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Inhibition of Mitogen-Activated Protein Kinase Phosphatase-1 (MKP-1) Increases Experimental Stroke Injury

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Abstract

Background and Purpose—Activation of mitogen-activated protein kinases (MAPKs), particularly c-jun-N-terminal kinases (JNK) and p38 exacerbate stroke injury by provoking pro-apoptotic and pro-inflammatory cellular signaling. MAPK phosphatase-1 (MKP-1) restrains the over-activation of MAPKs via rapid de-phosphorylation of the MAPKs. We therefore examined the role of MKP-1 in stroke and studied its inhibitory effects on MAPKs after experimental stroke.

Methods—Male mice were subjected to transient middle cerebral artery occlusion (MCAO). MKP-1 knockout (KO) mice and a MKP-1 pharmacological inhibitor were utilized. We utilized flow cytometry, immunohistochemistry (IHC), and Western blots analysis to explore MKP-1 signaling and its effects on apoptosis/inflammation in the brain and specifically in microglia after stroke.

Results—MKP-1 was highly expressed in the nuclei of both neurons and microglia after stroke. MKP-1 genetic deletion exacerbated stroke outcome by increasing infarct, neurological deficits and hemorrhagic transformation. Additionally, delayed treatment of MKP-1 pharmacological inhibitor worsened stroke outcome in wild type (WT) mice but had no effect in MKP-1 KO mice. Furthermore, MKP-1 deletion led to increased c-jun-N-terminal kinase (JNK) activation and microglial p38 activation after stroke. Finally, MKP-1 deletion or inhibition increased inflammatory and apoptotic response as evidenced by the increased levels of interleukin-6 (IL-6), tumor necrosis factor α (TNFα), ratio of p-c-jun/c-jun and cleaved caspase-3 following ischemia.

Conclusions—We have demonstrated that MKP-1 signaling is an endogenous protective mechanism in stroke. Our data imply that MKP-1 possesses ant-apoptotic and anti-inflammatory properties by simultaneously controlling the activities of JNK and microglial p38.

Keywords

Cerebral ischemia; MKP-1; MAPK

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Introduction

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine protein kinases consisting of several members including extracellular signal-regulated kinase ½ (ERK½), c-jun-N-terminal kinases (JNK) and p38 (Nithianandarajah-Jones et al., 2012). MAPKs have crucial roles in signal transduction from the cell surface to the nucleus and regulate cell death and survival in both physiological and pathological conditions (Nozaki et al., 2001). The importance of MAKPs in stroke, especially JNK and p38, has been well documented in the literature. More specifically, JNK activation has been shown to increase stroke injury via enhancement of neuronal apoptosis and both genetic and pharmacological inhibition of JNK improved outcomes after stroke (Kuan et al., 2003; Cui et al., 2007). P38 signaling activation exacerbates stroke-induced inflammatory responses and also leads to worse stroke outcomes (Barone et al., 2001).

MAPK phosphatases (MKPs) inactivate MAPKs rapidly and directly. At least 10 MKPs have been identified, with MKP-1 being the archetypal member of the MKP family (Liu et al., 2007). MKP-1 is widely expressed in the CNS and is induced immediately in response to stressors (Horita et al., 2010). It is regulated at both transcriptional and post-translational levels to restrain MAPKs activity (Lawan et al., 2013). MKP-1 limits the activation of MAPKs through de-phosphorylation on regulatory threonine and tyrosine residues (Lawan et al., 2013). MKP-1 may have preferential activity for JNK and p38 among the three MAPKs (JNK, p38 and ERK½) (Farooq and Zhou, 2004).

Interestingly, MKP-1 has been implicated in the pathology of neurological disorders. A recent study demonstrated that MKP-1 is neuroprotective in Huntington's disease models, an effect attributed to its inhibition of JNK and p38 (Taylor et al., 2013). Additionally, MKP-1 directly inhibits microglia activation (Eljaschewitsch et al., 2006), a major contributor to the inflammatory response after ischemic injury. MKP-1 has been implicated in neuroprotection in several other models as well (Eljaschewitsch et al., 2006; Koga et al., 2012). Therefore, we hypothesized that MKP-1 would play a neuroprotective role after stroke by reducing ischemia-induced over-activation of MAPKs. In the present study we utilized mice deficient in MKP-1 and a MKP-1 inhibitor and evaluated infarct damage, behavioral deficits, and apoptotic/inflammatory signaling after stroke.

Methods

Animals

The current study was performed in accordance with the NIH guide for the care and use of laboratory animals. All protocols were approved by the Center for Laboratory Animal Care of University of Connecticut Health Center. MKP-1 KO mice were backcrossed into a C57BL/6J background for more than 9 generations (a kind gift from Dr. Carole Pilbeam at UConn Health Center). WT Littermates were used as controls for MKP-1 KO mice in stroke outcome studies. C57BL/6J WT mice used in MKP-1 inhibitor experiments were purchased from Charles River. There were no significant differences in size, gross physical or behavioral abnormalities between KO and WT control mice. Age- and weight- matched mice (20 to 25g, 9 to 12 weeks of age) were used in all experiments.

Middle cerebral artery occlusion

Focal transient cerebral ischemia (60 minutes middle cerebral artery (MCA) occlusion) was induced in MKP-1 KO and WT mice followed by reperfusion according to our previous publications (McCullough et al., 2013). After 60 min occlusion, the mice were re-anesthetized and reperfused by suture withdrawal. During the ischemic period, body temperature was controlled at a physiological level using a heating pad with feedback thermo-control system (FST). Cerebral blood flow and physiological parameters were evaluated as described previously (McCullough et al., 2013). Mice in sham group underwent the same procedure except occluding the MCA. Animals were randomized into stroke and surgical sham cohorts. Investigators who performed the procedures were blinded to drug treatment and genotypes.

