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β-adrenoceptor mediated surgery-induced production of pro-inflammatory cytokines in rat microglia cells

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ABSTRACT

Immunological changes initiated by major operative injury may result in inflammatory responses in both peripheral and central nervous system, which may lead to organ dysfunction. Recent studies indicate that β-adrenergic receptors (β-ARs) may mediate production of pro-inflammatory cytokines in the brain. In the present study propranolol (β-AR antagonist), but not prazosin (α1-AR antagonist), antagonized surgical trauma induced pro-inflammatory cytokine production in microglia cells isolated from rats. β-AR activation in the absence of pro-inflammatory stimuli increased IL-1β, TNF-α and IL-6 mRNA and protein expressions in the primary microglia cell culture. Isoproterenol (β-AR agonist) treatment induced a time- and concentration-dependent increase of IL-1β in cells. Both ERK1/2 and P38 MAPK inhibitor, but not PKA and JNK1/2 inhibitor abrogated isoproterenol-induced IL-1β and IL-6 production in microglia cells. In conclusion, the results suggest that β-ARs possess pro-inflammatory properties by modulating the functions of microglia cell.

1. Introduction

Surgery is a significant contributor to the initial systemic inflammatory response in hospitalized patients. Systemic inflammation can lead to dysfunction of the organ which is remoted from the site of traumatic injury. Elevated inflammatory cytokines in the brain during traumatic injury may have potent effects on the alteration of physiological, behavioral and cognitive processes (Garcia-Bueno et al., 2008). However, the mechanisms of how these cytokines are induced during surgery remain unclear.

Several studies have reported that pretreatment with β-blockers improved outcomes in patients having operative or traumatic injuries (Lindenauer et al., 2005; Mangano et al., 1996; Pasternack et al., 1989). Arbabi recently showed that the use of β-blockers resulted in improved outcomes in traumatic patients, especially in patients with brain injuries (Arbabi et al., 2007). However, the underlying mechanisms have not been clearly elucidated. While activation of β-ARs can significantly inhibit cytokine production induced by pro-inflammatory stimuli such as lipopolysaccharide (LPS), recent reports have shown that activation of β-ARs in the absence of such stimuli can increase pro-inflammatory cytokine production in different cell types. Activation of β2-ARs leads to the up-regulation of IL-6 in macrophages, skeletal muscles, myocytes, pituicytes and cardiac fibroblasts (Christensen et al., 1999; Frost et al., 2004; Yin et al., 2006). Recent reports demonstrate that β-AR mediates the induction of IL-1β in the brain during stress exposure (Blandino et al., 2006). Central β-AR activation may lead to increased inflammatory cytokine production in the brain following peripheral immune challenge (Johnson et al., 2008). These results suggest that activation of β-ARs may have pleiotropic effects on cytokine production in the brain. Although most works find that pro-inflammatory cytokines were up-regulated in different brain regions during major surgery or stress, the primary cellular source of those cytokine is still not clarified.

Microglia cells are resident brain mononuclear phagocytes and have functions similar to macrophages including phagocytosis, antigen presentation, production of cytokines, chemokines, eicosanoids, complement components, matrix metalloproteinases, oxidative radicals and nitric oxide (Aloisi, 2001). Evidence suggested that the central β-AR mediated increase in IL-1β in the brain may result from the direct action on microglial cells (Blandino et al., 2006; Maruta et al., 1997). We sought to determine if the β-ARs expressed by microglia cells are involved in pro-inflammatory cytokine production under major surgery.

Here, we present a series of experiment to examine the hypothesis that activation of β-AR on microglia cell mediates the induction of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) in the brain after surgical trauma stress.

2. Material and methods

2.1. Animals

Experiments were performed on adult male Sprague–Dawley (SD) rats (Experimental Animal Center, Shanghai Medical College of Fudan

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University, China) weighing 200–220 g. Rats were housed in temperature-controlled (22 ± 2 °C) and light-controlled (12:12 h light–dark cycle) room with free access to food and water. Prior to experimental manipulation, rats were allowed to acclimate to the housing facilities. All experimental protocols and animal handling procedures were approved by the Animal Care and Use Committee (ACUC) of Fudan University, and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHEW Publications, NIH, 80-23).

