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# The effects of IL-32 on the inflammatory activation of cultured rat primary astrocytes

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ABSTRACT

A new family of cytokine IL-32 has been implicated in pro-inflammatory immune responses several human diseases such as rheumatoid arthritis, inflammatory bowel diseases and vasculitis. In this study, we investigated the role of IL-32 in the inflammatory activation of cultured rat primary astrocytes. Treatment of IL-32 increased ROS production and augmented lipopolysaccharide-induced increased production of nitric oxide as well as the expression of iNOS. IL-32 also induced the expression of MMP-9 but not MMP-2 in rat primary astrocytes. The increased expression of these inflammatory mediators was accompanied by the increased mRNA expression encoding iNOS, MMP-9 and TNF-α. ERK1/2 and p38, two essential regulators of pro-inflammatory signaling in rat primary astrocytes were activated by IL-32 as evidenced by increased phosphorylation. The results from the present study suggest that IL-32 may play a role in the regulation of neuroinflammatory responses in several neurological disease conditions such as ischemia and Alzheimer's disease.



## 1. Introduction

Astrocytes, which constitute more than 50% of the entire brain, plays important role in a myriad of physiological and pathological processes in the brain such as maintenance of blood–brain barrier, trophic supports for neurons, maintenance of extracellular milieu as an ideal environment for the continued neural transmission as well as regulation of inflammatory responses against pathological brain insults and invading pathogens. Due to the critical involvement of neuroinflammatory processes in the CNS diseases such as neurodevelopmental disorders, the effects of cytokines in the regulation of physiology of glial cells draw much attention. It is relatively well known that cytokines such as IL-1β, TNF-α and IFN-γ induces inflammatory responses in astrocytes [1–3]. Resting or immunostimulated astrocytes express immune modulatory molecules including MHC class II and induce the expression of various cytokines and chemokines such as GM-CSF, TNF-α, IL-6 and RANTES. In addition, reactive astrocytes produce higher amount of ROS and RNS including nitric oxide and peroxynitrite, which may involve the upregulation of enzymes involved in the production of ROS and RNS including NADPH oxidase and iNOS as well as the decreased antioxidant defense capacity in the cell. Other inflammatory mediators such as COX-2 and MMP-9 are upregulated in response to inflammatory stimuli and these molecules may participate in secondary inflammatory responses in the brain as well as in direct neurotoxic responses.

IL-32, which is a recently described strong inflammatory cytokines, has been associated with the induction of inflammatory responses in various cell types by secreting TNF-α, IL-6 and IL-1β. Although structurally distinct from any known cytokine families, its role in inflammatory diseases such as rheumatoid arthritis, vasculitis, ulcerative colitis and Crohn's disease [4], has been suggested based on its action on the up-regulation and secretion of pro-inflammatory cytokines [5]. For example, it has been suggested that IL-32β increased adhesion of immune cells to the activated endothelial cells, possibly via the induction of vascular cell adhesion molecules and inflammatory cytokines. These results suggest that IL-32 may play an essential role in the regulation of vascular inflammation [6].

However, there is no report yet whether IL-32 may modulate inflammatory responses in brain. As a first step to investigate the role of IL-32 in the regulation of neuroinflammation in various pathological conditions such as ischemia and Alzheimer's disease, we investigated whether IL-32 induces inflammatory activation of astrocytes.

## 2. Materials and methods

### 2.1. Materials

Purified recombinant IL-32 was prepared as described [7]. The recombinant IL-32 was purified with high-performance liquid

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chromatography and tested on a *Limulus* amebocyte lysate (LAL) assay. The endotoxin level was lower than 0.5 EU per 1 μg of protein, as determined by the LAL method. Lipopolysaccharide (LPS) (026:B6) was purchased from Sigma (St. Louis, MO). Glucose-free DMEM, penicillin–streptomycin and fetal bovine serum (FBS) were from Gibco BRL (Grand Island, NY). All other reagents were obtained from Sigma (St. Louis, MO).