Drug treatment

Three doses of *E)*-2-Benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI) (Molina et al., 2009), a MKP-1 inhibitor were intraperitoneally injected at 2, 24 and 48 hour after stroke onset. The first dose of the inhibitor was given 2 hours after the onset of stroke to see if MKP-1 could be a viable target for stroke treatment. Each dose was 2.5mg/kg and dissolved in saline containing 5% dimethyl sulfoxide (DMSO) and 1.25% Tween 20. Control mice received the equal amount of vehicle. For doses amount, our calculations were based on mice body size and EC_{50} data of BCI reported for MKP-1 in *in* vitro assay (Molina et al., 2009).

Behavior Measurement

Neurological deficits were scored at 72 hours after stroke on a four-point scale as described previously (McCullough et al., 2013). 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling.

Infarct measurement

Briefly, after the animals were sacrificed at 72 hours after stroke, the brains were immediately removed and cut into 5 individual 2-mm slices. Brain slices were stained with 1.5% 2, 3, 5-triphenyltetrazolium (TTC) at 37 °C for 8 minutes. Images were digitalized, and the infarct volumes (corrected for edema) were analyzed using computer software (Sigmascan Pro5) as previously described (McCullough et al., 2013).

For **hemoglobin assay, immune-blotting, immunohistochemistry, flow cytometry, enzyme-linked immunosorbent assay (ELISA) analysis, cerebral angioarchitecture and statistics analysis**, please refer to supplemental materials.

Results

Stroke induced MKP-1 expression

Six hours after the onset of ischemia, a significant increase of MKP protein expression in stroke mice was observed when compared to shams by both Western blot (Figures 1A, n=3 p/g, $p<0.05$ sham versus stroke) and immunohistochemistry analysis (Figures 1B, n=3

p/g, p<0.05 sham versus stroke). Additionally, we found that MKP-1 co-localized with the microglial marker IBA1 and neuronal marker NeuN (Figures 1C and D). MKP-1 was found to be primarily localized in the nuclei as its staining co-localized with DAPI (Figures 1C and D).

Genetic deletion of MKP-1 aggravated stroke injury

MKP-1 KO mice had significantly larger infarcts in the cortex, striatum and total hemisphere (Total: WT 40.6±2.1% versus KO 55.1±1.5%, n=7 p/g, p<0.05) 72 hours after stroke onset (Figure 2A). These larger infarcts were also reflected in the neurological deficits scores (ST1). Furthermore, genetic deletion of MKP-1 increased hemorrhagic transformation as measured by hemoglobin assay 72 hours after stroke (WT 114.9±21.2 μg/hemisphere versus KO 260.9±44.2 μg/hemisphere, n=7 p/g, p<0.05) (Figure 2C). There was no difference in mortality rates between MKP-1 KO and WT mice after stroke (1 out 8 in each group).

No differences were seen in mean arterial pressure, blood glucose, pO2, pCO2, or pH between the KO and WT controls. In addition, local cerebral blood flow as measured by laser Doppler flow, was equivalently reduced during ischemia and restored equally in early reperfusion in both genotypes (ST1). Furthermore, no significant differences in cerebral angioarchitecture were seen between the KO and WT controls (SF2).

Post-stroke pharmacological inhibition of MKP-1 exacerbated stroke outcome

We then employed an MKP-1 pharmacological inhibitor, BCI to confirm the deleterious effects of MKP-1 genetic deletion after stroke. In line with the results using the genetic approach, BCI significantly worsened infarct volume assessed 72 hours following the onset of ischemia in WT mice (n=6 vehicle/n=8 BCI) in cortex, striatum and total hemisphere (total: vehicle 43.5±2.6% versus BCI 56.8±3.5%, p<0.05) (Figure 2B). Additionally, neurological deficits were aggravated in the BCI treated mice (ST2). Furthermore, BCI treatment significantly increased hemorrhagic transformation as measured by hemoglobin assay 72 hours after stroke (vehicle 105.6±20.5 μg/hemisphere versus BCI 193.1±16.9 μg/hemisphere, n=6 vehicle/n=8 BCI, p<0.05) (Figure 2D).

To verify the specificity and mechanism of the inhibitor in our model, we injected BCI to MKP-1 KO mice and studied outcome. In the absence of MKP-1, BCI had no effects in infarct volume or neurological deficits scores (n=8 vehicle/n=7 BCI) (Figure 2E and ST2).

MKP-1 genetic deletion increased the levels of p-JNK after stroke

Here we focused on p-p38, p-ERK½ and p-JNK expression in MKP-1 KO mice as studies showed these three MAPKs are possible substrates of MKP-1 (Farooq et al., 2004). Our Western results showed that p-JNK level in the ischemic hemisphere was significantly up-regulated in MKP-1 KO mice compared to WT controls 6 hour after the onset of stroke (Figure 3A and 3C, p<0.05 WT stroke versus KO stroke n=3 p/g). However, p-p38 and p-ERK½ levels in the ischemic hemispheres did not differ between the MKP-1 KO and WT controls 6 hours after stroke (Figure 3B, 3D, SF2, for p-p38, n=2 in WT sham, KO sham and KO stroke; n=3 in WT stroke, For p-ERK½, p>0.05 WT stroke versus KO stroke, n=2 p/g).

