Author's response to reviews

Title: Electroacupuncture at different frequencies (5Hz and 25Hz) ameliorates cerebral ischemia-reperfusion injury in rats by activating p38 MAPK-mediated anti-apoptotic signaling pathways

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Author's response to reviews: see over
Dear Dr. Rowles:

Thank you for your dealing with the manuscript entitled “Electroacupuncture at different frequencies (5Hz and 25Hz) ameliorates cerebral ischemia-reperfusion injury in rats by activating p38 MAPK-mediated anti-apoptotic signaling pathways”, coded as MS: 1767418987155096. The manuscript has been revised and compiled as the reviewers’ suggestions.

Our point-by-point reply to the reviewers’ comments is described as follows:

**Reviewer: Jakob Troppmair**

1. If p38 is involved in protective signaling and damage normally is visible after 1 day of reperfusion (neurological impairment, increased infarct area) p38 activation is required very early on. Thus suggesting that p38 activity found of day 7 was responsible for the protection may be true but not substantiated by any experimental data. This also has to be seen in the light that the authors also do not provide any evidence (e.g. through the use of inhibitors) that p38 signaling is really responsible for the protective effect observed. Thus if the paper should be published in its current form, the limitations clearly should be stated and the claims should be tuned down.

**Ans.:** (1) Previous studies have reported that mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathways are involved in the neuroprotective effects of electroacupuncture (EA) against cerebral ischemia/reperfusion (I/R) injury in mild transient focal cerebral ischemia [1, 2]. The MAPK family consists of JNK, ERK1/2, and p38 MAPK proteins, which play crucial roles in diverse cellular functions [3]. Studies have shown
that phospho-p38 MAPK expression in the ischemic cortex or hippocampus provides neuroprotective effects against cerebral I/R injury by activating antiapoptotic (Bcl-2, Bcl-xL, and cAMP response element-binding protein (CREB)) signaling pathways in focal [4], global ischemia [5], hypoxic [6], and anesthetic [7] preconditioning models. A study done by Luo et al. has also demonstrated that the activation of Akt exerts neuroprotective actions against ischemic brain injury by activating the CREB signaling pathway in a neonatal rat model of cerebral hypoxia-ischemia [8].

2. In order to clarify the relationship between neuroprotection and the involvement of signaling pathways, we performed quantitative immunoblot of phospho-MAPK, total MAPK, phospho-Akt (p-Akt) and total Akt. The representative western blot images of p-JNK, JNK, p-ERK1/2, ERK1/2, p-p38 MAPK, p38 MAPK, p-Akt and Akt were shown in Figure 3A, and we have drawn the bar graphs to show the ratios of phospho-MAPK/MAPK and p-Akt/Akt in Figures 3B, 3C, 3D, and 3E.

3. Our immunoblot analysis results indicate that p-p38 MAPK was derived from total p38 MAPK, and that p-p38 MAPK, but not p-JNK, p-ERK1/2, or p-Akt, expression was markedly downregulated in the cytosolic fraction in the cortical penumbra; however, this expression was effectively restored by EA at acupoints, at frequencies of 5Hz (EA-5Hz) and 25Hz (EA-25Hz), 7 d after reperfusion. Based on previous reports and our results, we reasonably deduce that EA-5Hz and EA-25 Hz both provide neuroprotection against cerebral I/R injury by activating p38 MAPK signaling in the ischemic cortical penumbra 7 d after reperfusion.
(4) We have recomposed the Discussion section from lines 13 p.19 to lines 5 p.21 according to the reviewer’s comments.

2. In the discussion of the apoptotic pathways there is no non-cytochrome c mitochondrial pathway, which leads to the activation of caspase-3.

**Ans.:** Studies have shown that cytosolic cytochrome c expression significantly increased at 4 h, peaked at 1 d, and thereafter gradually decreased at 7 d in the ischemic area after transient middle cerebral artery occlusion (MCAo) in rats [9, 10]. In the present study, cytosolic cytochrome c was detected in the ischemic cortical penumbra at 7 d after mild transient MCAo and results revealed that EA-5Hz and EA-25Hz exert nonsignificant inhibitory effects on cytosolic cytochrome c expression. Our findings are consistent with those of the previous studies, which showed nonsignificant differences in the cytosolic cytochrome c among the sham, model, and treatment groups at 7 d after mild transient MCAo [10, 11]. Previous studies have also demonstrated that caspase-3 plays a key role in the execution of apoptosis and significantly increased at 6 h, peaked at 24 h and decreased at 3-7 d in the penumbra after transient MCAo [12-14]. In the present study, we found that cytosolic cleaved caspase-3 expression was significantly higher in the model group than in the sham group in the ischemic cortical penumbra 7 d after mild transient MCAo. Our results are in line with an early study that used a model of mild transient focal cerebral ischemia to show the expression of cleaved caspase-3 7 d after reperfusion [11]. Based on these findings, we suggest that the time course of detection (7 d after
reperfusion) and the type of cerebral ischemia model (mild transient MCAo) may contribute to causing the different expression patterns of cytochrome c and cleaved caspase-3 between the sham and model groups. However, further investigations are needed to clarify this issue.

3. The DAPI/nuclear pCREB co-staining should be shown by preparing an overlay of the two images rather than using arrows

Ans.: In Figure 9 (p-CREB/DAPI costaining), the merged image produced using a fluorescent microscope (CKX41, Olympus) was an overlay image of the p-CREB and DAPI images.

Reference:


endothelial cells from apoptosis: a mechanism for the protective effect in stroke?


Please handle our manuscript at your convenience. Thank you for your kindly help.

Sincerely yours,

Chin-Yi Cheng