### 2.2. Astrocyte cell culture and treatment

Rat primary astrocytes were prepared as described [8]. In brief, 2 day-old neonatal Sprague Dawley rats were sacrificed and brains were removed. A single cell mixture was prepared by mild trypsin-DNAse I digestion and trituration processes and the cells were passed through a 100-mm mesh filter in DMEM containing 10% fetal bovine serum. The cells were seeded at a density of two cortices/75 $cm^2$ flask. The cells were grown in DMEM/F12 containing 10% fetal bovine serum until they reach confluency. The cells were harvested with trypsin–EDTA and re-plated on 12- or 6-well plates and were grown in above media for 6–7 days before experiments. The cells were usually 95% pure with small amount of microglial contamination. Cells ($5 \times 10^5$) were treated with IL-32 diluted in culture media with or without LPS (10 ng/ml) stimulation. In experiments investigating the role of IL-32 on MMPs and tPA activity, cells were washed two times with serum free media before treatment to remove the contaminating MMPs and tPA activity from serum.

### 2.3. Measurement of ROS production

Cultured rat primary astrocytes were incubated with 2,7-dihydrodichlorofluorescein diacetate(DCF-DA, 5 μg/ml) in PBS for 10 min and then rinsed with the same solution. After 30 min incubation at room temperature, the fluorescence of DCF was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm using a fluorescence microplate reader (Spectramax Gemini EM, Molecular Devices, CA). Autofluorescence was subtracted from the reading by determining the fluorescence intensity of the cells not loaded with DCF-DA.

### 2.4. Determination of NO

NO production from the immunostimulated cells was determined by Greiss reaction measuring nitrite, a stable oxidation product of NO, as described previously [8]. In brief, nitrite levels were determined by adding the Griess reagent (mixing equal volumes of 0.1% napthylethylenediaminedihydrochlroride and 1% sulfanilamide in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was determined using an ELISA reader (SPECTRAMAX 190, Molecular Devices, CA).

### 2.5. Gelatin zymography

The activity of MMP-9 and MMP-2 was determined semi-quantitatively by zymography as described by us [9]. In brief, samples were mixed with 4 x SDS sample buffer (8% w/v SDS, 40% glycerol, 200 mM Tris–HCl, pH 6.8, and 0.02% bromophenol blue) devoid of reducing agent (dithiothreitol) to minimize irreversible denaturation of MMPs activity. Protein bands were resolved by electrophoresis at 180 V for 1 h on 8% polyacrylamide gel containing 0.1% SDS and gelatin at a final concentration of 1 mg/ml. Gels were washed twice in 2.5% Triton X-100 for 30 min each to remove the SDS and then incubated for 24 h at 37 °C in reaction buffer (20 mM Tris–HCl, 166 mM $CaCl_2$, pH 7.6). After staining the gel with 0.1% Coomassie Brilliant Blue R-250, gelatinolytic activities were visualized as a clear band in the uniformly stained background.

### 2.6. Casein zymography

tPA and PAI-1 activity was measured by casein zymography and one phase inverse zymography, respectively as described previously [10,11]. Briefly, samples were mixed with zymography buffer and loaded on 10% polyacryl amide gel containing casein (1 mg/ml, Sigma, St. Louis, MO) and plasminogen (13 μg/ml; American Diagnostica, Stanford, CT, USA). The gel was washed with 2.5% Triton-X for 30 min and the gel was washed with 0.1 M Tris-buffer (pH8.0) and further incubated in the 0.1 M Tris-buffer over 13 h to allow caseinolysis to happen. And the gel was stained with Coomassie brilliant blue (R250) and destained with 20% methanol and 10% acetic acid. In case of one-phase inverse zymography, gels were processed as above and urokinase (12.5 ng/ml) was added into 0.1 M Tris buffer (pH8.0) to allow caseinolysis except the region of the gel containing plasminogen activator inhibitors. The gel was incubated for 6 h and was stained as described above. The band was appeared as Coomassie Blue-stained dark bands over lightly stained bluish backgrounds. The gel was analyzed using LAS 3000 (Fuji, Japan).