Loss of MKP-1 led to p38 activation in microglia after stroke

As MKP-1 has been suggested to control inflammation by inhibiting p38 and ERK in microglia (Eljaschewitsch et al., 2006; Yingqian et al., 2011), we specifically investigated the change of p-p38 and p-ERK levels in microglia in the absence of MKP-1. Interestingly, using flow cytometry, we found that the percentage of microglia positive for p-p38 was indeed increased 6 hours after stroke in MKP-1 KO mice (Figures 4A and 4B) (WT stroke 8.6±2.0% versus KO stroke 16.1±2.1%, $p<0.05$, n=4 in WT sham, WT stroke and KO sham; n=3 in KO stroke). Furthermore, p-MK2, a downstream molecule of p-p38, was also activated in microglia in KO stroke mice (WT stroke 4.4±0.7% versus KO stroke 7.5±0.4%, $p<0.05$, n=4 in WT sham; n=3 in WT stroke, KO sham and KO stroke) (Figure 4A and 4C). However, we did not see any change in the percentage of microglia positive for p-ERK½ (n=3 p/g) (Figures 4A and 4D).

MKP-1 deletion activated apoptotic cell death pathway after stroke

As anti-apoptosis is an important property of MKP-1, we then studied the activation of apoptotic signaling pathways in the MKP-1 KO mice after stroke. C-jun is a direct downstream target of JNK and critical mediator of apoptotic cell death. Consistent with changes of activated JNK, the ratio of p-c-jun/c-jun was significantly higher in MKP-1 KO mice when compared to WT controls at 6 hours after the onset of stroke (n=3 p/g, $p<0.05$) (Figures 5A and 5B) although p-c-jun and c-jun levels were not significantly changed in MKP-1 KO mice (Quantified data not shown). Additionally, we observed that MKP-1 deletion up-regulated levels of cleaved-caspase 3 compared to WT controls at 6 hours after the onset of stroke (n=2 in sham and n=3 in stroke, $p<0.05$) (Figure 5C and 5D) although cleaved-caspase 8 levels did not differ between the MKP-1 KO stroke mice and WT controls (n=3 p/g) (Figures 5E and 5F).

MKP-1 inhibition led to aggravated inflammatory response after stroke

As previous compelling evidence and our present data demonstrating increased microglial p38 in KO mice have suggested MKP-1 is a key inhibitor of inflammatory response, we investigated serum cytokine levels of IL-6, IL-10 and TNFα levels in the MKP-1 inhibitor treated mice after stroke. MKP-1 inhibitor treatment enhanced the inflammatory response as indicated by the higher levels of IL-6 (vehicle stroke 42.1±9.3 pg/ml versus BCI stroke 102.0±13.9 pg/ml, $p<0.05$, n=3 in vehicle sham, BCI sham and vehicle stroke; n=4 in BCI stroke)(Figure 6A) and TNF-α (vehicle stroke 68.9±18.2 pg/ml versus BCI stroke 170.6±32.2 pg/ml, $p<0.05$, n=3 in vehicle sham and BCI stroke; n=4 in vehicle stroke; n=2 in BCI sham) (Figure 6B) assessed at 72 hours after stroke. However, no difference was seen in IL-10 levels between vehicle treated and drug treated groups (Figure 6C).

Discussion

The present study identified a novel molecular signaling pathway in stroke disease by making the following significant new findings. First, we demonstrated the MKP-1 is induced after stroke; we showed that MKP-1 is highly expressed in neurons and microglia following stroke with its expression primarily restricted in nuclei. Second, we found that pharmacological inhibition or genetic deletion of MKP-1 was detrimental in cerebral

ischemia by increasing infarcts, neurological deficits and HT. Third, we showed that MKP-1 deletion significantly up-regulated p-JNK signaling after cerebral ischemia, as indicated by changes in p-JNK and p-c-Jun/c-Jun levels in the ipsilateral hemispheres. Fourth, MKP-1 deletion led to activation of p38 and its downstream molecule MK2 signaling in microglia. Finally, we revealed that MKP-1 pathway inhibition resulted in activation of both pro-apoptotic and pro-inflammatory molecules including cleaved-caspase-3, IL-6 and TNFα.

The exacerbated stroke injury in MKP-1 KO mice and BCI-treated WT mice implied that MKP-1 normally mediates neuroprotective signaling. MKP-1, a phosphatase of MAPKs, is rapidly induced in nuclei by many of the same stress conditions that activate MAPKs (Liu et al., 2007). In a neuroblastoma cell line subjected to hypoxia/reoxygenation, knock-down of MKP-1 using SiRNA enhanced pJNK levels and neuronal death that was rescued by JNK inhibitor. In other studies MKP-1 over-expression reduced levels of pJNK, the expression of proapoptotic genes, and decreased cell death (Koga et al., 2012). These data suggested that MKP-1 signaling enhances survival under hypoxic stress, consistent with our results. In a most recent study, it was proposed that MKP-1 may enhance hippocampal neuronal apoptosis in a global ischemia model as it inhibited C/EBPβ, which has been shown to have anti-apoptotic properties (Rininger et al., 2012). However, in a focal cerebral ischemia model, C/EBPβ loss-of-function was in fact neuroprotective (Kapadia et al., 2006). It is very likely MKP-1's functions are dependent on the particular cellular and pathological contexts. Our pharmacological approach clearly confirmed our results obtained using the genetic deletion model, suggesting MKP-1 signaling is pro-survival in stroke.

To the best of our knowledge, this is the first study in which a pharmacological MKP-1 inhibitor was employed in a stroke model. BCI functions by locking its substrate phosphatases in low activity conformation (Molina et al., 2009). Of note, it has been shown that, besides inhibiting MKP-1, BCI also has shown effects on MKP-3 in a transgenic zebrafish chemical screen model (Molina et al., 2009). However, in our study, BCI completely lost its detrimental effect at the absence of MKP-1, suggesting that MKP-1 was the specific target of this compound in our stroke model. It is plausible that MKP-3 plays a less important role in neuroprotection in stroke. MKP-3 resides in the cytosol and is an ERK-specific MKP. It is often induced by neuronal differentiation but not by mitogens or stress (Muda et al., 1996). Indeed, in a Huntington disease model, MKP-3 manipulation was not effective in cellular survival/death and had no effects on JNK and p38 activities (Taylor et al., 2013). Our data and others indicate that MKP-1 may possess a unique profile of enzymatic and neuroprotective activities in stroke compared with MKP-3 (Taylor et al., 2013).

