Targeting TNFα-mediated cytotoxicity using thalidomide after experimental cardiac arrest in rats: An exploratory study

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Received December 4, 2021; Accepted March 15, 2022

DOI: 10.3892/etm.2022.11307

Abstract. Cardiac arrest (CA) results in a central and systemic cytokine and inflammatory response. Thalidomide has been reported to be neuroprotective by selectively decreasing TNFα synthesis. We hypothesized that thalidomide would decrease the systemic and organ-specific TNFα/cytokine response and biomarkers of injury in rats subjected to 10 min CA. Naïves, CA treated with vehicle (CA) and CA treated with thalidomide (50 mg/kg; CA+T) were studied (n=6 per group). TNFα and key cytokines were assessed at 3 h after resuscitation in the cortex, hippocampus, striatum, cerebellum, plasma, heart and lung. Neuron specific enolase (NSE), S100b, cardiac troponin T (cTnT) and intestinal fatty acid binding protein (IFABP) were used to assess neuronal, glial, cardiac and intestinal damage, respectively. CA increased TNFα and multiple pro-inflammatory cytokines in plasma and selected tissues with no differences between the CA and CA+T groups in any region. NSE, S100b, cTnT and IFABP were increased after CA or CA+T vs. in the naïve group (all P<0.05) without significant differences between the CA and CA+T groups. In conclusion, CA resulted in a TNFα and cytokine response, with increased biomarkers of organ injury. Notably, thalidomide at a dose reported to improve the outcome in *in vivo* models of brain ischemia did not decrease TNFα or cytokine levels in plasma, brain or extracerebral organs, or biomarkers of injury. Although CA at 3 h post resuscitation produces a robust TNFα response, it cannot be ruled out that an alternative dosing regimen or assessment at other time-points might yield different results. The marked systemic and regional cytokine response to CA remains a potential therapeutic target.

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Key words: heart arrest/pathology, brain ischemia, cardiopulmonary resuscitation, post-cardiac arrest syndrome, neurons/drug effects, microglia/drug effects, thalidomide/pharmacology, tumor necrosis factor-α

Introduction

Overall mortality and morbidity after cardiac arrest (CA) remain high, despite improvements in resuscitation and critical care. Neurocognitive disabilities are frequently observed in survivors from CA. Histological damage including neuronal cell loss was characterized in multiple experimental global ischemia-reperfusion insults (1). Several selectively vulnerable regions have been identified, namely hippocampus, cerebellar Purkinje neurons, lamina IV cortical layer and striatum. While early ischemic brain injury is the result of energy failure, neuro-inflammation could contribute, or even represent a major cause of delayed neuronal death. Tumor necrosis factor alpha (TNFα) is a pivotal cytokine that can induce neuronal apoptosis and/or necroptosis, and increase neuroinflammation (2-4).

CA also triggers a sepsis-like inflammatory response with expression of TNFα up-regulated in cerebral ischemia. Systemic selective anti-TNFα therapies improved early recovery from CA, in both small and large animal models (4,5). Our prior studies in multiple models of CA in rats, identified a unique early cytokine response specifically in striatum, showing a dramatic >100-fold increase of TNFα vs. other brain regions including hippocampus, where no increase in TNFα was seen (6-8).

The striatum is a region with selective vulnerable neuronal death in our model (9). Surprising is the fact our prior studies showed TNFα localized immunohistochemically in neurons rather than microglia (6,7). Thus, unique TNFα production in striatal neurons may mediate the region-specific neuronal loss in striatum after CA. Thus, region-specific neuronal therapies may be required to best target neuronal death after CA.

Among several readily translatable potential strategies for CA to target TNFα, thalidomide, an inhibitor of TNFα protein synthesis is readily capable of crossing the blood brain barrier (BBB) (10). Thalidomide has been reported to selectively decrease TNFα (11-13) and shown to be neuroprotective via destruction of TNFα mRNA *in vivo* (14) and *in vitro* (15).

Specifically, thalidomide and its derivatives, including both lenalidomide and pomalidomide, termed immunomodulatory imide drugs (IMiDs), are a class of drugs that target the 3'-untranslated region (3'-UTR) of TNFα mRNA, inhibiting TNFα production (16). In this exploratory study, we hypothesized that thalidomide would attenuate (1) systemic TNFα levels, (2) neuroinflammation as reflected by brain tissue TNFα levels, (3) extra-cerebral organ TNFα levels, and (4) markers of organ injury after prolonged CA in rats. We have also assessed (5) complex cytokine response to CA to elucidate the potential downstream effect of thalidomide on other cytokines. Naïve rats and rats treated with vehicle served as controls.

Materials and methods

Institutional approval. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (Protocol no. 13021161; 'Neuroinflammation after prolonged cardiac arrest'). We used our previously established model of ventricular fibrillation (VF) CA (17).

Preparation phase. In brief, adult male Sprague-Dawley rats (350-400 g) were obtained from a licensed vendor (Hilltop Lab Animals, Scottdale, PA) and housed under 12/12 h light/dark in a holding facility for at least two days prior to the experiment. Water was provided ad libitum until the experiment. Standard chow was removed 12 h prior to experiment. On the day of the experiment, rats were anesthetized with 4% isoflurane (Baxter) in pure oxygen in a plexiglass jar, intubated with a 14-gauge cannula (Becton Dickinson), and mechanically ventilated (Harvard Ventilator 683, Harvard Rodent Apparatus) with tidal volume 8 ml/kg, PEEP 3 cm H_2O and respiratory rate 30-40/min to maintain normocapnia. Anesthesia was maintained with 2% isoflurane (FiO_2 of 0.5).

CA and resuscitation phase. Three groups (n=6 per group) were studied: i) Naïve rats; ii) rats subjected to VFCA without thalidomide (CA); and iii) rats subjected to VFCA with thalidomide (CA+T). Naïve rats were deeply anesthetized with isoflurane 4% over 4 min, midline laparotomy and sternotomy were performed, and rats were perfused transcardially with 250 ml of ice-cold heparinized normal saline.

