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Regional TNF α mapping in the brain reveals the striatum as a neuroinflammatory target after ventricular fibrillation cardiac arrest in rats★

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Abstract

Cardiac arrest (CA) triggers neuroinflammation that could play a role in a delayed neuronal death. In our previously established rat model of ventricular fibrillation (VF) CA characterized by extensive neuronal death, we tested the hypothesis that individual brain regions have specific neuroinflammatory responses, as reflected by regional brain tissue levels of tumor necrosis factor (TNF) α and other cytokines. In a prospective study, rats were randomized to 6 min (CA6), 8 min (CA8) or 10 min (CA10) of VF CA, or sham group. Cortex, striatum, hippocampus and cerebellum were evaluated for TNF α and interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-6, IL-10, IL-12 and interferon gamma at 3 h, 6 h or 14 d after CA by ELISA and Luminex.

Immunohistochemistry was used to determine the cell source of TNF α . CA resulted in a selective TNF α response with significant regional and temporal differences. At 3 h after CA, TNF α -levels increased in all regions depending on the duration of the insult. The most pronounced increase was observed in striatum that showed 20-fold increase in CA10 vs. sham, and 3-fold increase vs. CA6 or CA8 group, respectively ($p < 0.01$). TNF α levels in striatum decreased between 3 h and 6 h, but increased in other regions between 3 h and 14 d. TNF α levels remained twofold higher in CA6 vs.

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Conflict of interest statement

All other authors report no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.resuscitation.2014.01.033>.

shams across brain regions at 14 d ($p < 0.01$). In contrast to pronounced TNF α response, other cytokines showed only a minimal increase in CA6 and CA8 groups vs. sham in all brain regions with the exception that IL-1 β increased twofold in cerebellum and striatum ($p < 0.01$). TNF α colocalized with neurons. In conclusion, CA produced a duration-dependent acute TNF α response, with dramatic increase in the striatum where TNF α colocalized with neurons. Increased TNF α levels persist for at least two weeks. This TNF α surge contrasts the lack of an acute increase in other cytokines in brain after CA. Given that striatum is a selectively vulnerable brain region, our data suggest possible role of neuronal TNF α in striatum after CA and identify therapeutic targets for future experiments.

Keywords

Cardiac arrest; Resuscitation; Cytokines; Neuroinflammation; TNF α

1. Introduction

Overall mortality after cardiac arrest (CA) remains high, despite improvements in resuscitation and critical care.¹ Neurocognitive disabilities are frequently observed in survivors from CA. Histological damage including neuronal cell loss was characterized in multiple experimental global ischemia–reperfusion insults. Several selectively vulnerable regions have been identified, namely hippocampus, cerebellar Purkinje neurons, lamina IV cortical layer and striatum.^{2,3} Both the systemic inflammatory response to CA and the local central nervous system (CNS) specific response may contribute to the ultimate histological damage.

CA and resuscitation result in a systemic inflammatory response that has been compared to sepsis.⁴ Cytokines produced by multiple cells including resident CNS cells are considered a hallmark of inflammatory reaction to an insult. Systemic and CNS levels of various cytokines including tumor necrosis factor (TNF) α , interleukin (IL)-1, IL-6, IL-8, IL-10, and others are increased after CA.^{4–7}

We have recently established a rat model of ventricular fibrillation (VF) CA and characterized extensive neuronal death with characteristic regional pattern.⁸ We hypothesized that neuroinflammation after CA might also show a regional pattern and be related to the duration of the insult. Thus, we used ELISA and Luminex methods to explore regional brain tissue levels of multiple cytokines after 6, 8 and 10 min of VF CA in cortex, hippocampus, striatum and cerebellum at 3 and 6 h after restoration of spontaneous circulation (ROSC) to study the early inflammatory response to ischemia and reperfusion. We furthermore assessed TNF α levels in rats that survived for 2 weeks. We have used immunocytochemistry to identify the cell sources of TNF α .

2. Methods

The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. A total of 45 adult male Sprague–Dawley rats (350–400 g; Hilltop Lab Animals, Scottdale, PA) were used as described in detail previously. In brief,

isoflurane-anesthetized rats were intubated and mechanically ventilated, while maintaining normothermia. After cannulations, rats were subjected to 6 min (CA6), 8 min (CA8) or 10 min (CA10) of untreated VF CA or sham, and 3 or 6 h of survival ($n = 6/\text{group}$). Based on initial results, we also studied an additional group of 6 min CA and 14 d outcome ($n = 3$). We also performed additional experiments to identify the cellular source of TNF α in the CA10 group.