MKP-1 serves as a convergence point in the MAPK pathway (Boutros et al., 2008). In our work, we found MKP-1 deletion increased the levels of both p-JNK and p-p38 in microglia and while no effect was observed on p-ERK. Other studies also found that MKP-1 was specific for JNK and p38. In mouse embryonic fibroblasts, MKP-1 deletion had no effects on ERK activation during serum stimulation (Dorfman et al., 1996). Additionally, MKP-1 deficiency enhanced LPS stimulated JNK and p38 activities but had little effect on ERK activity in primary macrophage cell culture (Zhao et al., 2006). In a Huntington disease

model in rats, MKP-1 afforded its neuroprotection through functioning as a phosphatase for pJNK and p-p38, not p-ERK (Taylor et al., 2013). Therefore, ours and others work (Zhao et al., 2006), imply that under most physiological as well as pathological conditions, JNK and p38, but not ERK, are the preferred targets of MKP-1.

In this study, we evaluated the effect of MKP-1 in apoptosis after stroke as the majority of published work suggests an anti-apoptotic role of this molecule in stressed neurons. In cultured sympathetic neurons, MKP-1 overexpression led to JNK inhibition and reduced apoptosis induced by NGF (nerve growth factor) withdrawal while MKP-1 knockdown accelerated NGF withdrawal-induced death (Kristiansen et al., 2010). As described previously, MKP-1 suppressed neuronal apoptosis through JNK signaling inhibition in neuroblastoma cell line culture in a hypoxia-reoxygenation model (Koga et al., 2012). In line with others work, our work demonstrated an induction of MKP-1 in the nuclei of neurons 6 hours after stroke and our data additionally showed the ratio of p-c-jun/c-jun was increased in MKP-1 KO stroke mice, so did cleaved caspase-3, the executioner caspase in apoptotic cell death pathway. Activated caspase-8 was not changed with MKP-1 deletion, suggesting the extrinsic apoptotic pathway, which is activated by caspase-8 (Tait et al., 2010), may play a less important role than the intrinsic pathway in MKP-1/JNK apoptotic signaling. The underlying mechanisms warrant further investigation; however, our data clearly showed that MKP-1 serves as an endogenous suppressor of apoptotic cell death after stroke.

Our work also revealed a critical role of MKP-1 in restraining inflammatory response after stroke. Compelling evidence has shown that MKP-1 is a key negative modulator of innate immune responses, as well as a regulator of adaptive immune responses (Korhonen et al., 2013). For instance, MKP-1 KO macrophages produced excessive amounts of cytokines, including TNFα, IL-6, and IL-10 (Zhao et al., 2006). In patients with multiple-sclerosis, MKP-1 co-localized with invading microglia and MKP-1 inhibited microglia activation in NMDA induced toxicity models in co-culture of brain slice and microglia (Eljaschewitsch et al., 2006). Interestingly, we found MKP-1 was highly expressed in the nuclei of microglial cells after stroke and MKP-1 inhibition significantly induced increased levels of pro-inflammatory cytokines IL-6 and TNFα mice 6 hours after stroke. Additionally, our flow cytometry data demonstrated that MKP-1 deletion specifically enhanced p-p38 levels and its downs stream molecule p-MK2 in microglia, while no effect was detected when p-38 was simply examined in the whole stroke hemispheres. Our results suggest MKP-1 specifically targets p38 in microglia and its anti-inflammatory effects are mediated by p38. Microglial cells are the main resident immune cells in the central nervous system and is activated rapidly (within minutes) by cerebral ischemia (Patel et al., 2013). Microglia activation plays critical roles in brain ischemia, inflammation and neurodegeneration (Patel et al., 2013). p38 activation in microglia through various pathways has been demonstrated to be essential for IL-1, IL-6, TNFα, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) expression (Koistinaho and Koistinaho, 2002). Indeed, pharmacological inhibition of p-p38 restrained microglia activation and reduced stroke injury (Piao et al., 2003). The mechanisms are not completely clear but it is thought that p38 enhances the stabilization and promotes translation of mRNAs which encode the pro-inflammatory molecules. Taken together, our work showed MKP-1 may limit microglia activation induced inflammatory response by inhibiting microglial p38 in stroke. Of note, TNFα is known to enhance

activation and trafficking of neutrophils (Pouliot et al., 1999), which is well documented to aggravate BBB disruption in the ischemic brain by releasing matrix metalloproteinases (MMPs) (McCullough et al., 2013). Therefore, the exacerbated inflammatory response seen with MKP-1 inhibition may also shed light onto the increased hemorrhagic transformation observed in both the KO mice and inhibitor treated mice following stroke.

In summary, we demonstrated the detrimental effects of MKP-1 inhibition in cerebral ischemia. Our data suggested that MKP-1 simultaneously targets p-JNK and microglial p-p38 signaling after stroke, subsequently inactivating pro-apoptotic and pro-inflammatory pathways. Compelling evidence has now suggested that both inflammation and apoptotic responses are major contributors of neuronal death after stroke, particularly in the delayed phase. Our work implies targeting MKP-1 could have potential value for stroke treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Mitogen-activated protein kinases (MAPKs) are known to be detrimental in stroke
- MAPK phosphatase-1 (MKP-1) pharmacological/ genetic inhibition exacerbated injury
- MKP-1 signaling may be an endogenous protective mechanism in stroke