In rats subjected to VFCA, arterial (PE50) and venous (PE90) femoral catheters were inserted via cut-downs for blood pressure monitoring and drug administration. For VFCA, 5F pacing catheter was introduced via the jugular vein to the conjunction of right atrium and right ventricle.

Electrocardiogram (ECG) and mean arterial pressure (MAP) were continuously monitored and recorded (Polygraph, Grass Instruments). Rectal temperature was controlled at 37.0±0.5˚C with a temperature controlled operating table, overhead heating lamp and a fan. After surgery, the FiO_2 was reduced to 0.3 and isoflurane was gradually weaned to 0% over 10 min in rats scheduled for CA.

No-flow was then induced by a 2-minute impulse of 12 V/50 Hz alternating current and ensured by ECG readings and reduction in MAP <10 mmHg. The pacing catheter was then removed and jugular vein ligated. After 10 min of VFCA, manual chest compressions were started at a rate ~360/min along with mechanical ventilation with 100% oxygen. Epinephrine (Abbott) 0.01 mg/kg was given with start of compressions. Additional epinephrine 0.005 mg/kg was given at 1 min resuscitation time (RT). Sodium bicarbonate (Abbott) 1 mEq/kg was also given at start of resuscitation. At 2 min after the start of resuscitation (2 min RT), defibrillation was attempted with biphasic 10 J impulse (Zoll M series defibrillator; Zoll). If unsuccessful, subsequent shocks were delivered every 30 seconds, with maximum 5 attempts over 4 min resuscitation effort. Return of spontaneous circulation (ROSC) was defined as sustained supraventricular rhythm with MAP >50 mmHg. In rats subjected to thalidomide treatment (CA+T group), 50 mg/kg thalidomide (Enzo Life Sciences; cat. no. BML-T115-0100) dissolved in dimethyl sulfoxide (DMSO) was administered i.p. at 5 min after initiation of resuscitation. This dose was selected based on prior studies reporting benefits (18-21). Control rats (CA group) received an identical volume of DMSO at the corresponding timepoint.

Postoperative care. After ROSC, rats were mechanically ventilated with FiO_2 1.0, Vt 8 ml/kg, PEEP 5 cmH_2O and respiratory rate adjusted to maintain normocapnia. Epinephrine infusion was titrated to maintain MAP >65 mmHg. Controlled normothermia (36.5-37.5˚C) was maintained for 3 h. At 3 h RT, serum samples were obtained, rats were deeply anesthetized with isoflurane 4%, and perfused transcardially with 250 ml of ice-cold heparinized normal saline. Rats were then decapitated, hearts and brains removed and dissected into four regions of interest: cortex, hippocampus, striatum, and cerebellum. Plasma, heart, and lung samples were also obtained. Individual tissue samples were then snap-frozen in liquid nitrogen and stored in -70˚C freezer until further processing.

TNFα and cytokine measurements. Tissues were then processed for cytokine assessment for interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, interferon γ (IFNγ) and granulocyte-macrophage colony stimulating factor (GMCSF) using Luminex-200 multiplex analyzer (Luminex) using a rat-specific kit (Millipore). All values were corrected for protein concentration. The tissue was homogenized in phosphate-buffered saline (PBS) by using Dounce homogenizer for 20 strokes. The homogenate was then sonicated for 10 seconds for three times with an interval of 20 seconds, followed by centrifugation at 16,000 x g for 30 min. The supernatant was used for TNFα analysis. Protein levels in the supernatant was measured using the bicinchoninic acid (BCA) protein kit (Thermo Fisher Scientific, Inc.). TNFα concentrations that were read as 'out of range below' were nominally assigned a value at one hundredth of the lowest level on the calibration scale.

Biomarkers of organ-specific injury. Biomarkers of neuronal injury (neuron-specific enolase, NSE) and glial injury (S100b) were assessed using kits (MyBioSource kits catalogue nos. MBS262217 and MBS849461, respectively; MyBioSource, Inc.) according to manufacturer's instructions. Myocardial injury was assessed using rat-specific cardiac troponin T (cTnT) ELISA kit (MyBioSource catalogue no. MBS730382). Intestinal injury was assess using rat specific ELISA kit for

Table I. Physiologic and biochemical profile after CA.

Variable	BL	RT5	RT30	RT60	RT120	RT180	P-value
HR, bpm							
CA	324±11	272±86	324±40	364±27	374±15	390±20	0.88
CA+T	344±25	302±22	340±32	384±43	378±46	390±31	
MAP, mmHg							
CA	86±4	146±18	75±8	70±5	80±12	85±16	0.21
CA+T	91±12	133±22	72±4	66±7	79±18	81±12	
pHa							
CA	7.39±0.04	7.31±0.13	7.18±0.04	7.43±0.03	7.48±0.02	7.45±0.01	<0.0001
CA+T	7.40±0.02	7.31±0.09	7.18±0.04	7.35±0.08	7.38±0.04	7.39±0.03	
paO_2, mmHg							
CA	189±31	343±50	354±44	366±31	369±68	370±45	0.75
CA+T	227±15	363±73	413±102	324±90	397±79	379±75	
$paCO_2$, mmHg							
CA	37±6	33±10	42±6	34±4	29±5	33±3	0.10
CA+T	38±5	34±7	39±4	35±7	39±6	40±6	
BE, mEq/l							
CA	-2.1±1.4	-9.5±3.8	-11.9±2.2	-1.4±2.5	-1.8±2.7	-0.8±1.4	0.36
CA+T	-1.1±2.4	-8.9±2.0	-13.0±0.7	-5.3±3.8	-1.7±4.8	-0.7±4.8	
Lactate, mmol/l							
CA	1.4±0.8	9.8±1.6	9.4±2.6	4.5±1.8	3.1±0.9	2.5±0.7	0.82
CA+T	1.4±0.5	10.3±1.0	8.4±2.7	3.9±0.7	2.8±0.7	3.1±1.0	
Hct, %							
CA	43±2	46±3	50±3	48±3	45±4	47±4	0.33
CA+T	43±3	45±2	47±5	45±4	47±1	48±3	
Glucose, g/dl							
CA	166±31	105±42	121±28	142±41	145±24	161±46	0.76
CA+T	178±35	86±14	94±22	125±34	190±26	199±60	

Differences among groups (P-value) were examined using Generalized Estimating Equations models for treatment (RT5-RT180 in CA vs. CA+T). BL, baseline; RT, resuscitation time (min); HR, heart rate; MAP, mean arterial pressure; BE, base excess; Hct, hematocrit; CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group).

intestinal fatty acid binding protein (IFABP; MyBioSource catalogue no. MBS024910).