2.2. Drugs and treatments

All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. The drugs were dissolved in sterile, endotoxin-free saline. The rats ($n = 6$–8 per group) were injected intraperitoneally with 2 or 10 mg/kg propranolol or prazosin 30 min before surgery. Propranolol and prazosin were dissolved at a concentration of 5 mg/ml. Isoproterenol (Iso), H89, U0126, SB203580 and SP600125 were obtained from Cell Signaling Technology (Beverly, MA, USA). All doses were chosen based on prior studies in our laboratory (Wang et al., 2007) or doses reported in the literature (Tan et al., 2007; Yin et al., 2006).

2.3. Surgical trauma stress model

The surgical trauma stress was performed as previously described (Wang et al., 2007). Briefly, rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). Animals were then incised longitudinally to a length of 6 cm along the dorsal median line and 5 cm along the abdominal median line. After surgery, the wounds were sutured and the animals were kept warm under standard housing conditions.

2.4. Isolation and verification of microglia cells from the CNS

Mononuclear cells were isolated from the CNS of SD rats as described (de Haas et al., 2007; Ponomarev et al., 2005). Briefly, rats were anesthetized and perfused intracardially with PBS (0.1 M phosphate buffer, pH 7.4, containing 0.9% NaCl). The brain was homogenized, and mononuclear cells were isolated using 40/70% discontinuous Percoll gradients. The viability was assessed by trypan blue exclusion. The total number is counted and adjust to 1×10^6 cells/rat. To determine microglia purity, cells were stained with antibodies against CD11b. Microglia (1×10^6 cells/staining condition) were suspended in 50 μl FACS buffer (1× PBS + 10% FBS (fetal bovine serum)) and pre-incubated for 15 min on ice prior to staining. Microglia cells were then incubated with monoclonal antibodies against PE-CD11b (BD Pharmingen™, 1:50) and FITC-CD45 (BD Pharmingen™, 1:100) or isotype controls (BD Pharmingen™, 1:50) for 15 min at 4 °C. Cells were rinsed 2 times in FACS buffer. The purity of microglia cell is almost 98% as determined by FACS.

2.5. Real-time PCR for mRNA quantification

The mRNA of the pro-inflammatory cytokines was measured using real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA from the rat cells was extracted with the Trizol reagent (Invitrogen, USA). SYBR Green qRT-PCR detection (iCycler iQ® real-time PCR detection system, Bio-Rad, CA, USA) was used to quantify the relative abundance of target mRNAs in the samples. The size and sequence of each primer and the number of cycles used are given in Table 1.

The relative amount of each mRNA was normalized to the housekeeping gene GAPDH. The relative mRNA levels of gene expression were determined using the threshold cycle (CT) and arithmetic formulas. The CT from the GAPDH was subtracted from the CT for each animal model (ΔCT) and then the control group for each protein of interest was set to 1 for reference. The relative mRNA levels of IL-β, IL-6 and TNF-α were found by subtracting the control ΔCT from each animal ΔCT. These values were entered into the equation $2^{-\Delta CT}$ to solve for the relative exponential PCR amplification of each gene for each animal.

2.6. Pro-inflammatory cytokine ELISA

To evaluate pro-inflammatory cytokine expression after surgery, microglia cells from the whole brain were isolated and intracellular levels of cytokines were measured. The IL-1β, IL-6 and TNF-α protein concentrations were measured by using ELISA kits according to the manufacturer's instructions (RapidBio Lab, CA, USA) and the absorbance was determined with an ELISA plate reader at 450 nm (Bio-Tek Instrument). Total protein content from each sample was measured using the Bradford method. The data were expressed as either pg/100 μg of total protein or precise concentration.