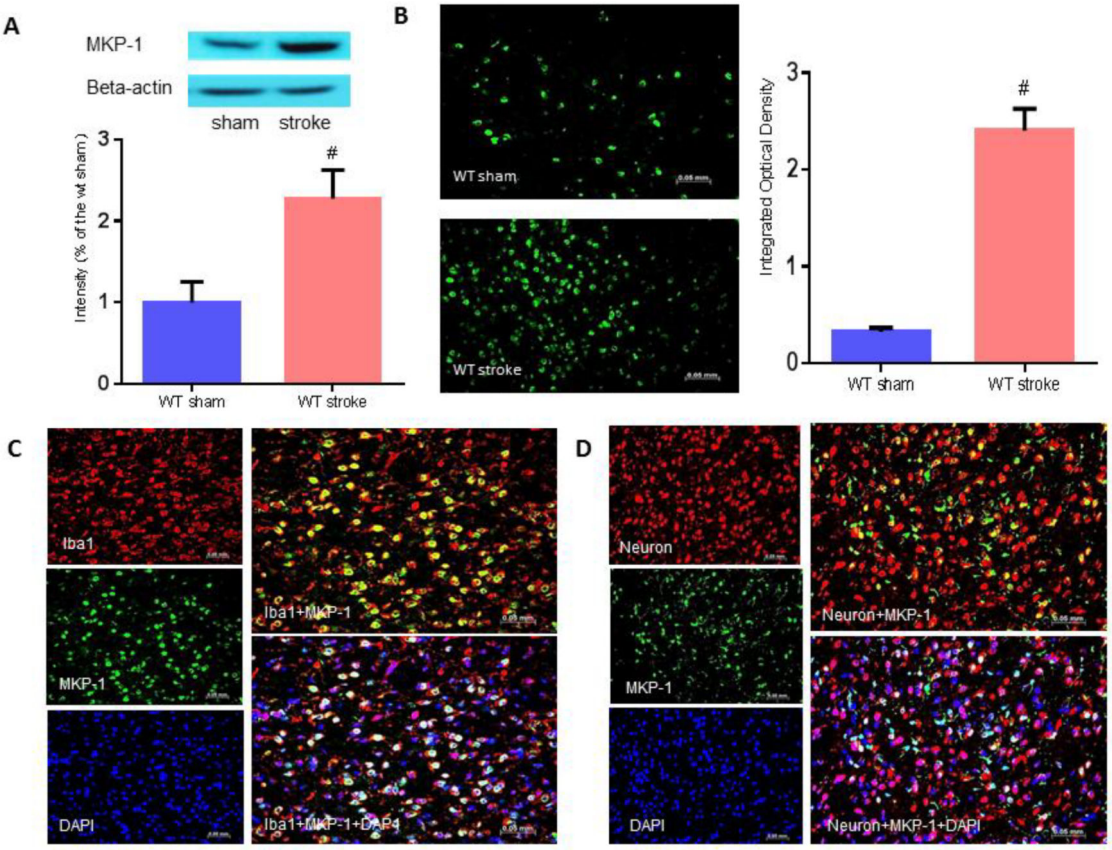

Fig. 1. MKP-1 was induced 6 hours after the onset of stroke.

A and B, Representative images and quantified data of Western blot and immunohistochemistry(IHC) analysis for MKP-1 expression; C and D, MKP-1 co-localized with nuclei of microglia and neurons. Samples for Western blots and IHC were prepared from brains collected at 6 hours after the stroke (60 minutes MCAO) onset. n=3 p/g. #p<0.05: WT sham versus WT stroke (Student t-test). Data were presented as Mean± SEM.