Statistical analysis. The analyses were performed using IBM SPSS Statistics 26.0 software (IBM Corp.). The samples size calculations were based off the results published by us previously. We hypothesized that thalidomide would reduce TNFα levels in the striatum by 50%. Using alpha=0.05 and power=80%, number of rats per group needed was five. Anticipating ~20% mortality in our model, we randomized 6 rats per group.

Data were tested for normality using the Kolmogorov-Smirnov test. Hemodynamic and biochemical data are presented as mean ± standard deviation (SD). Differences between groups (P-value) were tested using Generalized Estimating Equations models for treatment (RT5-RT180 in CA vs. CA+T). Cytokine levels are presented as median and the first and the third quartile. Differences in plasma cytokines were assessed using one-way analysis of variance (ANOVA) with post hoc Tukey's test. Differences in extracerebral organs cytokines at three hours after CA were assessed using Kruskal-Wallis test followed by Dunn's test with post hoc Bonferroni test. The differences in biomarkers of organ-specific damage between groups were evaluated with a one-way ANOVA with Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Survival. One rat in each group died. One rat in the CA group died from hemodynamic collapse at RT 10 min, whereas one rat in the CA+T group did not achieve ROSC. Data from both rats were excluded from further analyses.

Biochemical and hemodynamic profiles. As anticipated in our model, CA induced profound metabolic acidosis with increased lactate that illustrated the severity of the insult. The metabolic derangements progressively resolved by the 180 min RT. There

Table II. Biomarkers of brain and extracerebral organ injury after CA.

Variable	Naïve	CA	CA+T
NSE, ng/ml	0.01±0.02	1.19±0.16[a]	1.13±0.22[a]
S100b, pg/ml	22±1	196±26[a]	177±37[a]
cTnT, pg/ml	0.64±0.38	1.20±0.06[a]	1.17±0.30[a]
IFABP, pg/ml	239±73	368±39[a]	393±70[a]

[a]P<0.05 vs. naïve; no significant differences for CA vs. CA+T. Differences among groups were examined using one-way analysis of variance with post hoc Tukey's test. NSE, neuron specific enolase; S100b, S100 calcium binding protein beta; cTnT, cardiac troponin-T; IFABP, intestinal fatty acid binding protein; CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group).

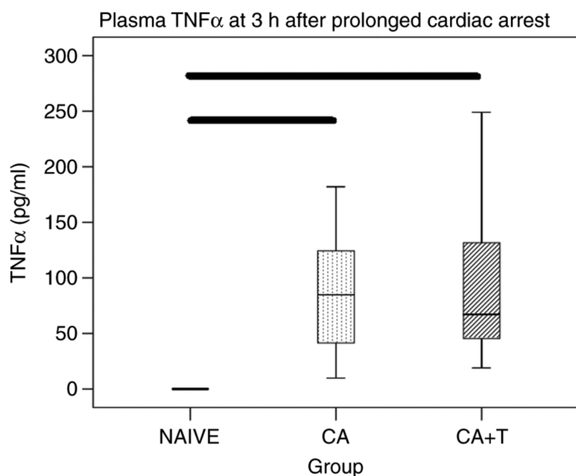

Figure 1. TNFα levels in plasma at 3 h after prolonged ventricular fibrillation CA in rats. Boxes represent the 25-75% interquartile range, the line across the box represents the median, and the whiskers are min-max values. The thick horizontal line signifies P<0.05 between groups (one-way ANOVA with post hoc Tukey's test). CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group).

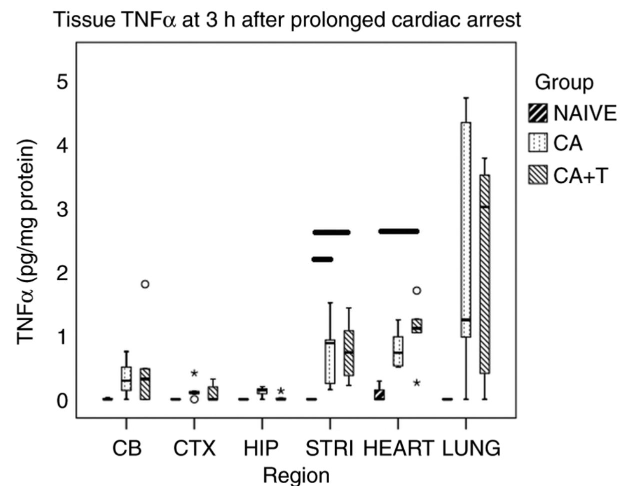

Figure 2. TNFα levels in selected brain regions and extracerebral organs at 3 h after prolonged ventricular fibrillation CA in rats. Boxes represent the 25-75% interquartile range, the line across the box represents the median, and the whiskers are min-max values. The thick horizontal line signifies P<0.05 between groups (Kruskal-Wallis test followed by Dunn-Bonferroni post-hoc test). The round marker and the asterisk represent outliers ('out' and 'far out' values). CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group); CB, cerebellum; CTX, cortex; HIP, hippocampus; STRI, striatum.

were no major differences in hemodynamic or biochemical profiles between CA and CA+T groups except pHa (Table I).