2.7. Primary microglia cell culture preparation

Primary cultures of microglial cells were generated from mixed cell cultures of the forebrains of 2- to 3-day-old SD rats as described previously (Tanaka et al., 1997). Mixed glial cultures contained oligodendrocytes, microglia and astrocytes. After culture for one week, mixed glial cultures were shaken for 2 h at 180 rpm. The supernatants containing 90% microglia were plated onto uncoated tissue culture plates. 15 min after plating, nonadherent cells (predominantly astrocytes and oligodendrocytes) were removed by three washes with PBS. Microglia were maintained in DMEM with 5% FBS. Microglia cells were seeded at a density of 1×10^6 cells in 1 ml in a 12-well tissue culture plate. For inhibitor experiments, cells were treated with the indicated drug for 30 min prior to treatment with Iso. The purity of the microglial culture was almost 99%, as determined by the immunocytochemical staining of microglia-specific markers (data not shown).

2.8. Western blotting analysis

After appropriate stimulation, cells were washed twice with ice-cold PBS and extracted in the lysis buffer for 45 min on ice. Equal amounts of protein were subjected to SDS-PAGE. The separated proteins were transferred to a PVDF membrane. After incubating in blocking reagent (1× PBS, 4% BSA, 0.05% Tween-20) at 37 °C for 60 min to block non-specific binding, the membranes were then incubated with primary antibodies, anti-ERK1/2 (1:1000), phosphorylated ERK1/2 (1:1000), p38 (1:1000), phosphorylated p38 (1:1000), JNK (1:500), and phosphorylated JNK (1:500) (Cell Signaling Technology, Beverly, MA, USA). After incubating with HRP conjugated secondary antibodies, protein was visualized using an enhanced SuperSignal West Pico Chemiluminescent system (Pierce).

2.9. Statistical analysis

Data were presented as mean ± SEM. Statistical analyses were performed using a two tailed Student's *t*-test when comparing two groups, an analysis of variance (ANOVA) with a post-hoc Newman–Keuls test for comparisons across multiple groups. $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. The purity of microglia cells isolated from whole brain

Microglia cells were identified on the basis of their specific surface expression profile of CD11b and CD45 ($CD11b^+$/CD45low) using flow activated cell sorting (FACS), as described by others (de Haas et al.,

Table 1
Sequences of the forward and reverse primers and PCR conditions used for RT-PCR.

Genbank accession numbers	Target gene	Primers	PCR conditions (temperature/time)			Predicted size (bp)
			Denature	Anneal	Extend	
NM_017008	GAPDH	Forward: 5′-cccttcattgacctcaactac-3′ Reverse: cttctccatggtggtgaagac-3′	94 °C/45 s	60 °C/1 min	72 °C/1 min	217
NM_031512	IL-1β	Forward: 5′-agagcttcaggaaggcag-3′ Reverse: 5′-tgttgttcatctcgaagcct-3′	94 °C/45 s	58 °C/1 min	72 °C/1 min	220
NM_012589	IL-6	Forward: 5′-gacaaagccagagtccttca-3′ Reverse: 5′-actaggtttgccgagtagac-3′	94 °C/45 s	58 °C/1 min	72 °C/1 min	229
X66539	TNF-α	Forward: 5′-cgagatgtggaactggcaga-3′ Reverse: 5′-ctacgggcttgtcactcga-3′	94 °C/45 s	58 °C/1 min	72 °C/1 min	259

2007; Frank et al., 2006). Microglial forward (cell size) and side (granularity) scatters of light indicated a highly homogeneous population of cells (Fig. 1A). In concurrence with previous published studies, CD11b and CD45 expression levels clearly distinguished microglia from other events (Fig. 1B). The microglia purity, defined as the percentage of all living cells that showed $CD11b^+$/CD45low expression, appeared about 97.6% (upper left quadrant, Fig. 1B).