### 2.7. Western blot

The expression of iNOS and the activation of ERK1/2 and p38 MAPKs were determined by Western blot using antibodies specific for iNOS and phosphorylated forms of ERK1/2 and p38. Cells were treated with various concentrations of IL-32 in the presence or absence of LPS and were harvested with 2× sample buffer (4% w/v SDS, 20% glycerol, 200 mM DTT, 0.1 M Tris–HCl, pH 6.8, and 0.02% bromophenol blue). The samples were fractionated by 10% SDS–PAGE and electrotransferred to nitrocellulose (NC) membrane. The NC membrane was blocked with polyvinylalcohol and then was incubated at room temperature for 2 h with mAb against iNOS (Upstate, Lake Placid, NY, USA), *p*-ERK1/2 or *p*-p38 (Cell Signaling Technology, Beverly, MA, USA) which was diluted at 1:3000 in 5% Blotto (Santa Cruz Biotechnology Inc., Santa Cruz, CA). After three 10 min washes with PBS containing 0.2% Tween-20 (PBS-T), the NC membranes were incubated with peroxidase-labeled secondary antibodies at room temperature for 2 h. After extensive washing with PBS-T, the membranes were developed by enhanced chemiluminescence (Amersham, Buckinghampshire, UK). As loading controls, Western blot was performed using antibodies against total ERK1/2 and p38 (Cell signaling Technology) as well as β-actin (Sigma) in appropriate dilution.

### 2.8. Semiquantitative RT-PCR

Total RNA was extracted from rat primary astrocytes using Trizol reagent (GibcoBRL, Grand Island, NY). Reverse transcription was performed for 45 min at 45 °C with 2 μg of total RNA using *Maxime* RT PreMix Kit (iNtRON Biotechnology, Seoul) according to the manufacturer's protocol. Oligo $(dT)_{15}$ was used as a primer for this reaction. The samples were then heated at 94 °C for 5 min to terminate the reaction. The cDNA obtained from 0.5 μg total RNA was used as a template for PCR amplification of MMP-2 (accession number: U65656),-9 (U24441), iNOS (U03699), tPA (M23697), PAI-1 (M24067), TNF-α X66539), TGF-β (X52498) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, M17701) mRNA as described previously. The following Primers were used for amplification reaction:

for MMP-2
forward primer 5′-ACCTGGATGCAGTCGTGGAC-3′
reverse primer 5′-AGTGTGGAGCACCAGGGCAGC-3′

for MMP-9,
forward primer 5′-TAAGGTATTCAGTTACTCCTACTGGAA-3′
reverse primer 5′-CCTCTCTAGCACACATGCACTT-3′

for iNOS,
forward primer 5′-CAAGAGTTTGACCAGAGGACC-3′
reverse primer 5′-TGGAACCACTCGTACTTGGGA-3′

for tPA
forward primer 5′-TCAGATGAGATGACAGGGAAATGCC-3′
reverse primer 5′-ATCATACAGTTCTCCCAGCC-3′

for PAI-1
forward primer 5′-GCTCCTGGTCAACCACCTTA-3′
reverse primer 5′-CCCCACAAAATTCAAGACCA-3′

for TNF-α
forward primer 5′-TAGCCCACGTCGTAGCAAAC-3′
reverse primer 5’-GGAGGCTGACTTTCTCCTGG-3′

for TGF-β
forward primer 5′-GACGTCACTGGAGTTGTCCG-3′
reverse primer 5’-CACTTCCAACCCAGGTCCTT-3′

for GAPDH,
forward primer 5′-TCCCTCAAGATTGTCAGCAA-3′
reverse primer 5′-AGATCCACAACGGATACATT-3′

PCR mixture contained 1 pmol of each forward and reverse primer and 5 μl of each cDNA and *Maxime* PCR PreMix Kit (i-Taq). For amplification, the following PCR incubation times were used: 94 °C for 5 min followed by 28 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min. At the end of entire cycle, an additional extension step was performed at 72 °C for 10 min. The amplified PCR products were electrophoresed and analysed on 1.2% agarose-gel. The expected size of the amplified DNA fragments (in bp) was 419 for MMP-2, 300 for MMP-9, 654 for iNOS, 385 for tPA, 308 for PAI-1, 240 for TNF-α, 335 for TGF-β and 308 for GAPDH.