VF CA was induced by a 12 V/50 Hz alternating current. Epinephrine 20 $\mu\text{g}/\text{kg}$ (40 $\mu\text{g}/\text{kg}$ in CA10 group) and bicarbonate 1 mmol/kg were given at start of CPR. Additional epinephrine 10 $\mu\text{g}/\text{kg}$ (20 $\mu\text{g}/\text{kg}$ in CA10 group) was given 75 s after the start of CPR. Resuscitation was performed with mechanical ventilation (FiO_2 1.0) and manual chest compressions (200/min). At 2 min of CPR, rats were defibrillated (5 J, monophasic); defibrillation attempts were repeated every 30 s at 10 J.

2.1. Sham groups

Identical surgical procedures as in the CA rats were performed in shams, but CA was not induced ($n = 6$). Intensive care was the same as in the CA groups.

2.2. Post-resuscitation care

Rats were weaned from the ventilator at 20 min after ROSC and extubated 60 min after ROSC. After decannulation, rats were placed in cages and kept normothermic. Three or 6 h after ROSC, or 14 d in long-term outcome experiments, rats were sacrificed with an isoflurane overdose and perfused with normal saline. Brains were removed and samples from cortex, hippocampus, striatum and cerebellum were snap-frozen.

2.3. Cytokine measurements

Tissues were then processed for TNF α measurement by enzyme-linked immunosorbent assay (ELISA TNF α kit, R&D Systems) according to manufacturer's instructions. A Luminex-200 multiplex analyzer (Luminex, Austin, TX) was used to simultaneously measure 10 different cytokines using a rat-specific kit (Millipore, Billerica, MA). All values were corrected for protein concentration.

2.4. Immunocytochemistry

Samples were mounted in OTC media and 10 μm sections were cut and mounted on Superfrost slides (Fisher). Tissue sections were fixed for 10 min in acetone, rinsed with 1 $\times$ TBST and blocked in 3% normal horse serum for 30 min. Slides were washed in 1 $\times$ TBST and incubated overnight in primary antibody (TNF- α 1:40, R&D Systems, NeuN 1:100, Millipore). Sections were then rinsed 3 times with 1 $\times$ TBST and incubated for 1 h in secondary antibody (donkey anti-goat Alexa 488 for TNF- α , horse anti-rabbit Alex 594 for NeuN, both at 1:400 dilution). Sections were rinsed, coverslipped with aqueous mounting media and visualized for co-localization. For Iba-1 staining, the adjacent 10 μm sections were used, but fixed in 4% paraformaldehyde for 10 min. The same procedure was used above with a 1:250 dilution for Iba-1 (Wako Chemicals) and a goat anti-rabbit Alexa 594 as the secondary antibody.

2.5. Statistical analysis

Physiologic and biochemical data from the resuscitation phase were pooled from all groups based on CA duration. Data were tested for normality using the Kolmogorov–Smirnov test. Cytokine levels are presented as median and the first and the third quartile. Group comparisons were made with the Kruskal–Wallis test or the Mann–Whitney test as appropriate. A Bland–Altman plot was constructed to define the association between TNF α levels obtained by ELISA vs. Luminex.⁹ Spearman's rank correlation coefficient was used as a measure of linear relationship between two sets of data. Our significance level was a two-sided p -value of <0.05 . All calculations were performed with PASW Statistics 18.0 (SPSS Inc., Chicago, USA).

3. Results

There were no significant group differences at baseline. As expected, CA resulted in significant duration-dependent derangements in multiple biochemical parameters (Table 1 – Supplemental File).

Rats in the CA10 group could not be weaned from the ventilator. Therefore, the cytokine profiles could only be studied at 3 h timepoint.

Cytokine levels after 3 h and 6 h of observation time are presented in Figs. 1–3. Significant differences between groups were detected for TNF α and IL-1 β and these results are presented in detail below. IL-1 α levels were too low to be detectable (data not shown). IL-4 levels were also low (data not shown).

3.1. TNF α

Three hours after CA, group differences for TNF α were detected in the striatum ($p = 0.003$ for Luminex, $p = 0.008$ for ELISA). TNF α was higher in the CA6 vs. sham ($p = 0.002$ for Luminex, $p = 0.015$ for ELISA), and in the CA8 vs. sham ($p = 0.002$ for Luminex, $p = 0.002$ for ELISA). There were no differences between CA6 vs. CA8 group, respectively. The increase was greatest in the CA10 group (CA10 vs. CA8, $p = 0.009$) (Fig. 1).