Plasma TNFα. CA resulted in an anticipated marked increase in plasma TNFα (both naïve vs. CA and naïve vs. CA+T P<0.05). However, no significant differences were found in plasma TNFα between CA and CA+T treatment groups (Fig. 1).

TNFα in individual brain regions. In general, brain cytokines in naïve rats were low or undetectable. Although the TNFα levels were increased after CA in most surveilled brain regions, no statistically significant difference was found between any groups for CA, CA+T and naïve in the cerebellum (P=0.236), cortex (P=0.297), or in the hippocampus (P=0.067). As anticipated from our prior studies, TNFα was significantly increased after CA in the striatum vs. naïve (P<0.05) but was not found to be significantly different between CA and CA+T (P=1.0), failing to support our hypothesis (Fig. 2).

TNFα in extracerebral organs. TNFα levels were numerically increased after CA or CA+T vs. naïve in lung tissue but this trend did not reach statistical significance (P=0.077). There were no differences in lung TNFα between CA and CA+T. In contrast, the myocardial tissue levels of TNFα were significantly increased after CA+T vs. naïve (P<0.05) but were not found to be significantly different between CA and CA+T (Fig. 2).

Biomarkers of organ-specific injury. Rats subjected to CA had significantly increased biomarkers of end-organ injury over naïves across all the organs sampled: NSE (P<0.05), S100b (P<0.05), cardiac troponin T (P<0.05), and IFABP (P<0.05), without significant differences between CA and CA+T groups (Table II).

Cytokine response in individual brain regions. Rats subjected to CA showed a significant increase of IL-6 in the hippocampus and TNFα in the striatum over naïve rats, with no differences between CA and CA+T groups (Table III).

Cytokine response in plasma and extracerebral organs. CA resulted in statistically significant increases of selected cytokines in plasma, namely IL-1β, IL-6, IL-10 and IL-12. None of these cytokines were affected by thalidomide administration. Increases of selected cytokines in heart (IL-1α, IL-1β, IL-6, TNFα) and lung (IL-1α, IL-1β, IL-6, IFNγ) were also not affected by thalidomide (Table IV).

Discussion

We previously reported that this model of 10 min of VFCA results in extensive neuronal death and dramatic increases in cytokines. In the current study, we confirmed our findings of early marked increases in TNFα levels in the striatum,

Table III. Cytokine profile in individual brain regions at 3 h after CA.

Variable	CTX, pg/mg protein	HIP, pg/mg protein	STRI, pg/mg protein	CEREB, pg/mg protein
IL-1a				
N	0.00 (0.00-1.97)	0.08 (0.00-1.25)	0.00 (0.00-0.00)	0.00 (0.00-0.65)
CA	1.43 (0.00-3.11)	2.56 (1.12-4.47)	2.91 (0.00-4.44)	0.47 (0.00-2.17)
CA+T	0.00 (0.00-1.47)	2.04 (0.10-4.35)	3.40 (1.47-4.72)	1.38 (0.00-1.76)
IL-1b				
N	2.90 (2.27-4.42)	6.45 (3.40-7.96)	6.90 (2.23-10.62)	7.87 (7.10-9.02)
CA	8.02 (4.85-11.77)	7.93 (7.25-14.23)	7.12 (6.38-14.44)	9.72 (4.84-14.44)
CA+T	6.81 (3.58-9.76)	8.29 (6.15-10.0)	10.42 (7.28-11.76)	12.73 (9.78-13.51)
IL-2				
N	6.12 (1.41-50.59)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	4.98 (4.08-5.47)
CA	0.00 (0.00-14.04)	4.55 (0.00-9.10)	0.00 (0.00-26.60)	5.75 (0.00-52.85)
CA+T	0.00 (0.00-22.33)	0.00 (0.00-2.82)	24.28 (2.83-40.66)	4.08 (0.00-10.12)
IL-4				
N	0.02 (0.01-0.02)	0.02 (0.01-0.02)	0.03 (0.01-0.04)	0.02 (0.02-0.02)
CA	0.02 (0.02-0.02)	0.02 (0.01-0.02)	0.02 (0.02-0.04)	0.02 (0.01-0.02)
CA+T	0.02 (0.02-0.03)	0.02 (0.01-0.02)	0.02 (0.02-0.04)	0.02 (0.02-0.02)
IL-6				
N	0.00 (0.00-0.00)	0.00 (0.00-0.34)	1.01 (0.00-2.05)	0.41 (0.06-0.90)
CA	2.88 (1.05-10.92)	1.52 (0.25-3.05)	1.88 (0.63-3.47)	2.29 (0.92-6.82)
CA+T	2.20 (0.61-3.28)	1.34 (0.81-2.32)	2.66 (1.91-4.40)	3.96 (2.65-4.41)
IL-10				
N	5.72 (1.06-9.15)	1.75 (0.00-4.14)	17.48 (4.27-28.86)	1.55 (0.00-4.12)
CA	9.41 (5.96-10.83)	1.90 (1.19-4.99)	13.52 (11.28-24.75)	4.56 (0.00-15.32)
CA+T	13.76 (7.75-18.65)	4.83 (2.35-6.94)	18.51 (8.88-23.71)	5.45 (1.49-7.99)
IL-12				
N	0.00 (0.00-2.45)	1.13 (0.00-2.48)	0.00 (0.00-0.00)	1.33 (0.08-2.91)
CA	1.56 (0.62-3.06)	1.50 (0.76-3.55)	0.00 (0.00-0.57)	0.00 (0.00-1.49)
CA+T	2.31 (0.90-4.05)	1.04 (0.39-2.4)	1.37 (0.58-3.19)	0.97 (0.00-2.35)
IFNγ				
N	0.48 (0.10-1.12)	0.33 (0.10-0.48)	0.33 (0.18-1.32)	0.42 (0.37-0.50)
CA	0.38 (0.00-0.57)	0.26 (0.11-0.37)	0.31 (0.03-0.78)	0.24 (0.09-0.54)
CA+T	0.40 (0.00-0.65)	0.27 (0.12-0.49)	0.54 (0.18-1.32)	0.50 (0.25-0.64)
TNFα				
N	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-0.16)
CA	0.10 (0.04-0.27)	0.14 (0.04-0.18)	0.88 (0.20-1.22)[a]	1.29 (0.07-0.63)
CA+T	0.00 (0.00-0.25)	0.00 (0.00-0.08)	0.74 (0.29-1.25)[a]	0.31 (0.00-1.14)

