg were achieved. Arterial blood gas values were controlled using the pH-stat strategy (PaCO2 of 31 – 40 mmHg).

Following decannulation, rats remained anesthetized with 1.0 – 1.5% isoflurane, intubated and ventilated for one hour (rectal temperature of 36.5°C). Sodium bicarbonate was administered to treat acidosis, and calcium was injected to prevent a drop of ionized calcium concentration below 1 mmol/l, if necessary. The heparin-induced anticoagulation was allowed to dissipate spontaneously without supplemental administration of protamine. When the animals resumed spontaneous ventilation, the tracheas were extubated and the rats put for continuous observation into a transparent, oxygen-enriched box for 12 hours, with free access to water and food. Animals were returned to their cages on the first postoperative day and housed in their familiar groups. On the respective day of sacrifice, rats were anesthetized, brains removed and immediately frozen in methylbutane on dry ice in a tissue-freezing medium.
**Immunohistochemistry**

To detect NFκB-positive neurons, an immunohistochemical double staining was performed. NFκB was labeled using an antibody raised against the p65 subunit (NFκB phospho S276 antibody rabbit polyclonal to NFκB p65, BIOZOL, Eching, Germany) and visualised using diamino-benzidine (DAB, DakoCytomation, Denmark A/S, Glostrup, Denmark). Neuronal structural protein N (NeuN) was labeled using mouse anti-neuronal nuclei (NeuN) Monoclonal antibody (CHEMICON International Inc., Temecula, California, USA). NFκB-positive neurons were counted in the motor cortex and hippocampus within five high-magnification (x 400) fields per region.

**Neurologic testing**

Preoperatively and on the day of sacrifice, the animals underwent standardized functional neurological testing as previously described. Briefly, motor function was scored using the performance on a beam, performance on a rotating grid, time hanging on a rope (prehensile traction), movement symmetry and gait. Consequently, each of the animals tested received score values between 0 and 18 with 0-1 representing normal neurologic function.

**Western Blotting**

With Western Blotting, the amount of cerebral COX-2 and IκBα protein was determined. Primary antibodies in immunoblotting were polyclonal rabbit anti-human COX-2 (Cayman Chemical, Ann Arbor, USA) and monoclonal mouse anti-human phosphor-IκBα (Ser32/36) (Cell Signaling Technology Inc., Beverly, USA) and monoclonal mouse anti-human beta-actin (β-actin) (Sigma, Saint Louis, USA).

Protein signals for COX-2 and phosphorylated IκBα were normalized for beta-actin (β-actin) signals, with β-actin developed on the original blottings (Kodak 1D Image Analysis Software, Eastman Kodak Company, Rochester, USA).

**Histologic Examination**

To validate neuronal damage, frozen brains were cut in 10 µm sections and stained with HE. All slides were scored by an investigator blinded to experimental groups. Two brain regions were chosen for evaluation of neuronal cell damage: motor cortex and hippocampus. Five high-magnification (x 400) fields per region were examined, counting the number of eosinophilic neurons within.

**Enzyme-linked immunosorbent assay, ELISA**

Serum samples of all groups, harvested at the day of sacrifice, were analyzed for IL-6 and TNFα levels, according to the manufacturers instructions, using commercially available ELISAKits, with the minimum detectable level at < 5pg/mL (Qantikine®, R&D Systems, Wiesbaden-Nordenstadt, Germany).

**Statistics and Data management**

Physiologic data were analyzed using general linear models, with the between-groups factor post-DHCA survival time, the within-group factor time2, and their interaction term (time2 × post-DHCA survival time). Effects of time levels were analyzed quadratically, focusing on biphasic changes of all variables during the observation period. Post hoc values at certain times were analyzed using factorial ANOVA followed by Bonferroni t-tests.

Cerebral inflammatory parameters, and neurologic and histologic outcomes were analyzed using the Kruskal-Wallis test combined with post hoc Mann-Whitney U test to compare groups.

Statistical analyzes were performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

**Results**

Four animals were excluded from further data analysis due to either insufficient venous return (three animals) or due to problems with the inflow cannula during CPB (one rat in the day 3 group). These rats were replaced to keep sample size equal.

Histologic Examination showed more NFκB-positive neurons in the motor cortex over 28 days after DHCA, without a defined peak, compared to the untreated controls. In the hippocampus, the number of NFκB-positive neurons peaked on postoperative day one (Figure 1A and 1B, p < 0.05).