3.2. Effect of β-adrenergic receptor blocker and α1-adrenergic receptor blocker on surgical trauma stress-induced pro-inflammatory cytokine expression

Quantification of protein by ELISA (Fig. 2) showed increased expression of IL-1β, IL-6 and TNF-α levels in the microglia cells isolated from whole brain of rats 24 h post-surgical trauma. Propranolol, a β-AR antagonist pretreatment significantly attenuates IL-1β and IL-6 expression at 10 mg/kg in traumatic rats, on the other hand prazosin, a α1-AR antagonist did not produce any effect on the cytokine expression (Fig. 2). The findings suggested that surgery-induced pro-inflammatory cytokine expression is mediated by β-AR.

3.3. β-AR activation leads to pro-inflammatory cytokine production in neonatal rat microglia cell

Since β-AR antagonist could antagonize IL-1β and IL-6 production in microglia cells *in vivo*, we sought to determine if β-AR activation could directly modulate pro-inflammatory cytokine mRNA expression in the primary microglia cell culture. Microglia cells were treated with 1 μM isoproterenol (Iso), a β-AR agonist, and real-time quantitative PCR and ELISA were used to quantify the level of mRNA and protein expression at different time points. Treatment with 1 μM Iso significantly increased expressions of mRNAs for IL-1β, TNF-α and IL-6 (Fig. 3a). The up-regulation of IL-1β expression reached peak at 2 h after Iso stimulation, while IL-6 and TNF-α reached peak at 6 h and 12 h, respectively. The increase in mRNA expression was accompanied with protein secretion. IL-1β concentration increased 2 h after Iso stimulation and reached the peak 12 h later; IL-6 secretion showed the same pattern as IL-1β except the peak point was 6 h after Iso stimulation. TNF-α secretion increased 12 h later and remained high 24 h after Iso administration. (Fig. 3b). The results indicate that β-AR activation leads to a significant up-regulation of pro-inflammatory cytokine expression in microglia cells.

3.4. Dose-dependent effect of Iso on IL-1β mRNA expression

Microglia cells were stimulated with various concentration of Iso, and then mRNA samples were isolated at 2 h. Iso induced a concentration-dependent increase of the mRNA level of IL-1β (Fig. 4).

3.5. ERK and p38 MAPK, but not JNK signal pathway plays a role on Iso-induced IL-1β and IL-6 expression

To define the downstream signaling events of the β-AR pathway, we examined the possible involvement of protein kinase A (PKA) and mitogen activated protein kinase (MAPK). Stimulation with 1 μM Iso significantly increased extracellular regulated protein kinase (ERK) and p38 MAPK activation in microglia cells, but had no effect on JNK expression (Fig. 5A). Iso dose-dependently increased phosphorylation of ERK and P38 MAPK in microglia cells, without affecting the phosphorylation of JNK (Fig. 5B). Microglia cells were pretreated with