### 2.9. Statistical analysis

Data are expressed as the mean ± standard deviation (S.D.) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Newman-Keuls test as a post hoc test. Data with values of $p < 0.05$ were accepted as statistically significant.

## 3. Results

To investigate the effect of IL-32 on ROS production in rat primary astrocytes, we treated the cells with various concentration of IL-32 (Fig. 1A). IL-32 induced concentration-dependent increase in ROS production as determined by DCF-DA fluorescence. The maximum stimulation of ROS production was about 50% over control value that was observed at 1 μg/ml IL-32 stimulation, the highest concentration of IL-32 used in this study. We also treated rat primary astrocytes with 10 ng/ml LPS to induce ROS production (Fig. 1A) and co-treatment of IL-32 showed additive effects on LPS-stimulated ROS production (Fig. 1A).

We next investigated NO production in rat primary astrocytes stimulated with IL-32. Although IL-32 *per se* did not show noticeable changes in NO production or iNOS expression (Fig. 1B and C), IL-32 treatment in LPS-stimulated astrocytes significantly increased both NO production and iNOS expression. These results suggest that IL-32, albeit modest, induces oxidative stress in rat primary astrocytes.

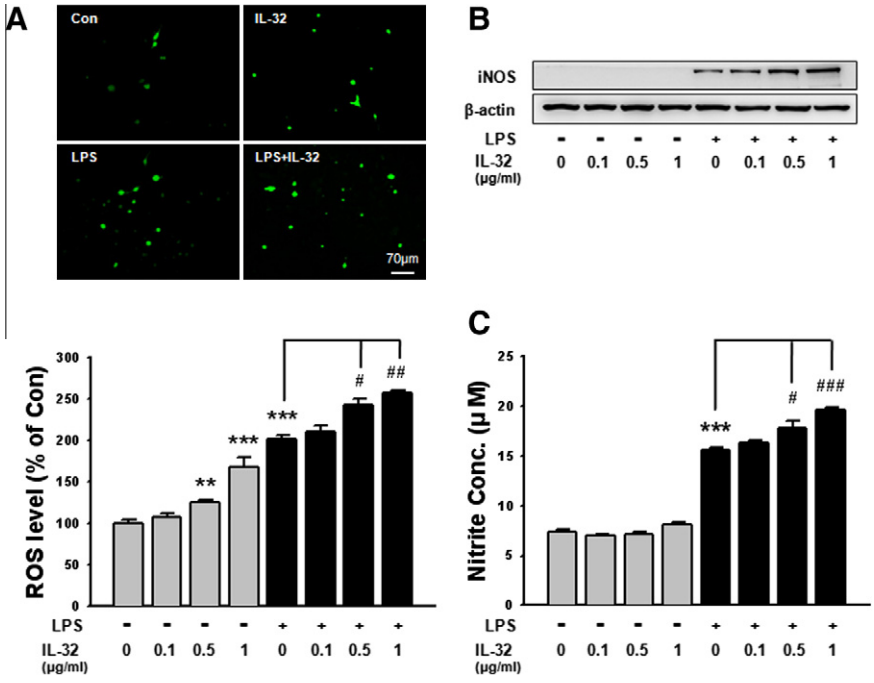


**Fig. 1.** Effects of IL-32 on intracellular accumulation of ROS and NO production in normal and LPS-stimulated rat primary astrocytes. Cells were treated with various concentration of IL-32 for 24 h in the presence or absence of LPS (10 ng/ml). (A) Representative photomicrographs of intracellular ROS production were monitored with a confocal microscope equipped with a laser at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Graphs represent intracellular ROS levels which were determined based on the DCF fluorescence as described in materials and methods. (B) The expression level of iNOS was determined by Western blot using specific antibody as described above. β-Actin levels were measured as a loading control. Results shown here are representative of at least four independent experiments, which gave similar results. (C) The concentration of nitrite was determined by the Griess assay as described above. Data were expressed as the means ± S.E.M. ($n = 4$).*Significantly different from control group: $^{**}p < 0.01$, $^{***}p < 0.001$. #Significantly different from the LPS-treated group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

To investigate whether IL-32 affects MMP-9 activity in rat primary astrocytes, we performed gelatin zymography using cell culture supernatants stimulated with IL-32. As shown in Fig. 2A, IL-32 treatment increased MMP-9 activity in rat primary astrocytes, which is more prominent 48 h after IL-32 treatment. Interestingly, LPS stimulation increased MMP-9 activity in rat primary astrocytes and the increase in MMP-9 activity was synergistically activated by co-treatment of IL-32 (Fig. 2A). The activity of MMP-2, which is a constitutively expressed subtype of MMPs, was not changed by LPS or IL-32. We previously reported that the activity of another important proteinase expressed in