Differences among groups were examined using the Kruskal-Wallis test followed by Dunn-Bonferroni test (adjusted significance, [a]P<0.05 vs. naïve). N, naïve; CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group); CTX, cortex; HIP, hippocampus; STRI, striatum; CEREB, cerebellum.

and also demonstrated that CA resulted in an early marked TNFα response both systemically, and in other target organs. Contrasting our hypothesis, however, thalidomide in a dose reported previously to improve outcome of *in vivo* models of brain ischemia did not decrease TNFα levels in the striatum, a brain region that showed the most pronounced response to ischemia in our model of experimental CA. Unfortunately, TNFα levels in plasma, other brain regions or extracerebral organs were also not attenuated by thalidomide. Selected pro- and anti-inflammatory cytokines increased in brain, plasma, heart or lung after CA were not affected by thalidomide. The reason for the failure of this approach to attenuate the increase in striatal and/or other levels of TNFα in our model and/or exhibit neuroprotective effects is unclear.

We used a dose previously reported to be effective in an experimental incomplete brain ischemia in mice. However, only pre-treatment with that dose was effective; post-treatment was not (20). To evaluate the potential of this approach

Table IV. Cytokine profile in extracerebral organs and plasma at 3 h after CA.

Variable	Heart, pg/mg protein	Lung, pg/mg protein	Plasma, pg/ml
IL-1a			
N	0.00 (0.00-0.00)	46.49 (44.14-52.17) (43.95-46.49)	0.00 (0.00-0.00)
CA	12.59 (9.97-18.80)[a]	1135.50 (699.93-1777.02)	266.41 (93.34-420.76)
CA+T	17.39 (12.43-32.68)[a]	851.2 (818.11-1231.17)[a]	216.04 (105.97-405.33)
IL-1b			
N	4.82 (3.23-6.12)	45.21 (43.90-47.95)	2.68 (0.07-11.99)
CA	54.57 (34.17-75.14)[a]	1,421.24 (559.38-1,887.66)[a]	255.81 (141.62-619.25)
CA+T	59.50 (37.90-79.31)[a]	1,135.49 (784.93-1,534.19)[a]	330.60 (229.21-863.37)[a]
IL-2			
N	118.71 (86.42-142.46)	0.00 (0.00-0.00)	179.61 (80.27-1495.17)
CA	84.71 (65.65-120.83)	13.38 (1.95-18.84)	1033.3 (661.27-1,903.21)
CA+T	148.08 (49.9-194.49)	24.15 (0.00-64.48)	1,053.10 (672.88-2,526.03)
IL-4			
N	0.28 (0.12-0.25)	0.02 (0.02-0.04)	0.17 (0.14-1.02)
CA	0.07 (0.04-0.25)	0.03 (0.02-0.04)	0.38 (0.32-0.87)
CA+T	0.19 (0.02-0.25)	0.04 (0.03-0.05)	0.41 (0.26-0.76)
IL-6			
N	0.00 (0.00-1.85)	0.00 (0.00-0.00)	2.31 (0.00-7.29)
CA	61.26 (26.02-117.38)	131.05 (20.75-165.54)[a]	6,316.17 (2,533.21-8462.35)[a]
CA+T	129.84 (73.25-333.64)[a]	119.86 (86.35-169.09)[a]	6,756.38 (2,561.31-7775.65)[a]
IL-10			
N	145.01 (96.93-194.58)	10.74 (1.91-16.33)	0.00 (0.00-0.00)
CA	75.55 (48.46-157.16)	17.02 (4.06-24.36)	925.86 (453.14-1390.16)[a]
CA+T	133.14 (27.23-147.75)	16.22 (13.02-16.84)	868.66 (597.01-1336.45)[a]
IL-12			
N	8.24 (6.54-12.78)	21.69 (18.22-30.02)	1,479.62 (1,142.40-1704.93)
CA	9.45 (6.81-15.84)	32.29 (27.04-67.66)	4,754.44 (2,533.69-9116.59)[a]
CA+T	10.26 (7.93-13.91)	31.53 (17.88-63.99)	4,049.25 (2,830.96-7046.72)[a]
IFNγ			
N	3.04 (2.26-3.55)	0.37 (0.25-0.48)	1.89 (0.35-7.90)
CA	1.35 (0.90-2.05)	0.63 (0.50-2.40)	17.39 (7.74-190.14)
CA+T	3.01 (0.55-3.35)	0.78 (0.70-1.50)[a]	30.86 (12.58-105.17)
TNFα			
N	0.00 (0.00-0.21)	0.00 (0.00-0.00)	0.00 (0.00-0.00)
CA	0.73 (0.52-1.05)	1.24 (0.49-4.50)	84.74 (25.68-153.13)[a]
CA+T	1.11 (0.65-1.48)[a]	3.01 (0.20-3.64)	67.14 (32.24-190.30)[a]

Differences among groups were examined using Kruskal-Wallis test followed by Dunn-Bonferroni test (adjusted significance, [a]$P<0.05$ vs. naïve). N, naïve; CA, cardiac arrest (control group); CA+T, cardiac arrest plus thalidomide (treatment group).

in a clinically relevant translational CA model, we used a post-treatment paradigm.