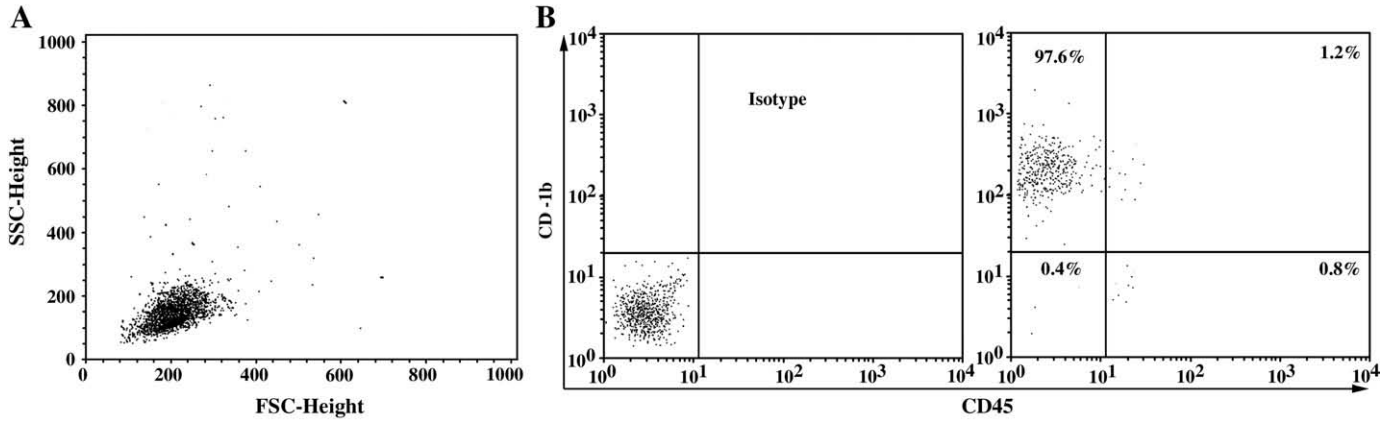

Fig. 1. Flow cytometric analysis of the purity of isolated microglia cells. (A) Light scattering properties of isolated microglia. Cells analyzed by flow cytometry exhibited highly uniform light scattering properties. Forward light scatter is indicative of cell size, while side light scatter is indicative of internal cell complexity or granularity. (B) Microglia cells were identified according to their specific $CD11b^+$/CD45 low surface expression using flow cytometry. Isolated microglia were stained with an isotype control (left) or antibody to $CD11b^+$ and CD45 (right) and analyzed by flow cytometry. The microglia purity is about 98% (upper left quadrant of Panel B). Figures presented are representative of three independent experiments.