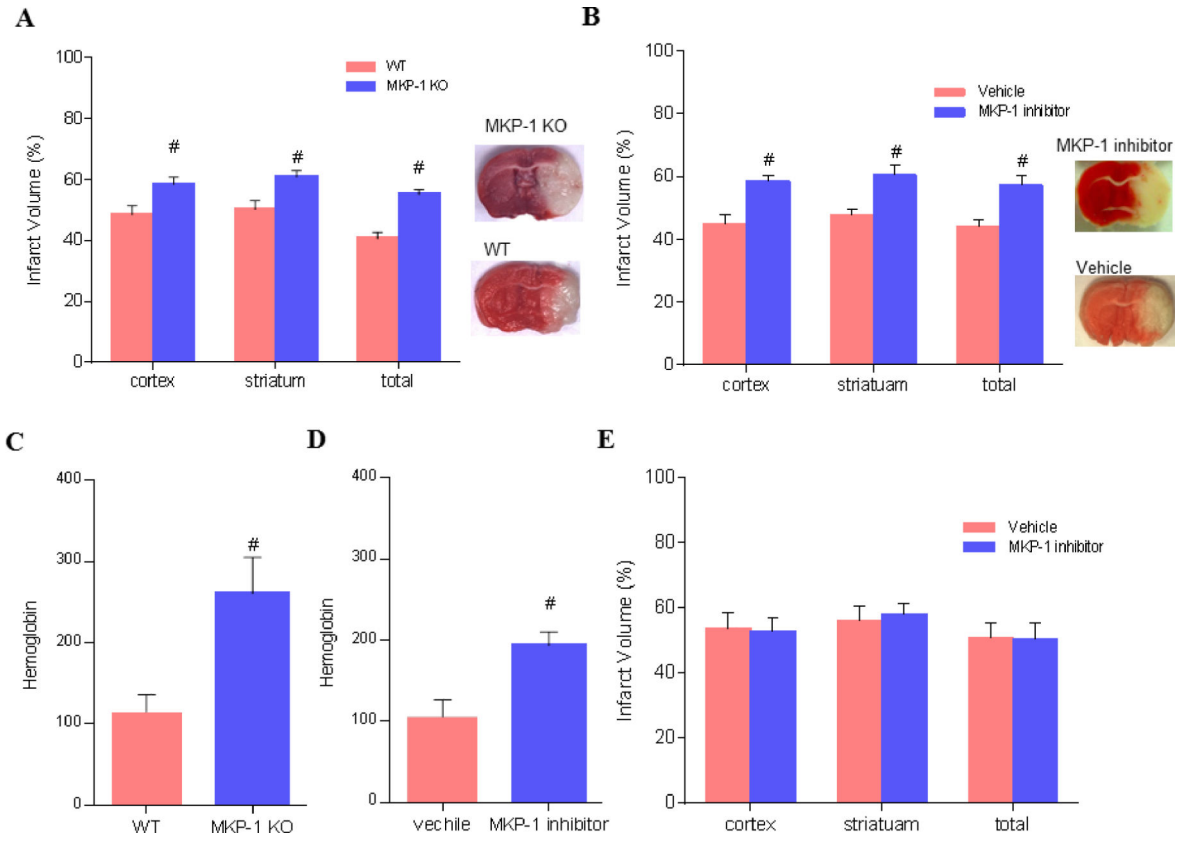

Fig. 2. Deletion or inhibition of MKP-1 exacerbated stroke outcome in mice.

A, Infarct volumes and representative TTC stained brain slices of MKP-1 KO mice and WT controls; n=7 p/g; B. Infarct volumes and representative TTC stained brain slices of mice treated with MKP-1 inhibitor BCI or vehicle. n=6 vehicle/n=8 MKP-1 inhibitor; C and D, Hemoglobin content was higher in MKP-1 KO and MKP-1 inhibitor treated stroke groups at 72 hours after stroke; The unit for the hemoglobin concentration is μg/per hemisphere. in figure C, n=7 p/g; in figure D, n=6 vehicle/n=8 MKP-1 inhibitor; E, BCI had no effects in infarcts in MKP-1 KO mice. n=8 vehicle/n=7 MKP-1 inhibitor. BCI (2.5mg/kg per dose) were intraperitoneally injected at 2, 24 and 48 hour after stroke onset (60 minutes MCAO). Control animals received the equal amount of vehicle. Cortical, striatal, and total hemisphere infarction volumes assessed at 72 hours after stroke onset were calculated as the percentage of non-ischemic hemisphere. # $p<0.05$: versus controls. Student t-test was used to compare means. Data were presented as Mean±SEM.

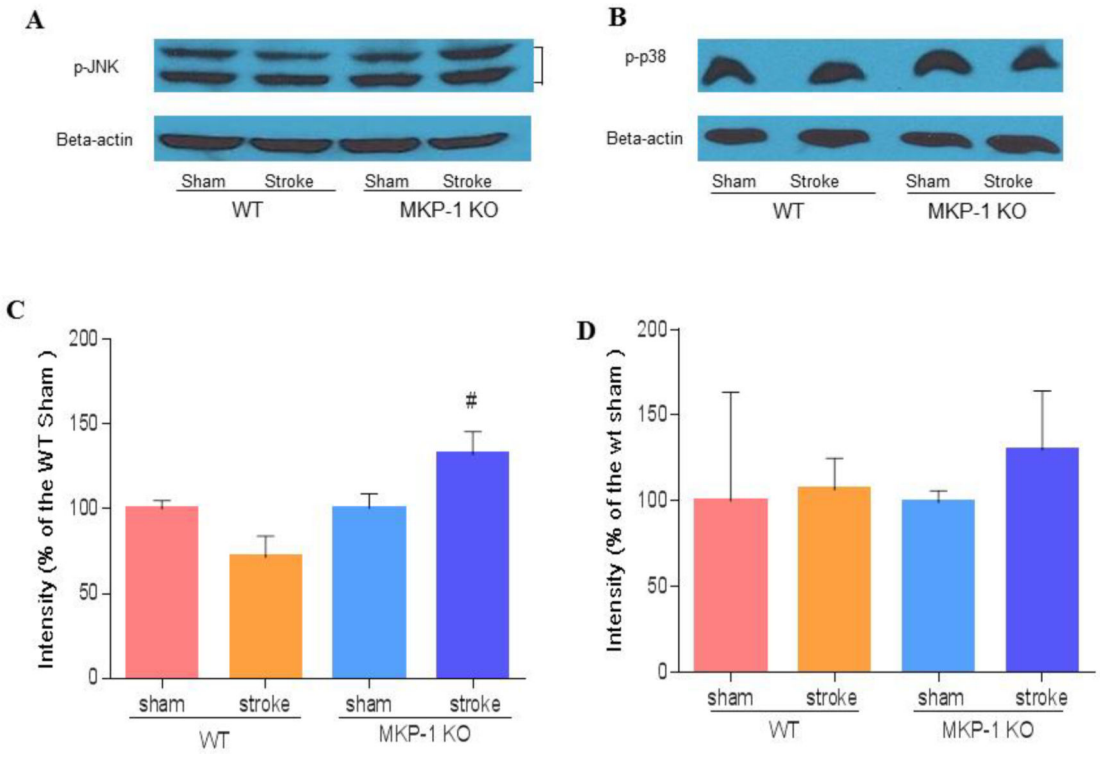

Fig. 3. Deletion of MKP-1 up-regulated p-JNK 6 hours after stroke.

A, B, C and D, Representative Western blots and quantified densitometry data of p-JNK and p-p38; Brains were collected 6 hours after onset of MCAO or sham operation. C: n=3 p/g; D: n=2 WT sham, KO sham and KO stroke; n=3 WT stroke; # $p<0.05$: WT stroke versus MKP-1 KO stroke (one-way ANOVA with post-hoc test). Data were presented as Mean± SEM.