Early brain cytokine response to global brain ischemia has been documented by us (6,7) and others (22). Microglia are deemed to be a major source of brain TNFα in the later phases after the insult or in neuro-inflammatory diseases in which thalidomide was shown to be effective, e.g., Alzheimer's disease (23). However, we previously reported that in the early phase post resuscitation, TNFα is produced by neurons (6,7). This observation has been supported by others (24,25). It is conceivable that neuronal origin of TNFα in this early phase of reperfusion could contribute to the lack of a definitive effect of thalidomide that primarily targets glia cells in brain (16). Also, thalidomide selectively targets microglia-mediated neuroinflammatory response rather than astrocytes (26). We reported that the cytokine production at the early stages of post-CA syndrome is not mediated by microglia but rather neurons and astrocytes (7).

Most studies focused on neuronal loss in hippocampus, a selectively vulnerable region with extensive neuronal

degeneration after cerebral ischemia. In recent studies focused on the neuro-inflammatory response to CA, however, we noted dramatic regional dependence of the cytokine response in brain after CA (6,8). These studies suggest the potential need for a paradigm shift in the approach to the development of neuroprotective therapies in CA-namely, region specific therapies tailored to individual brain regions. Dopaminergic neurons in the striatum may represent an alternative, selectively vulnerable region in the prolonged CA setting. The early surge of TNFα in the striatum as described by us earlier was selected as a primary target structure in our current study.

TNFα has also been documented to be increased after ischemia-reperfusion also in plasma and extracerebral organs.

In rats subjected to a shorter, 6 min VFCA, increased levels of TNFα, IL-6, and IL-10 were observed in the jejunum from 6 h until 7 d but not in serum in rats (27). Similarly, Wender *et al* (28) did not observe increased serum TNFα levels past 24 h in a rat CA model. In contrast, Yang *et al* (29) reported that serum IL-6 and TNFα levels were both increased at 6 h after 6 min VFCA in rats.

In a porcine model of VFCA, systemic TNFα was detected early (15 min after reperfusion), and peak plasma levels coincided with myocardial depression (30) and with poor survival (31). Zhu *et al* (32) recently reported early increases of both serum TNFα and IL-6 levels that continued to rise over 6 h.

In an experimental model of intestinal ischemia-reperfusion injury, thalidomide was effective to decrease both systemic and tissue (intestine, lung) TNFα levels, effectively ameliorating biomarkers of injury, edema and resulting histologic damage. However, pre-treatment with a large dose (400 mg/kg p.o.) was used (33). Not all studies reported decrease of systemic TNFα with thalidomide treatment. After a lipopolysaccharide challenge resulting in massive cytokine response, neither plasma nor hepatic TNFα levels were decreased with thalidomide (34,35).

Our study has several limitations. Although thalidomide has been shown to cross the BBB, most studies documenting the salutary effects of thalidomide were performed in models with markedly disrupted BBB, (e.g., traumatic brain injury, stroke (20) or lipopolysaccharide-induced chronic neuroinflammation). Mohammed *et al* (36) reported a salutary effect of thalidomide was elicited by direct injection of the drug into the hippocampus, obviating the need for transport across the BBB. In this light, it is possible that newly synthetized derivatives of thalidomide, e.g., 3,6'-dithiothalidomide or pomalidomide, that penetrate BBB more easily, could have been more effective in ameliorating neuronal injury (37-39).

We also did not perform a dose response in our model. Instead, we selected for our study the highest (and the only effective) dose used in other studies (18,19,21). Importantly the same dose given to naïve rats did not elicit notable adverse effects (19). In that regard, pomalidomide has been shown to produce a TNFα inhibitory effect that is 50,000 times greater than thalidomide, and thus might have more potential in a CA scenario, where a rapid inhibitor effect is needed in the setting of an intact BBB (16). Also, the time-course of TNFα response in traumatic brain injury and models of chronic neuroinflammation seems to be delayed (40), providing a more favorable scenario for thalidomide or its derivatives to exert the effects on the injured brain even with oral administration (16). However, the lack of effect of thalidomide on increases in TNFα levels in plasma and extracerebral organs argue against brain penetration as the explanation for failure to effect target engagement and/or reduce secondary injury.

We explored TNFα levels only at a singular timepoint (3 h RT). We chose this timepoint based on results from our prior study using VFCA model in which we have explored both early (3 and 6 h RT) and late periods (14 days). Earlier timepoints showed more robust cytokine response (6). Moreover, TNFα-as the major target for thalidomide effects-peaks early, perhaps within the first hour after reperfusion (22). We have also tested a single, high dose of thalidomide shown previously to be beneficial and safe. However, we cannot rule out that alternative dosing regimen or assessments at other time-points might yield different results. Biomarkers of end-organ injury were also assessed at an early timepoint. Despite this limitation, we were able to document significant increases in CA groups over naïve controls.

We studied healthy young male rats only. More pronounced systemic cytokine response was observed in aged rats subjected to asphyxial CA (41). A significant difference in plasma cytokine response to CA between sexes have been observed by others (30).

Finally, we observed a trend toward reduced TNFα levels in the hippocampus in rats treated with thalidomide (P=0.067). We cannot rule out a possible effect in that brain region that would need to be explored with a larger sample size to appropriately test that hypothesis given the modest increase in TNFα seen in that brain region vs striatum in our model.

In conclusion, this exploratory study suggests that TNFα is increased early after CA systemically, in the brain and in extracerebral organs. Thalidomide, used early after reperfusion at high-dose previously showed to confer benefits, failed to decrease TNFα levels or other increased cytokines assessed at this timepoint in our experimental CA model. Biomarkers of end-organ injury were markedly increased after CA without any effect of thalidomide. Early systemic and organ-specific cytokine response after CA remains a valuable therapeutic target for future interventions in both acute and longitudinal studies.

Acknowledgements

The authors would like to thank Mr. Manuel S. Lombardero (Senior Statistician, Department of Epidemiology, School of Public Health, University of Pittsburgh, Pittsburgh, PA, USA) for his invaluable help with the statistical analyses and reviewing of the manuscript.