In the cortex, group differences in TNF α could be demonstrated at 3 h after CA with Luminex ($p = 0.036$). While absolute values were markedly lower than in striatum, TNF α was increased in the CA6 group vs. sham ($p = 0.026$) and in the CA8 group vs. sham ($p = 0.041$).

In the cerebellum, group differences were only detected with ELISA. TNF α was significantly higher in the CA10 vs. CA6 and sham ($p = 0.01$).

Six hours after CA, TNF α was still significantly increased in the striatum by ELISA ($p = 0.047$) but not by Luminex method ($p = 0.053$). The difference was statistically significant only for CA6 vs. sham ($p = 0.04$) but not for CA8 vs. sham ($p = \text{ns}$). There were no differences between CA6 and CA8 groups ($p = \text{ns}$).

14 d after CA, TNF α levels were increased in CA6 vs. sham group across all brain regions: cerebellum ($p = 0.009$), hippocampus ($p = 0.004$), striatum ($p = 0.018$) and cortex ($p = 0.004$).

TNF α levels were compared in the CA6 group at 3 h, 6 h and 14 d after CA to determine a time course of TNF α production. No significant group differences were detected in any brain region (Fig. 2).

3.2. IL-1 β

A group difference of IL-1 β was detected in the striatum 3 h after CA ($p = 0.046$), IL-1 β was increased in the CA6 vs. sham ($p = 0.015$), but not in the CA8 group. This increase in IL-1 β in the striatum was still present at 6 h after CA ($p = 0.019$). IL-1 β was significantly increased in the CA6 group ($p = 0.009$) and in the CA8 group ($p = 0.026$) vs. sham at the 6 h timepoint. A group difference for IL-1 β was also present in the cerebellum 6 h after CA ($p = 0.031$). IL-1 β was increased in the CA6 group vs. sham ($p = 0.009$, $p = \text{ns}$ for CA8 vs. sham) (Fig. 3).

3.3. Cytokine response

Surprisingly, other cytokines, namely IL-2, IL-4, IL-6, IL-10, IL-12 and IFN- γ did not show statistically significant regional differences or group differences at 3 or 6 h after CA (Fig. 3).

3.4. Correlation of TNF α levels

Bland–Altman plot was constructed to correlate TNF α levels obtained by ELISA and Luminex, respectively. The results show in general good agreement between the two methods ($p = 0.011$) (Fig. 4).

3.5. Immunohistochemistry

TNF α showed co-localization with NeuN, a marker for neurons, across multiple brain regions, namely striatum, CA1 region of the hippocampus, and hilar neurons of the dentate gyrus. There was a lack of co-localization with astrocytes (Fig. 5). Adjacent sections were used to visualize TNF α (Fig. 6, panel A) and microglia (Fig. 6, panel B) in the striatum at 3 h. The different regional cell morphology and density suggest that microglia do not contribute to the TNF α production at this early timepoint.

4. Discussion

This study was designed to explore the regional pattern of brain tissue cytokines early after VF CA to identify possible therapeutic targets. We used our established VF CA model characterized by neurologic impairment and extensive histological damage in multiple brain regions, including hippocampus and striatum.⁸

We found that brain tissue levels of TNF α are increased as early as 3 h after CA, being the most prominent in the striatum. This early TNF α response in the striatum was more pronounced with prolonged durations of CA with a robust 20-fold increase in that brain

region. Two weeks after the insult, TNF α levels were still increased vs. shams, without regional differences.

This massive and sustained TNF α increase especially in the striatum contrasts a relative lack of increase in other cytokines (except IL-1 β) across brain regions, further suggesting the role of TNF α as a potential key player in the cytokine response, and as a possible therapeutic target. A close correlation of TNF α levels assessed by ELISA and Luminex methods strengthens the validity of our results. Surprisingly, immunohistochemistry identified neurons as a source of TNF α at 3 h after CA, rather than astrocytes or microglia.

There is a considerable body of literature exploring CNS inflammation in chronic neuroinflammatory diseases, and in stroke. In contrast, regional neuroinflammation after global ischemic insults has been relatively understudied. Traditionally, most neuropathology is focused on histological damage in the selectively vulnerable regions, namely hippocampus. We have shown previously that neurologic outcome could differ despite similar histological damage in hippocampus suggesting that other brain regions could be responsible for the neurologic deficits.¹⁰

Reperfusion after global ischemia resembles systemic inflammation and sepsis.⁴ Most studies have explored systemic rather than CNS response to global ischemia. Given the immune privilege of the CNS, significant differences between systemic and CNS responses may exist. Our data suggest that the cytokine response in brain is unique, region dependent, quite rapid and involving direct participation of neurons.