astrocytes, namely tPA, is differentially regulated by cAMP pathway or inflammatory stimulation [11]. Consistent with the previous results, LPS stimulation decreased tPA activity with the increased expression of tPA inhibitor PAI-1. Although IL-32 itself induced only marginal induction of PAI-1 and decrease in tPA activity, if any, cotreatment of IL-32 with LPS significantly induced the expression of PAI-1concomittant with the decrease in tPA activity, especially at 48 h after treatment (Fig. 2B and C).

We next examined the mRNA expression level of several inflammatory activation marker in rat primary astrocytes after IL-32 treatment. The mRNA level encoding iNOS, MMP-9, TNF-α and TGF-β was increased by IL-32 stimulation (Fig. 3). IL-32 stimulation also increased the LPS-induced up-regulation of those inflammatory markers in rat primary strocytes. Similar pattern was also observed with PAI-1 mRNA expression. The expression of mRNAs encoding MMP-2 and tPA, as well as GAPDH, was not changed by IL-32 or LPS stimulation (Fig. 3).

Several researchers including us demonstrated the essential role of MAPK pathway in inflammatory activation of astrocytes [8,9,12]. Therefore, we examined whether IL-32 stimulation affects the activation status of MAPK pathways (Fig. 4). Treatment of IL-32 increased the phosphorylation, and thus the activation status, of ERK1/2 and p38 without affecting total level of ERK1/2 and p38 (Fig. 4). Unfortunately, we cannot reliably detect phospho-JNK level in this study (data not shown). LPS stimulation increased the phosphorylation of ERK1/2 and p38 and cotreatment of IL-32 further increased the phosphorylation of ERK1/2 and p38 (Fig. 4), suggesting that IL-32 modulate inflammatory activation of astrocytes through the regulation of ERK1/2 and p38 pathway.

## 4. Discussion

In this study, we demonstrated that IL-32 induced inflammatory activation of rat primary astrocytes possibly via activation of ERK1/2 and p38 MAPK pathways. The inflammatory activation of astrocytes was evidenced by the increased production of ROS and NO as well as the increased protein and/or mRNA expression of several inflammatory marker proteins including iNOS, MMP-9 and TNF-α. In general, the response was most strong in the activation of MMP-9 activity suggesting the immediate early nature of MMP-9 expression after inflammatory stimulation and/or the specific of IL-32 stimulation on the regulation of MMP-9 expression. The inflammatory activation of rat primary astrocytes observed in this study supports the idea that IL-32 is a strong pro-inflammatory cytokine involved in induction of pro-inflammatory cytokines such as TNF-α and IL-1β [4,5,13] as well as in several human diseases including inflammatory bowel disease and rheumatoid arthritis [14,15]. The pro-inflammatory response of IL-32 in astrocytes suggests that receptor(s) for IL-32 should be expressed in astrocytes. IL-32 is a recently added member of cytokine and unfortunately, the race for identification of receptor molecule(s) for IL-32 is unsuccessful yet. The identification and cloning of receptor for IL-32 would greatly facilitate the analysis of the physiological role of IL-32 in brain.