Neurologic function was impaired at postoperative day one and returned to baseline at day three (Figure 2, p < 0.05).

Western Blot analysis for cerebral inflammatory marker COX-2 showed elevated levels during the first week after DHCA (Figure 3A, p < 0.05). IκBα protein expression in the brain peaked 14 days after DHCA (Figure 3B, p < 0.05).
Histologic outcome in the motor cortex and hippocampus was worse over 28 days following DHCA when compared to the untreated controls (Figure 4A and 4B, p<0.05).

Systemic IL-6 and TNFα levels were below the detectable limit in all five groups (data not shown).

**Discussion**

Both NFκB expression in the hippocampus and neurologic dysfunction peaked at postoperative day one following DHCA in the rats. NFκB expression in the hippocampus was within normal ranges at three days after DHCA, but still elevated in the motor cortex until postoperative day 28. Systemic IL-6 and TNFα were already not detectable at one day after DHCA. Neurologic function normalized within three days.

Inflammatory reaction might present an important therapeutic target to avoid or treat neurologic dysfunction following DHCA. Using the recently established model of DHCA in the rat, we have the option to trace cerebral inflammatory reaction as well as assessing neurologic outcome and to screen the effect of anti-inflammatory drugs and strategies before their use in humans. The major advantage of such a preclinical model is the availability of brain tissue to investigate inflammation directly at the target.

Several studies using cerebral injury models have provided important insights into inflammatory pathways in the brain; however, these results cannot be entirely transferred to the setting of CPB and DHCA. To characterize cerebral inflammation under these circumstances, we chose to investigate cerebral expression of NFκB along with IκBα and COX-2 for several reasons: NFκB has

| Table 1. Physiologic data during the operative procedure in all groups of 45 min of deep hypothermic circulatory arrest (DHCA). |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Survival [days] | pre-CPB | pre-DHCA | post-DHCA | CPB-end | post-CPB | time² x rewarming | time² |
| mean arterial pressure [mmHg] | 1 | 76±2 | 40±1 | 59±4 | 1) | 78±6 | n.s. | p < 0.001 |
| 3 | 80±2 | 38±2 | 57±6 | 8±8 | 1 | 78±6 | n.s. | p < 0.001 |
| 7 | 71±2 | 36±3 | 48±2 | 75±6 | 7 | 74±4 |
| 14 | 73±2 | 36±1 | 46±3 | 74±4 | 14 | 76±2 |
| 28 | 77±1 | 38±2 | 48±4 | 76±2 |
| hemoglobin [g/dl] | 1 | 14.3±0.5 | 7.2±0.4 | 6.8±0.4 | 7.3±0.4 | 10.8±0.2 | n.s. | p < 0.001 |
| 3 | 13.6±0.2 | 6.8±0.1 | 6.3±0.1 | 6.8±0.3 | 10.3±0.2 |
| 7 | 13.9±0.2 | 7.0±0.1 | 6.8±0.2 | 7.3±0.2 | 10.6±0.3 |
| 14 | 14.3±0.2 | 7.4±0.2 | 7.1±0.1 | 6.9±0.3 | 10.7±0.2 |
| 28 | 14.0±0.2 | 7.1±0.3 | 7.0±0.3 | 7.2±0.4 | 10.3±0.3 |
| PaO₂ [mmHg] | 1 | 178±20 | 474±16 | 413±14 | 375±20 | 156±9 | n.s. | p < 0.001 |
| 3 | 186±11 | 465±18 | 369±25 | 363±9 | 150±5 |
| 7 | 164±12 | 486±11 | 358±32 | 400±24 | 151±10 |
| 14 | 173±15 | 481±17 | 413±16 | 394±17 | 160±8 |
| 28 | 176±11 | 506±29 | 433±12 | 371±18 | 148±11 |
| PaCO₃ [mmHg] | 1 | 36±1 | 35±1 | 31±1 | 39±1 | 37±1 | n.s. | p < 0.001 |
| 3 | 36±2 | 34±1 | 31±1 | 39±1 | 38±1 |
| 7 | 36±1 | 35±1 | 30±1 | 38±1 | 35±1 |
| 14 | 33±1 | 36±1 | 31±1 | 39±1 | 36±2 |
| 28 | 36±2 | 36±2 | 32±1 | 41±1 | 37±2 |
| BE [mmol/l] | 1 | 1.9±0.4 | -1.5±0.5 | -5.2±0.4 | n.s. | p < 0.001 |
| 3 | 2.4±0.7 | -1.2±0.5 | -5.8±0.5 | 2 | 2 |
| 7 | 2.4±0.7 | -1.2±0.4 | -5.4±0.7 | 7 |
| 14 | 2.1±0.4 | -1.2±0.2 | -5.1±0.5 |
| 28 | 2.7±0.6 | -0.3±0.2 | -5.0±0.5 |
| Glucose [mg/dl] | 1 | 110±7 | 159±12 | 25±21 | 288±28 | 168±19 | n.s. | p < 0.001 |
| 3 | 125±7 | 168±12 | 253±13 | 350±30 | 195±13 |
| 7 | 125±4 | 165±19 | 273±26 | 296±35 | 181±22 |
| 14 | 118±9 | 162±11 | 257±20 | 339±30 | 202±15 |
| 28 | 118±8 | 155±14 | 258±17 | 315±27 | 160±5 |