4.1. Role of TNF α in brain injury

TNF α is an important mediator of disease in various acute brain insults including global and focal brain ischemia,¹¹ traumatic brain injury¹² and spinal cord injury,¹³ as well as in chronic brain diseases.¹⁴ TNF α is a major pro-inflammatory mediator, with the capacity to induce apoptosis, as well as to support regeneration and tissue repair.^{15,16} Whether the net effect is deleterious or reparative might depend upon concentration, local distribution and timing of TNF α production, and interaction with co-mediators.^{15,17}

Pretreatment with exogenous TNF α before experimental stroke exacerbated infarct size and neurological sequelae,¹⁸ whereas inhibition of TNF α reduced brain infarct volume.¹⁹ Pretreatment with systemic monoclonal anti-TNF α antibodies reduced infarct size and improved neurological outcome in experimental stroke in rats.²⁰

4.2. Time course and intensity of TNF α production after brain injury

TNF α has been shown to be produced early in reperfusion after global ischemia. In a rat model of asphyxial CA, serum levels of TNF α peaked during the first hours, with a return to baseline after 3 h.²¹ Our work builds on these findings. We found an early increase of TNF α in the striatum, with elevated levels without any regional difference 14 d after the insult. TNF α levels were not different after 6 min vs. 8 min of VF CA, but doubled after 10 min VF CA. This exponential increase in striatal TNF α after prolonged CA in our study might alter the net effect toward these deleterious results, supported by the fact that rats subjected to 10 min VF CA were unable to be weaned from mechanical ventilation.

4.3. Regional cerebral distribution of TNF α

In our study, the striatum proved a focus of the neuroinflammatory response after CA and showed the most prominent increase in TNF α . In contrast, TNF α significantly increased in the cerebellum only after 10 min VF CA.

Yasuda et al. recently explored longitudinal hippocampal cytokine signatures after global brain ischemia. TNF α levels were low during the first 5 days, followed by a significant increase between 7 and 21 days. Other cytokines were unchanged from shams at 6 h, consistent with our results. Striatum was not studied.²² We have observed a sustained twofold increase in TNF α at 14 d in the hippocampus. Saito et al. studied regional distribution of TNF α , IL-6 and IL-1 β after global ischemia, documenting an early duration-dependent increase of TNF α in the hippocampus and the striatum.²³ This is in contrast to our results, where TNF α levels in the hippocampus were low. However, the dependence on insult severity is supported by our data. There is evidence that TNF α may play a less important role in the hippocampal neuronal loss. Murakami et al. showed no differences between wild type TNF α mice and TNF α gene-deficient mice in hippocampal neuronal damage after global ischemia.²⁴ Our results are further supported by Tchelingirian et al. who identified increased TNF α levels in striatum after a stab lesion to the hippocampus.^{25,26} The TNF α increase seen in this study could parallel an early response to the evolving extensive hippocampal neuronal death also seen in our model and merits future exploration.

Teschendorf et al. studied longitudinal caspase response and neuronal cell death in a similar model of VF CA in rats. An early (6 h) caspase positivity was identified in striatum but not in hippocampus that showed delayed apoptotic cell death at 7 d.² This may suggest that in VF CA different brain regions have distinct response to global brain ischemia and neuronal death in striatum may evolve quickly and specifically involve TNF α , as suggested by our work. Ultimately, specific therapeutic interventions delivered at key time points after the insult may be needed to prevent neuronal death in different regions.

Multiple cell types were indicated as sources of intracerebral TNF α including glia, neurons or endothelial cells. Seminal experiments done by Liu et al. who examined the cerebral cortical levels of TNF α showed that transcription of TNF α starts 1 h after ischemia. Early upregulation was localized in neurons, after 5 days immunoreactivity was localized in inflammatory cells.²⁷ In contrast, Maddahi et al. identified the walls of cerebral arteries as the source of TNF α .²⁸ Our results identify neurons as a potential source of an acute production of TNF α in VF CA. This is surprising since most literature supports the pivotal role of microglia as a source of TNF α . Similar finding were also observed after exsanguination CA treated with deep hypothermia and delayed resuscitation with cardiopulmonary bypass.^{29,30} We cannot rule out that other cell types contribute to TNF α production at delayed timepoints. However, we focused on the source of early TNF production given the well recognized short time window believed to exist for successful therapeutic intervention in VF CA.³¹ The role of TNF α in our model will need to be determined in future experiments.