In various experimental scheme, it has been suggested that ERK1/2 phosphorylation induce the expression of inflammatory mediators such as iNOS and MMP-9 in rat primary astrocytes [9,12,16]. Interestingly, using an in vitro model of osteoclast precursor differentiation, it has been reported that IL-32 induces the phosphorylation of ERK1/2, which is stronger than that induced by RANKL [17], with only marginal effects on JNK and Akt pathway, if any. In addition, we also reported that inhibition of p38 pathway using a chemical inhibitor SB203580 inhibited IL-32-induced secretion of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in peripheral blood mononuclear cells [18]. These results suggest that the activation of ERK1/2 and p38 plays essential role in the inflammatory activation of astrocytes by IL-32, although the detailed kinetic and molecular biological experiments are needed to differentiate the specific role of those pathways on the regulation of individual pro-inflammatory mediators in rat primary astrocytes.

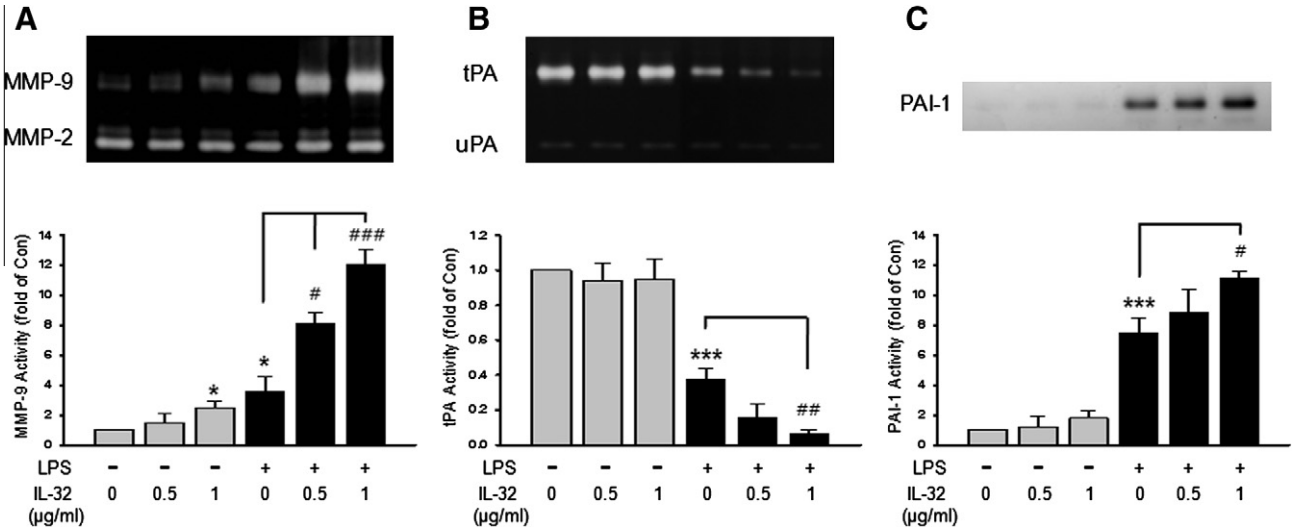


**Fig. 2.** Effects of IL-32 on MMPs and PA activities in normal and LPS-stimulated rat primary astrocytes. Cells were treated with various concentration of IL-32 in the presence and absence of LPS (10 ng/ml). After 48 h, culture supernatants were harvested and analyzed for (A) MMP-9 and MMP-2 activities using gelatin zymography, (B) tPA and uPA activities using casein zymography and (C) PAI-1 activity using one-phase inverse zymography as described above. Data are presented as the mean ± S.E.M. of four independent experiments. *Significantly different from control group: $^{*}p < 0.05$, $^{***}p < 0.001$. #Significantly different from the LPS-treated group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