Funding

This work was supported by the Laerdal Foundation (TD).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AAP, PMK and TD designed the study, analyzed and interpreted the data, and wrote the manuscript. JPS performed the experiments. KJF performed the Luminex assays and ELISAs. JPS and KJF confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (protocol no. 13021161; 'Neuroinflammation after prolonged cardiac arrest'), in compliance with ARRIVE guidelines and the AVMA euthanasia guidelines 2020.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Janata A, Drabek T, Magnet IA, Stezoski JP, Janesko-Feldman K, Popp E, Garman RH, Tisherman SA and Kochanek PM: Extracorporeal versus conventional cardiopulmonary resuscitation after ventricular fibrillation cardiac arrest in rats: A feasibility trial. Crit Care Med 41: e211-e222, 2013.
2. Badiola N, Malagelada C, Llecha N, Hidalgo J, Comella JX, Sabriá J and Rodríguez-Alvarez J: Activation of caspase-8 by tumour necrosis factor receptor 1 is necessary for caspase-3 activation and apoptosis in oxygen-glucose deprived cultured cortical cells. Neurobiol Dis 35: 438-447, 2009.
3. Liu Q, Qiu J, Liang M, Golinski J, van Leyen K, Jung JE, You Z, Lo EH, Degterev A and Whalen MJ: Akt and mTOR mediate programmed necrosis in neurons. Cell Death Dis 5: e1084, 2014.
4. Wang W, Xie L, Zou X, Hu W, Tian X, Zhao G and Chen M: Pomelo peel oil suppresses TNF-α-induced necroptosis and cerebral ischaemia-reperfusion injury in a rat model of cardiac arrest. Pharm Biol 59: 401-409, 2021.
5. Niemann JT, Youngquist ST, Shah AP, Thomas JL and Rosborough JP: TNF-α blockade improves early post-resuscitation survival and hemodynamics in a swine model of ischemic ventricular fibrillation. Resuscitation 84: 103-107, 2013.
6. Janata A, Magnet IA, Uray T, Stezoski JP, Janesko-Feldman K, Tisherman SA, Kochanek PM and Drabek T: Regional TNFα mapping in the brain reveals the striatum as a neuroinflammatory target after ventricular fibrillation cardiac arrest in rats. Resuscitation 85: 694-701, 2014.
7. Drabek T, Wilson CD, Janata A, Stezoski JP, Janesko-Feldman K, Garman RH, Tisherman SA and Kochanek PM: Unique brain region-dependent cytokine signatures after prolonged hypothermic cardiac arrest in rats. Ther Hypothermia Temp Manag 5: 26-39, 2015.
8. Uray T, Dezfulian C, Palmer AA, Miner KM, Leak RK, Stezoski JP, Janesko-Feldman K, Kochanek PM and Drabek T: Cardiac arrest induced by asphyxia versus ventricular fibrillation elicits comparable early changes in cytokine levels in the rat brain, heart, and serum. J Am Heart Assoc 10: e018657, 2021.
9. Janata A, Magnet IA, Schreiber KL, Wilson CD, Stezoski JP, Janesko-Feldman K, Kochanek PM and Drabek T: Minocycline fails to improve neurologic and histologic outcome after ventricular fibrillation cardiac arrest in rats. World J Crit Care Med 8: 106-119, 2019.
10. Huang YJ, Liao JF and Tsai TH: Concurrent determination of thalidomide in rat blood, brain and bile using multiple microdialysis coupled to liquid chromatography. Biomed Chromatogr 19: 488-493, 2005.
11. Sampaio EP, Sarno EN, Galilly R, Cohn ZA and Kaplan G: Thalidomide selectively inhibits tumor necrosis factor alpha production by stimulated human monocytes. J Exp Med 173: 699-703, 1991.
12. Klausner JD, Freedman VH and Kaplan G: Thalidomide as an anti-TNF-alpha inhibitor: Implications for clinical use. Clin Immunol Immunopathol 81: 219-223, 1996.
13. Alam HB: Translational barriers and opportunities for emergency preservation and resuscitation in severe injuries. Br J Surg 99 (Suppl 1): S29-S39, 2012.
14. Majumder S, Sreedhara SR, Banerjee S and Chatterjee S: TNF α signaling beholds thalidomide saga: A review of mechanistic role of TNF-α signaling under thalidomide. Curr Top Med Chem 12: 1456-1467, 2012.
15. Zhang L, Qu Y, Tang J, Chen D, Fu X, Mao M and Mu D: PI3K/Akt signaling pathway is required for neuroprotection of thalidomide on hypoxic-ischemic cortical neurons in vitro. Brain Res 1357: 157-165, 2010.
16. Jung YJ, Tweedie D, Scerba MT and Greig NH: Neuroinflammation as a factor of neurodegenerative disease: Thalidomide analogs as treatments. Front Cell Dev Biol 7: 313, 2019.
17. Nora GJ, Harun R, Fine DF, Hutchison D, Grobart AC, Stezoski JP, Munoz MJ, Kochanek PM, Leak RK, Drabek T and Wagner AK: Ventricular fibrillation cardiac arrest produces a chronic striatal hyperdopaminergic state that is worsened by methylphenidate treatment. J Neurochem 142: 305-322, 2017.
18. Andrade P, Visser-Vandewalle V, Del Rosario JS, Daemen MA, Buurman WA, Steinbusch HW and Hoogland G: The thalidomide analgesic effect is associated with differential TNF-α receptor expression in the dorsal horn of the spinal cord as studied in a rat model of neuropathic pain. Brain Res 1450: 24-32, 2012.
19. Choi JI, Kim WM, Yoon MH and Lee HG: Antiallodynic effect of thalidomide and morphine on rat spinal nerve ligation-induced neuropathic pain. Korean J Pain 23: 172-178, 2010.
20. Hyakkoku K, Nakajima Y, Izuta H, Shimazawa M, Yamamoto T, Shibata N and Hara H: Thalidomide protects against ischemic neuronal damage induced by focal cerebral ischemia in mice. Neuroscience 159: 760-769, 2009.
21. Ribeiro RA, Vale ML, Ferreira SH and Cunha FQ: Analgesic effect of thalidomide on inflammatory pain. Eur J Pharmacol 391: 97-103, 2000.
22. Saito K, Suyama K, Nishida K, Sei Y and Basile AS: Early increases in TNF-alpha, IL-6 and IL-1 beta levels following transient cerebral ischemia in gerbil brain. Neurosci Lett 206: 149-152, 1996.
23. Ryu JK and McLarnon JG: Thalidomide inhibition of perturbed vasculature and glial-derived tumor necrosis factor-alpha in an animal model of inflamed Alzheimer's disease brain. Neurobiol Dis 29: 254-266, 2008.
24. Liu T, Clark RK, McDonnell PC, Young PR, White RF, Barone FC and Feuerstein GZ: Tumor necrosis factor-alpha expression in ischemic neurons. Stroke 25: 1481-1488, 1994.
25. Pettigrew LC, Kindy MS, Scheff S, Springer JE, Kryscio RJ, Li Y and Grass DS: Focal cerebral ischemia in the TNFalpha-transgenic rat. J Neuroinflammation 5: 47, 2008.
26. Ryu JK, Jantaratnotai N and McLarnon JG: Thalidomide inhibition of vascular remodeling and inflammatory reactivity in the quinolinic acid-injected rat striatum. Neuroscience 163: 601-608, 2009.
27. Schroeder DC, Maul AC, Mahabir E, Koxholt I, Yan X, Padosch SA, Herff H, Bultmann-Mellin I, Sterner-Kock A, Annecke T, *et al*: Evaluation of small intestinal damage in a rat model of 6 Minutes cardiac arrest. BMC Anesthesiol 18: 61, 2018.
28. Wender M, Michalowska-Wender G and Szczech J: Minimal changes of TNF-alpha and MCP-1 expression in blood serum of rats subjected to experimental cardiac arrest. Folia Neuropathol 43: 109-111, 2005.
29. Yang M, Hua T, Yang Z, Chen L, Zou Y, Huang X and Li J: The protective effect of rhBNP on postresuscitation myocardial dysfunction in a rat cardiac arrest model. Biomed Res Int 2020: 6969053, 2020.
30. Niemann JT, Rosborough JP, Youngquist S, Shah AP, Lewis RJ, Phan QT and Filler SG: Cardiac function and the proinflammatory cytokine response after recovery from cardiac arrest in swine. J Interferon Cytokine Res 29: 749-758, 2009.
31. Youngquist ST, Shah AP, Rosborough JP and Niemann JT: High serum tumor necrosis factor levels in the early post-cardiac arrest period are associated with poor short-term survival in a swine model of ventricular fibrillation. J Interferon Cytokine Res 36: 575-579, 2016.