Table 1: Variables were obtained prior to cardiopulmonary bypass (pre-CPB), prior to DHCA (pre-DHCA), 5 min before cessation of CPB (CPB end), and 1 h after CPB (post-CPB). Data are presented as mean ± standard error. Some values were controlled within defined ranges at certain times and, therefore, they are not presented and statistically analyzed. (1) Mean arterial pressure was controlled above 50 mmHg by repeated 1-2 µg norepinephrine boli with a total of 26 ± 5 µg. (2) BE was controlled between 3 and 3 mmol/l by total doses NaHCO₃ of 75 ± 23 mmol/l. Respective p-values indicate statistically significant changes during the observation period within each group (time²) and between groups related to the time course (time² x group).
been identified as an important nuclear transcription factor up-regulated during cerebral ischemia and responsible for activating further pro-inflammatory parameters such as COX-2.9,10 The neuroprotective effect of hypothermia on cerebral injury is discussed to be related, at least in part, to IκBα inhibition;6 the activity of IκBα is increased in the brain of aged mice11 whereas IκBα activation is also crucial in the development of brain tolerance.12 Since IκBα activation regulates the expression of antiapoptotic, proapoptotic and proinflammatory genes,13 this important transcription factor presents a possible future therapeutic target. As it seems to function in both protective and damaging pathways, however, its expression pattern within the cardiac surgery setting needs to be carefully investigated first.

IKBα was investigated because of its regulatory influence on NFκB via two different mechanisms:10 it binds to NFκB directly and, thus, sequesters it in the cytosol of resting cells14 or it alleviates its transport out of the nucleus.15 COX-2, as the third component, presents an inducible enzyme regulated by NFκB9 that participates in inflammation-mediated cytotoxicity and plays physiological and pathological roles in mediating inflammatory responses and interactions between neurons, glia and endothelial cells within the central nervous system.16,10 Mediated through immediate early genes induced by neuronal excitation and an increase in intracellular calcium, COX-2 expression, in the early stages, adds to the ischemic damage.17 The relevance of COX-2 is further corroborated by studies that have shown a selective inhibition of COX-2 to lead to a reduction of infarct volume after focal cerebral ischemia, and also to a delay in hippocampal injury following global ischemia.16,18

Histologic outcome was assessed over the time period as a validated marker of cerebral damage. NFκB expression peaked at postoperative day one in the hippocampus and persisted to be up-regulated until postoperative day 28 in the motor cortex. This is in accordance with a study of intracerebral hemorrhage in rats, showing a robust and prolonged NFκB activation for at least seven days.10 The hippocampus has been shown to be the most ischemia-sensitive area in the brain.19 Therefore, this area is well studied in models of cerebral ischemia and of the most interest in the current study. COX-2 protein

Figure 1. Number of NFκB positive neurons in motor cortex and hippocampus counted in five high-magnification fields (x400) 1, 3, 7, 14 and 28 days following DHCA.

In the motor cortex, the number of NFκB positive neurons was elevated until day 28 compared to untreated controls (1A). In the hippocampus, the number of NFκB positive neurons is highest on postoperative day one (1B). Data are presented as box and whisker plot, $p<0.05$ vs. day 7, 14, 28, $p<0.05$ vs. all groups. The dashed lines mark the 5 and 95% confidence intervals of untreated control animals.

Figure 2. Neurologic outcome on postoperative days 1, 3, 7, 14 and 28 following 45min of DHCA.