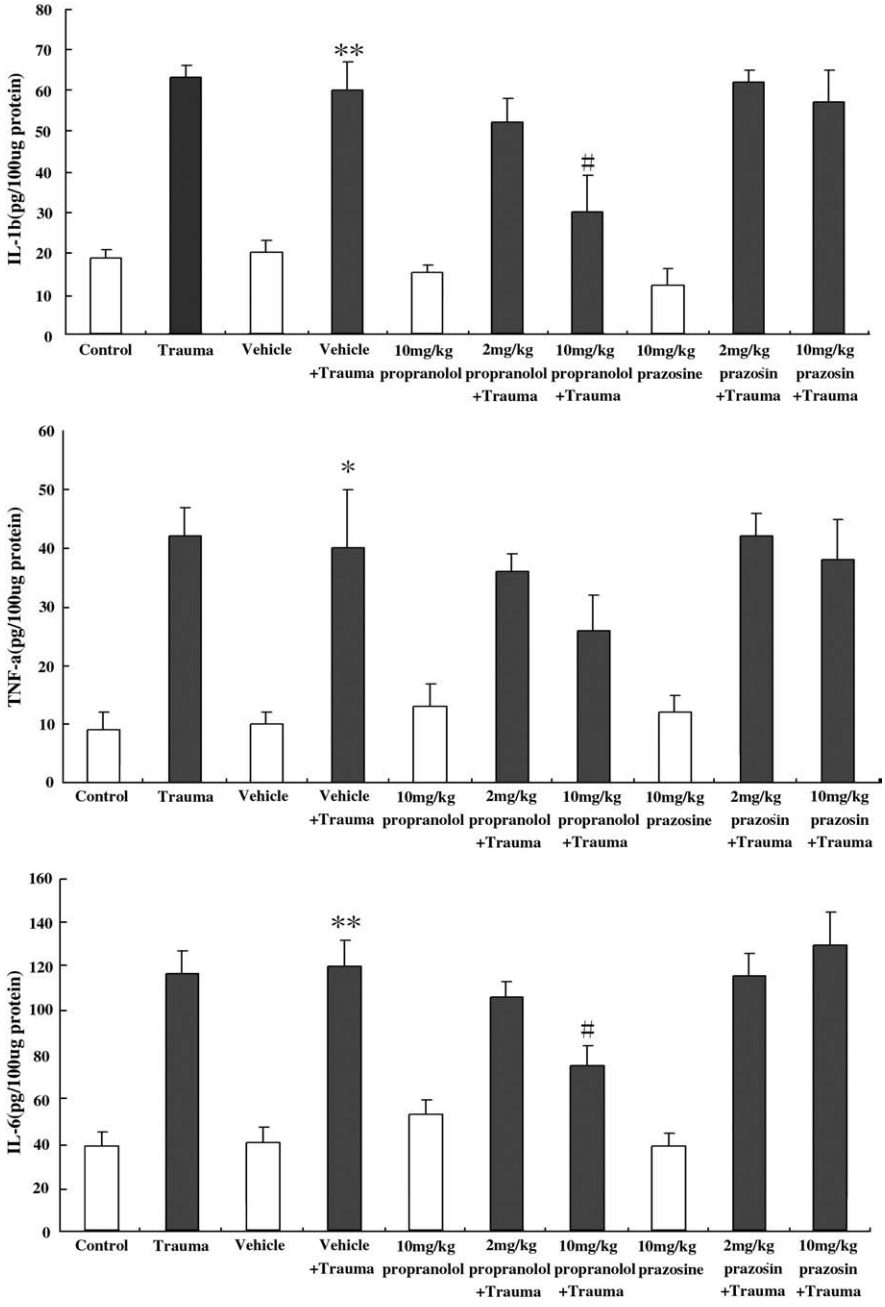

Fig. 2. Effect of β-AR or α1-AR antagonist on pro-inflammatory cytokine expression in microglia cells isolated from surgical trauma stressed-rats. Rats were injected i.p. with saline (vehicle), propranolol (β-AR antagonist), or prazosin (α1-AR antagonist) 30 min prior surgery. Animals were sacrificed 24 h later for measurement of IL-1β, TNF-α and IL-6 in isolated microglia cells from rats. All data are expressed as mean ± SEM ($n = 6$–8/group). *$P<0.05$, **$P<0.01$, vs vehicle group, #$P<0.05$, vs vehicle + trauma group.

H89 (10 μM, PKA inhibitor), U0126 (10 μM, ERK1/2 inhibitor), SB203580 (10 μM, P38 inhibitor), or SP600125 (10 μM, JNK inhibitor) for 30 min and then stimulated by 1 μM Iso for 2 h. Iso-stimulated IL-1β expression was partially antagonized in U0126 and SB203580 treated cells, while IL-6 expression was antagonized by U0126 and SB203580 (Fig. 5C) The results suggested that Iso-stimulated IL-1β and IL-6 expressions were mediated through ERK1/2 and P38 MAPK pathway, but not JNK and PKA pathway.

4. Discussion

In the present study we demonstrate that β-ARs mediate the induction of pro-inflammatory cytokines (IL-1β, TNF-α and IL-6) in microglia cells after surgical trauma stress. The β-AR antagonist but not α-AR antagonist abrogated surgical trauma induced pro-inflammatory cytokine expression in microglia cells isolated from rat brain. Furthermore, *in vitro* study showed that activation of β-AR unregulated pro-inflammatory cytokine expression in cultured microglia cells. This phenomenon is not mediated through the stimulation of PKA or JNK, but instead requires the stimulation of ERK1/2 and P38MAPK-dependent signaling pathway.

Sustained psychosocial stress or physical stress (surgery, infection, injury) results in immune disturbance, and may cause mental disorder associated with increased cytokine releases. It is not only systemic inflammation that can initiate the synthesis of pro-inflammatory cytokines in the brain but also stressors (Frank et al., 2007; O'Connor et al., 2003; Shintani et al., 1995). Although the mechanisms leading to CNS immune activation during stress and immune challenge remain unclear, evidence suggests that microglia are the primary cellular source of increased pro-inflammatory cytokine levels in the brain (Frank et al.,