IL-1 β was the only other cytokine with significantly increased levels after CA. Regional differences in IL-1 β were less pronounced. Other cytokines did not show significant changes

vs. shams at 3 h or 6 h after CA. Both TNF α and IL-1 β were shown to play a pivotal role in postoperative cognitive deficits.³² IL-1 β was specifically studied in a rat model of forebrain ischemia, with increased expression specifically in the hippocampus where IL-1 β co-localized with glia. In striatum, no specific co-localization was found, and the expression was much less pronounced.³³ The increased levels in striatum seen in our model could be explained by a different insult. Both TNF α and IL-1 β were also increased at 6 h after global brain ischemia, with slightly higher levels in hippocampus than in the striatum.²³ Intra-striatal injection of IL-1 β exacerbated striatal but not cortical neuronal death,³⁴ further stressing the need for in-depth studies of regional role of cytokines.

Hosomi et al. showed that attenuation of systemic TNF α response improved outcome after focal cerebral ischemia.³⁵ Systemic blockade of TNF α improved outcome from VF CA in swine. These improvements were mediated by effects on extracerebral organs.^{6,36} However, they could suggest that systemic TNF α targeting therapy could have beneficial effects both within and outside of the CNS.

Our study focused pre-dominantly on an early post-resuscitation period up to 6 h. Different regional cytokine profiles may be observed at delayed periods. However, our primary goal was to characterize an early cytokine response and identify potential therapeutic targets for future interventions. We did not assess systemic TNF α levels that were showed to be increased after CA and associated with an adverse outcome.^{4,37} Systemic TNF α may, however, originate from sources outside the CNS.⁷ We acknowledge that our findings represent an initial step in elucidating neuroinflammation after CA. The importance of our results described herein will need to be explored in future experiments targeting individual cytokines or complex neuroinflammatory cascades to achieve neurological benefits.

5. Conclusions

To our knowledge, this is a first exploratory study evaluating early regional cytokine profile in a clinically relevant VF CA model. CA induced cerebral cytokine production with a distinct spatial and temporal pattern and suggested neurons as an important early source of TNF α . Early after CA, the striatum showed marked increase in TNF α levels with highest levels after prolonged CA. 14 d after CA, brain tissue levels of TNF α remained increased over shams, but without regional differences. Our study suggests that therapeutic approach targeting TNF α early after CA deserves exploration and that the striatum rather than hippocampus might represent a therapeutic target.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

TNFα levels (pg/mg protein) at 3 h (a: ELISA and b: Luminex) and 6 h after CA (a and c: ELISA; b and d: Luminex). Values represent median and 25–75% interquartile ranges. The round and asterisk markers represent outliers of the respective groups. Brackets indicate statistical significance between the marked values ($p < 0.05$). CA, cardiac arrest.

Fig. 2.

Time course of TNFα levels at 3 h, 6 h and 14 d after 6 min CA vs. sham. Values represent median and 25–75% interquartile ranges. The round and asterisk markers represent outliers of the respective groups.

Fig. 3.

Cytokine levels at 3 h and 6 h after cardiac arrest (Luminex). Values represent median and 25–75% interquartile ranges. The round and asterisk markers represent outliers of the respective groups. Brackets indicate statistical significance between the marked values ($p < 0.05$). IL, interleukin; IFN, interferon; CA, cardiac arrest.

Fig. 4.

Bland–Altman plot of TNFα levels obtained by ELISA or Luminex methods, respectively.

Fig. 5. TNF α expression in striatum after prolonged 10 min cardiac arrest (a, TNF α expression; b, NeuN staining visualizing neurons; c, merge TNF α and NeuN; d, TNF α expression, e, GFAP staining visualizing astrocytes; f, merge TNF α and GFAP; magnification 20 $\times$). The TNF α -expressing cells co-localize with neurons (panel c) but not with astrocytes (panel f). The marker in panel a represents 40 μ m.

Fig. 6. TNF α positive cells (green, panel A) and microglia (red, panel B) in striatum show different morphology and cell density, further supporting our findings that microglia are unlikely to be a major source of the TNF α production at 3 h. Marker in panel A = 20 μ m.