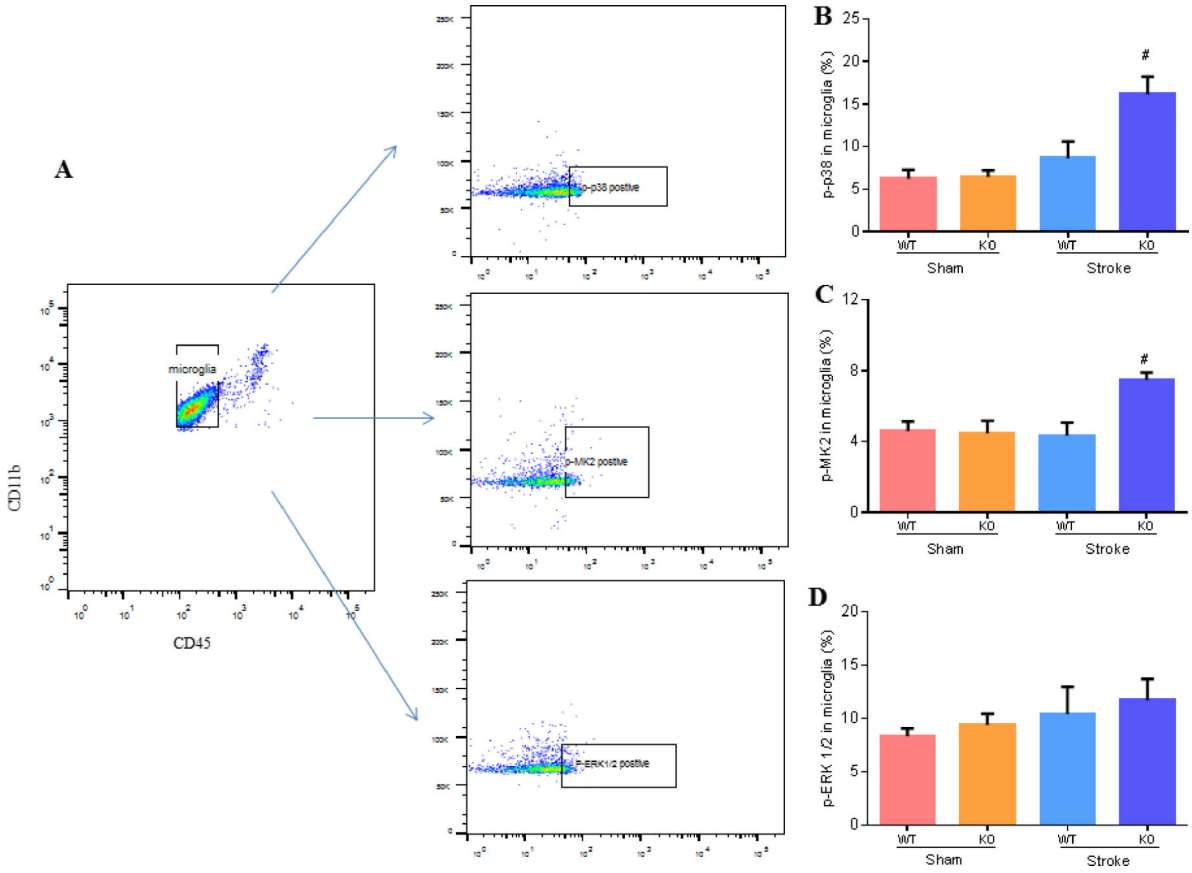

Fig. 4. MKP-1 deletion led to activation of p38 in microglia 6 hours after stroke.

A, Representative gating of microglia gating and gating of microglia that are positive for p-p38, p-MK2 and p-ERK½ respectively; Microglia was identified as cells expressing both low to intermediate levels of CD45 and positive for CD11b expression; B, C and D, Quantification of the percentages of microglia that are positive for p-p38, p-MK2 and p-ERK½ respectively. figure B: n=4 in KO sham, WT sham and WT stroke; n=3 in KO stroke. In figure C: n=4 in WT sham; n=3 in WT stroke, KO sham and KO stroke. In figure D: n=3 p/g. # $p<0.05$: WT stroke versus MKP-1 KO stroke (one-way ANOVA with post-hoc test). Data were presented as Mean± SEM.