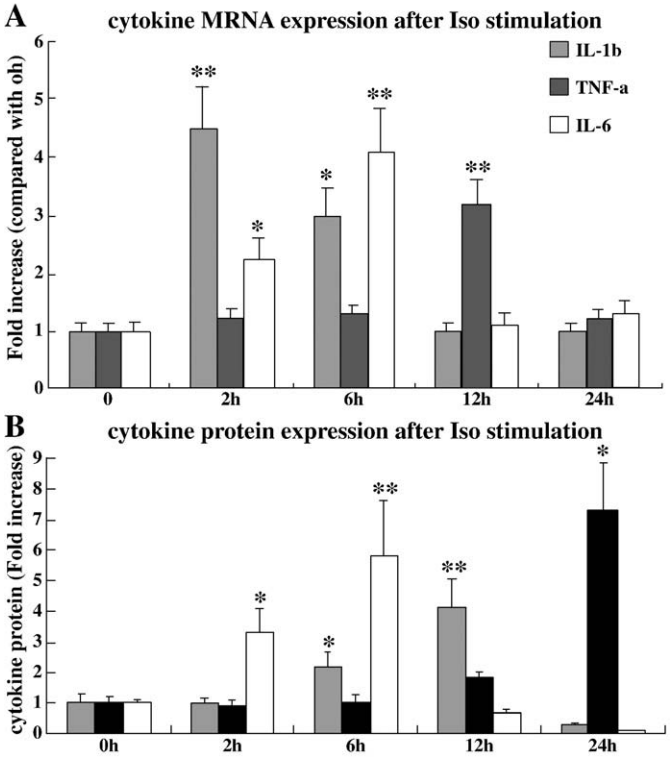

Fig. 3. β-AR stimulation leads to pro-inflammatory cytokine production in neonatal rat microglia cells. Microglia cells were treated with 1 μM β-AR agonist isoproterenol (Iso). (A) The total RNA from microglia cells was collected at different time points for the measurements of IL-1β, TNF-α and IL-6 mRNA by real-time PCR; (B) the supernatants were collected at different time points for measurements of IL-1β, TNF-α and IL-6 cytokine protein by ELISA. The results shown are representative of three independent experiments. Data are expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, vs untreated.

2007). By using a method that can isolate highly pure microglia cell from rat model rapidly (Sun et al., 2004), we are able to examine surgical stress-induced microglia activation. Consistent with recent evidence that restraint-stress activated microglia (Nair and Bonneau, 2006), surgical trauma stress increased the pro-inflammatory cytokine releases in the isolated microglia cells.

The catecholamines released during intense stress are important mediators in the stress-induced releases of pro-inflammatory cytokines in CNS. Microglia that express receptors for classical neurotransmitters, such as glutamate (Noda et al., 2000), GABA (Kuhn et al., 2004), and the

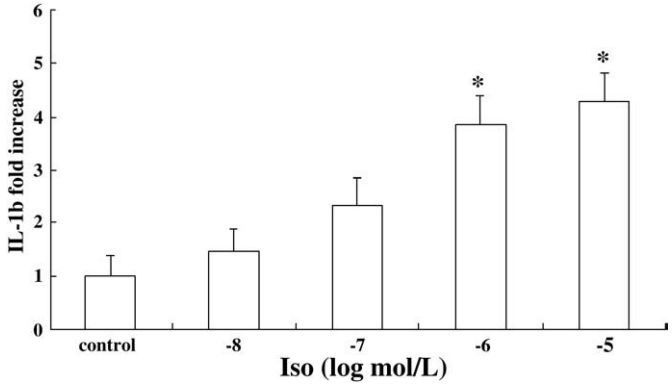

Fig. 4. Dose-dependent effect of Iso on IL-1β mRNA expression. Microglia cells were treated with various concentrations of Iso. The mRNA samples were collected at 2 h for IL-1β mRNA measurement. Results were obtained in three separate experiments $^{*}P<0.05$, vs untreated.