Neurologic function was impaired on postoperative day one compared to the preoperative baseline (unchanged in all groups at zero, not displayed). Data are presented as box and whisker plots. $*=p<0.05$ vs. baseline.
expression is up-regulated during the first postoperative week, with levels dropping back to normal by day 14, demonstrating a) that an upregulation of NFκB clearly modulates COX-2 expression and, therefore, the subsequent inflammatory cascade, and b) underlining the role of COX-2 as an enzyme of the acute-phase response to cerebral damage. Up-regulation of cerebral COX-2 mRNA expression together with elevated systemic IL-6 levels four hours after CPB, previously have been shown by Hindman et al. However, neither cerebral COX-2 protein nor a later time point was investigated in this study. IκBα protein expression was low on postoperative day one due to a higher turnover rate, resulting in higher levels of NFκB and lower levels of IκBα. Over time, a reduced NFκB activity led to a decreased turnover for IκBα and, consequently, to a peak of IκBα at postoperative day 14. Interestingly, levels of systemic IL-6 and TNFα, assessed with rat specific ELISA, were below detection limit already at one day after DHCA (data not shown). These results show that, despite a link between systemic and cerebral inflammatory reaction in the early postoperative phase following CPB, the up-regulation of cerebral inflammatory parameters NFκB, IκBα and COX-2 in our study is clearly independent of an ongoing systemic inflammatory reaction. Our findings underpin the role of the brain itself as an active participant in the inflammatory reaction to DHCA.

Surprisingly, neurologic function was just transiently impaired and returned to baseline already on postoperative day three. This might be due to the fact that the rats were young and healthy, lacking any of the co-morbidities typically seen in the cardiac surgery population.

**Figure 3.** Cerebral inflammatory parameters COX-2 and IκBα assessed by Western Blot 1, 3, 7, 14 and 28 days following DHCA. The ratio of cerebral COX-2 to Beta-Actin is increased during the first postoperative week compared to untreated controls (3A). The ratio of cerebral IκBα to Beta-Actin peaks on postoperative day 14 (3B). Data are presented as box and whisker plots. * = p<0.05 vs. untreated control § = p<0.05 vs. day 14. The dashed lines mark the 5 and 95% confidence intervals of untreated control animals.

**Figure 4.** Number of eosinophilic neurons in the motor cortex and the hippocampus counted in five high-magnification fields (x400) 1, 3, 7, 14 and 28 days following DHCA. In the motor cortex (4A) as well as in the hippocampus (4B), the number of eosinophilic neurons was elevated until day 28 compared to untreated controls. Data are presented as box and whisker plots. The dashed line marks the 95% confidence interval of untreated control animals. * = p<0.05 vs. untreated control animals.
However, the cerebral injury was severe enough to cause a significant histologic damage (Figure 4) visible already on postoperative day one and persisting until day 28.

Taking all these results together, postoperative day one presents with the highest NFκB expression, especially in the ischemia-sensitive region of the hippocampus, accompanied by neurologic dysfunction and histologic damage. Whether the elevated NFκB expression in this particular setting is of a neuro-destructive nature and whether the outcome on the first postoperative day is a suitable marker for long-term outcome cannot be answered with the current study.

To deduce clinical implications, caution is necessary, as contributors to adverse cerebral outcome in the clinical setting are missing in this preclinical scenario and some important limitations remain: to allow the long-term survival of the animals, the surgical trauma was kept at a minimum, with no median sternotomy, thoracotomy, direct cardiac cannulation or cardiac surgery being performed. Therefore, the marked inflammatory response generated by the surgical trauma or by reinfusion of blood from cardiotomy suction into the CPB circuit and its effect on cerebral inflammation could not be studied with this particular model. Based on the current study, we cannot tell if the cerebral inflammation and neurologic dysfunction is caused by the DHCA or by the deleterious effects of CPB. However, previous studies using this rodent model showed no consistent or reproducible cerebral injury following normothermic CPB alone, suggesting DHCA, the reperfusion injury or rewarming was the culprit in the current study.

In summary, postoperative day one presents with the highest NFκB expression, especially in the ischemia-sensitive region of the hippocampus, with the cerebral inflammatory reaction continuing without systemic analogue, accompanied by neurologic dysfunction and histologic damage following 45 minutes of DHCA in rats. Therefore, this day appears to be the best time window to assess the impact of potential neuroprotective strategies on cerebral NFκB expression and neurologic outcome in subsequent studies.

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