32. Zhu F, Zhong X, Zhou Y, Hou Z, Hu H, Liang L, Chen J, Chen Q, Ji X and Shang D: Protective effects of nicorandil against cerebral injury in a swine cardiac arrest model. Exp Ther Med 16: 37-44, 2018.
33. Camara-Lemarroy CR, Guzman-de la Garza FJ, Alarcon-Galvan G, Cordero-Perez P, Munoz-Espinosa LE and Fernandez-Garza NE: Effects of thalidomide and pentoxyphylline over local and remote organ injury after intestinal ischemia/reperfusion. Transplant Proc 42: 1624-1626, 2010.
34. Fernandez-Martinez E, Morales-Rios MS, Perez-Alvarez V and Muriel P: Immunomodulatory effects of thalidomide analogs on LPS-induced plasma and hepatic cytokines in the rat. Biochem Pharmacol 68: 1321-1329, 2004.
35. Ilhan N, Susam S, Gul HF, Bardas R and Ilhan N: Which one is more effective for the treatment of rat sepsis model: Thalidomide or etanercept? Bratisl Lek Listy 118: 283-287, 2017.
36. Mohammed RA, El-Yamany MF, Abdel-Rahman AA, Nassar NN and Al-Shorbagy MY: Role of pERK1/2-NFκB signaling in the neuroprotective effect of thalidomide against cerebral ischemia reperfusion injury in rats. Eur J Pharmacol 895: 173872, 2021.
37. Lin CT, Lecca D, Yang LY, Luo W, Scerba MT, Tweedie D, Huang PS, Jung YJ, Kim DS, Yang CH, *et al*: 3,6'-dithiopomalidomide reduces neural loss, inflammation, behavioral deficits in brain injury and microglial activation. Elife 9: e54726, 2020.
38. Batsaikhan B, Wang JY, Scerba MT, Tweedie D, Greig NH, Miller JP, Hoffer BJ, Lin CT and Wang JY: Post-injury neuroprotective effects of the thalidomide Analog 3,6'-Dithiothalidomide on traumatic brain injury. Int J Mol Sci 20: 502, 2019.
39. Tsai YR, Tweedie D, Navas-Enamorado I, Scerba MT, Chang CF, Lai JH, Wu JC, Chen YH, Kang SJ, Hoffer BJ, *et al*: Pomalidomide reduces ischemic brain injury in rodents. Cell Transplant 28: 439-450, 2019.
40. Baratz R, Tweedie D, Wang JY, Rubovitch V, Luo W, Hoffer BJ, Greig NH and Pick CG: Transiently lowering tumor necrosis factor-α synthesis ameliorates neuronal cell loss and cognitive impairments induced by minimal traumatic brain injury in mice. J Neuroinflammation 12: 45, 2015.
41. Secher N, Ostergaard L, Tonnesen E, Hansen FB and Granfeldt A: Impact of age on cardiovascular function, inflammation, and oxidative stress in experimental asphyxial cardiac arrest. Acta Anaesthesiol Scand 62: 49-62, 2018.