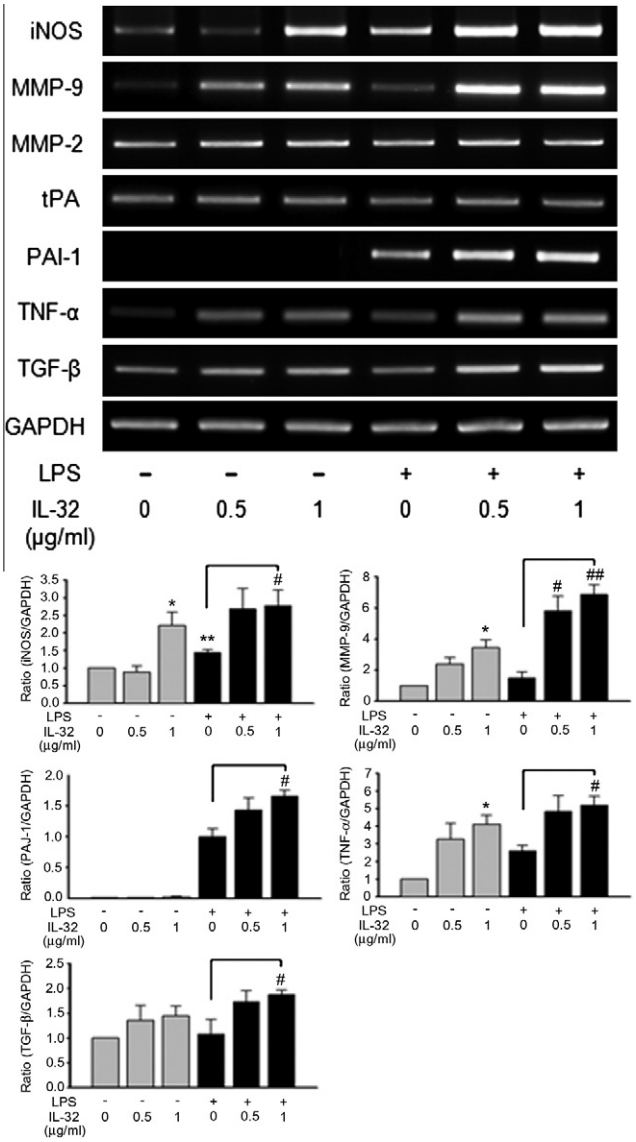


**Fig. 3.** Effects of IL-32 on the mRNA expression of pro-inflammatory mediators in normal and LPS-stimulated rat primary astrocytes. Cells were treated with various concentration of IL-32 in the presence or absence of LPS (10 ng/ml). After 48 h, the mRNA expression of iNOS, MMP-9, MMP-2, tPA, PAI-1, TNF-α and TGF-β was assessed by RT-PCR as described above. GAPDH levels were measured as an internal control. Data are presented as the mean ± S.E.M. of four independent experiments. *Significantly different from control group: $^{*}p < 0.05$, $^{**}p < 0.01$. #Significantly different from the LPS-treated group: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