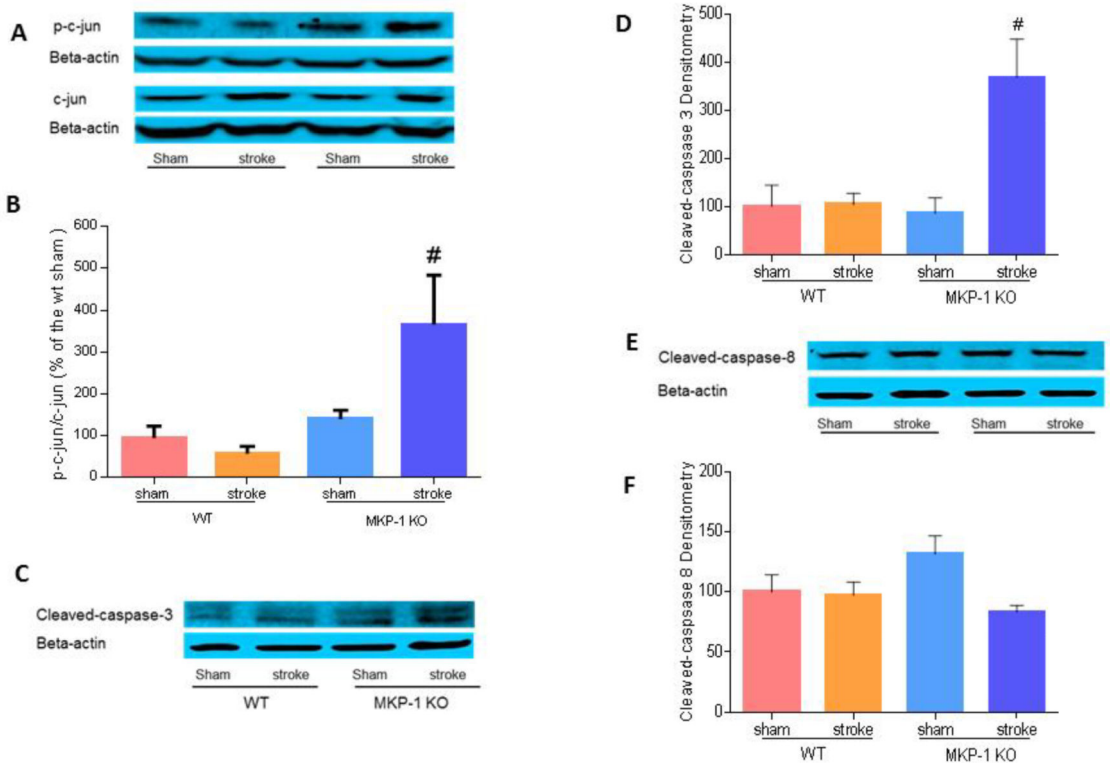

Fig.5. levels of p-c-jun, c-jun, cleaved-caspase 3 and cleaved-caspase 8 in MKP-1 KO mice 6 hours after stroke.

A,C and E, Representative Western blots of p-c-jun, c-jun, cleaved-caspase 3 and cleaved-caspase 8 in WT and MKP-1 KO mice; B, D and F, Quantified densitometry data of ratios of p-c-jun to c-jun, cleaved-caspase 3 and cleaved- caspase 8; Brains were collected 6 hours after onset of MCAO or sham operation. # $p<0.05$: WT stroke versus MKP-1 KO stroke (one-way ANOVA with post-hoc test); Data were presented as Mean± SEM. In figures B and F, n=3 p/g; In figure D, n=2 in sham; n=3 in stroke.

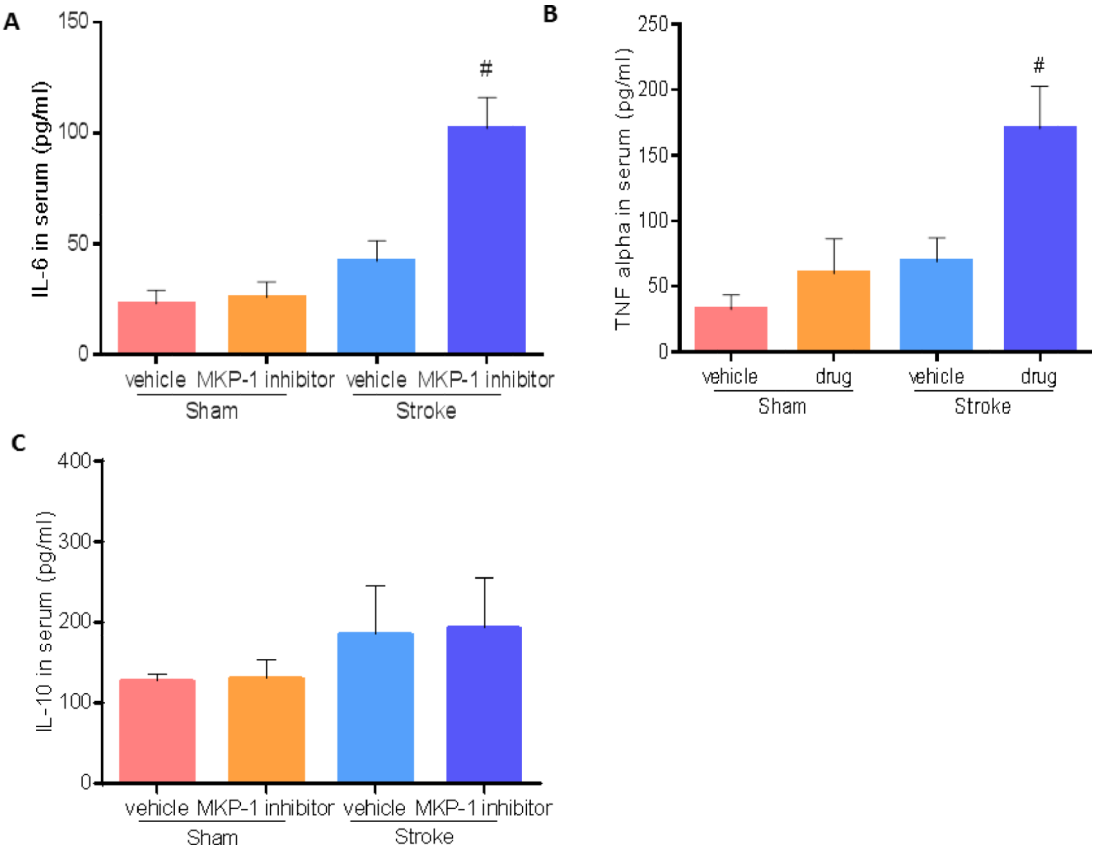

Fig. 6. Levels of IL-6, TNFα and IL-10 in MKP-1 inhibitor-treated mice 72 hours after stroke.

BCI (2.5mg/kg per dose) were intraperitoneally injected at 2, 24 and 48 hour after stroke onset (60 minutes MCAO). Control animals received the equal amount of vehicle. Serum was collected at 72 hours after stroke and cytokines were analyzed by ELISA kits. Figure A: n=4 in inhibitor stroke; n=3 in other groups. Figure B: n=3 in vehicle sham and inhibitor stroke; n=4 in vehicle stroke; n=2 in inhibitor sham. Figure C: n=3 in vehicle sham and stroke; n=4 in inhibitor stroke; n=2 in inhibitor sham. #$p<0.05$: vehicle stroke versus MKP-1 inhibitor stroke (one-way ANOVA with post-hoc test); Data were presented as Mean± SEM.