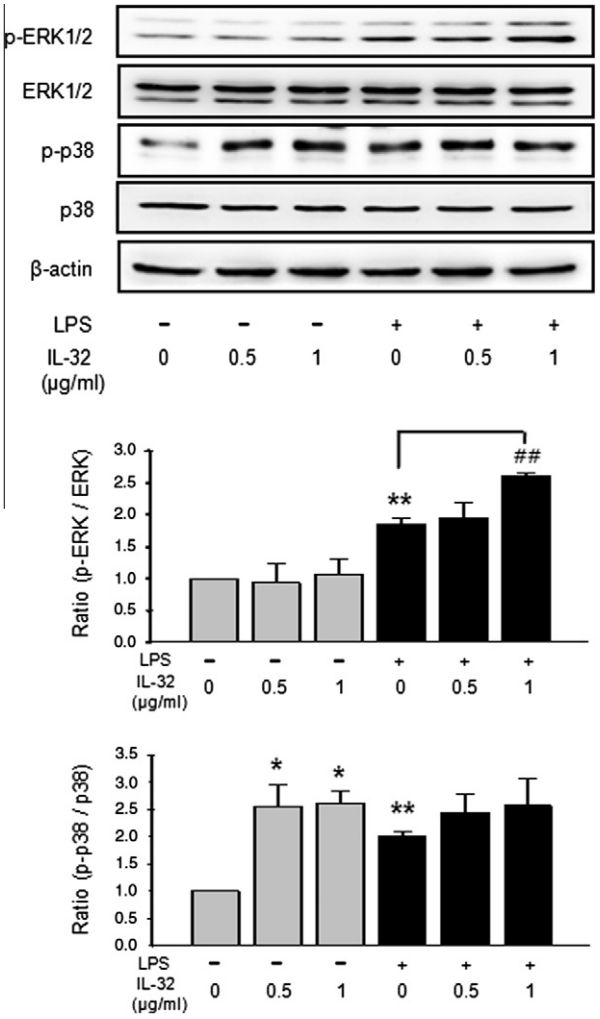


**Fig. 4.** Activation of ERK1/2 and p38 MAPK pathway by IL-32 in normal and LPS-stimulated rat primary astrocytes. Cells were treated with various concentration of IL-32 in the presence or absence of LPS (10 ng/ml) for 2 h. Cells were harvested for the Western blot analysis of the phosphorylation status of ERK1/2 and p38 as described above. As a loading control, the level of total ERK1/2, p38 as well as β-actin was also determined by Western blot. Data are presented as the mean ± S.E.M. of four independent experiments. * Significantly different from control group: $^{*}p < 0.05$, $^{**}p < 0.01$. #Significantly different from the LPS-treated group: $^{\#\#}p < 0.01$.

In A549 human lung epithelial cells, infected with influenza A virus or immune stimulated with poly(I:C)+IFN-γ, the expression of both IL-32 and iNOS is increased [19]. Interestingly, the overexpression of IL-32 increased iNOS expression with possible negative feedback modulation of IL-32 expression by NO [19]. IL-32 has been implicated in the manifestation of vasculitis [20,21]. Not only by mediating inflammatory responses in stimulate tissues but also by regulating vascular relaxation by excess NO, the induction of iNOS may affect the pathophysiological consequences of inflamed vasculature, which might also be true in ischemic brain injury. The role of IL-32 induced iNOS expression in astrocytes and possibly in brain endothelial cells on pathological outcomes of ischemic brain would be an intriguing topic to investigate further in the future.

In this study, the pro-inflammatory activity of IL-32 was relatively modest compared with classical pro-inflammatory cytokines such as IL-1. However, we used human recombinant IL-32 to stimulate rat primary astrocytes. Unfortunately, efforts to purify or clone murine IL-32 or IL-32 homologues were in vain. Considering the differences in specific activity of pro-inflammatory cytokines obtained from different species, the modest activity of IL-32 still implicates the physiological role of IL-32 in the modulation of immune response in brain.

Considering the strong pro-inflammatory responses elicited by IL-32 and the role of IL-32 in several inflammatory diseases such as vasculitis and rheumatoid arthritis as well as increasing importance of neuroinflammation in CNS disease such as ischemia and Alzheimer's disease, it is surprising that no reports are available as of yet regarding the role of IL-32 in brain. As far as we know, this is the first report implicating the role of IL-32 in the modulation of glial activation. Exploring the pathological role of IL-32, for example, investigating the role of IL-32 on microglial activation as well as determining the expression of IL-32 in neuroinflammatory tissues, may provide in depth understanding of the regulatory mechanism of inflammatory responses in brain, which is once regarded immune-privileged.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.09.099.

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