presence of functional adrenergic receptors were also demonstrated (Mori et al., 2002; Tanaka et al., 2002). The expressions of α1-AR, α2-AR, β1-AR, and β2-AR mRNAs were demonstrated in cultured rat microglial cells, while the expressions of α1B-AR, α1D-AR, and β3-AR mRNAs were not detectable (Mori et al., 2002). This suggests that there may be a signaling loop between microglia and neurons, and adrenergic receptors have a critical role in the regulation of microglia functions. The role of β-AR in microglia cell activation remains controversial. Several studies showed that β-AR activation could inhibit LPS-induced IL-6, TNF-α, IL-12 and nitric oxide production in cultured microglia cell, as well as ATP-stimulated P38MAPK phosphorylation and PMA-stimulated superoxide anion production (Morioka et al., 2009; Färber et al., 2005; Prinz et al., 2001; Chang and Liu, 2000; Colton and Chernyshev, 1996). On the contrary, other reports have shown that the activation of β-AR in the absence of such stimuli could activate microglia cells (Qian et al., 2009; Tanaka et al., 2002; Tomozawa et al., 1995).

In the present study, surgical trauma stress resulted in an increase in pro-inflammatory cytokine expression in central microglia cells. Blockade of β-AR, but not α1-AR, attenuated the induction of IL-1β, IL-6, and TNF-α in microglia cells isolated from rats with traumatic injuries. A recent study revealed that acute treatment of rats with the noradrenalin reuptake inhibitors (NRIs) desipramine and atomoxetine elicited anti-inflammatory actions in rat cortex following a systemic challenge with bacterial LPS (O'Sullivan et al., 2009). Several studies showed that exaggerated β-AR activation would result in the elevation of central pro-inflammatory cytokines (Blandino et al., 2006; Johnson et al., 2005, 2008). Johnson et al. found that central β-AR activation stimulated pro-inflammatory cytokine production in response to peripheral bacterial challenge or stressor (Johnson et al., 2005, 2008). However, they have not investigated the cellular source of cytokines. Our result demonstrated that β-AR mediated pro-inflammatory cytokine production in microglia cells after traumatic stress.

We have used pure microglial cells maintained in culture to analyze the regulation of IL-1β, IL-6 and TNF-α expression by β-adrenergic agonist isoproterenol. We have demonstrated that stimulation of β-AR alone (without additional immune stimuli) modified the expression of the gene coding for all three cytokines. Several laboratories have now demonstrated that activation of β-AR stimulates the induction of IL-6 and IL-1β mRNA in cultured macrophages and microglia (Hetier et al., 1991; Tan et al., 2007; Tomozawa et al., 1995), as well as other cell types, such as skeletal muscle (Frost et al., 2004), myocytes (Frost et al., 2004), pituicytes (Christensen et al., 1999) and cardiac fibroblasts (Yin et al., 2006).

The present results showed that β-AR activation up-regulated pro-inflammatory cytokine production in a PKA-independent manner. On the other hand, it is mediated through the stimulation of ERK1/2 and p38 MAPK. Tomozawa et al. found that β-AR mediated IL-1β induction in microglia may be due to the elevation of intracellular cyclic adenosine monophosphate (cAMP) (Tomozawa et al., 1995). This discrepancy may be due to the reason that they cultured in the presence of M-CSF to maintain survival of the microglia cells. Actually it has been reported that cAMP could inhibit M-CSF-induced ERK, JNK and p38MAPK activation in a time-dependent manner in macrophages (Zhu et al., 2008). Recent report from Tan et al. showed that the activation of β2-AR on macrophages led to IL-1β and IL-6 production via PKA-independent pathway that involved ERK1/2 and p38MAPK signaling (Tan et al., 2007). To date, PKA-dependent (Daaka et al., 1997) and PKA-independent pathway (Tan et al., 2007; Yin et al., 2006) has been reported to mediate β-AR-induced MAPK activation, depending on the cellular context. The PKA-dependent ERK1/2 phosphorylation has been shown to occur rapidly after receptor activation, peaking within 5 min. A second PKA-independent signal leading to ERK1/2 phosphorylation is slower in onset, peaking at 5 to 10 min and lasting at least 30 min (Shenoy et al., 2006). In the present study, the β-AR-dependent up-regulation of cytokines is mediated by ERK and p38MAPK pathways, independent of PKA, and

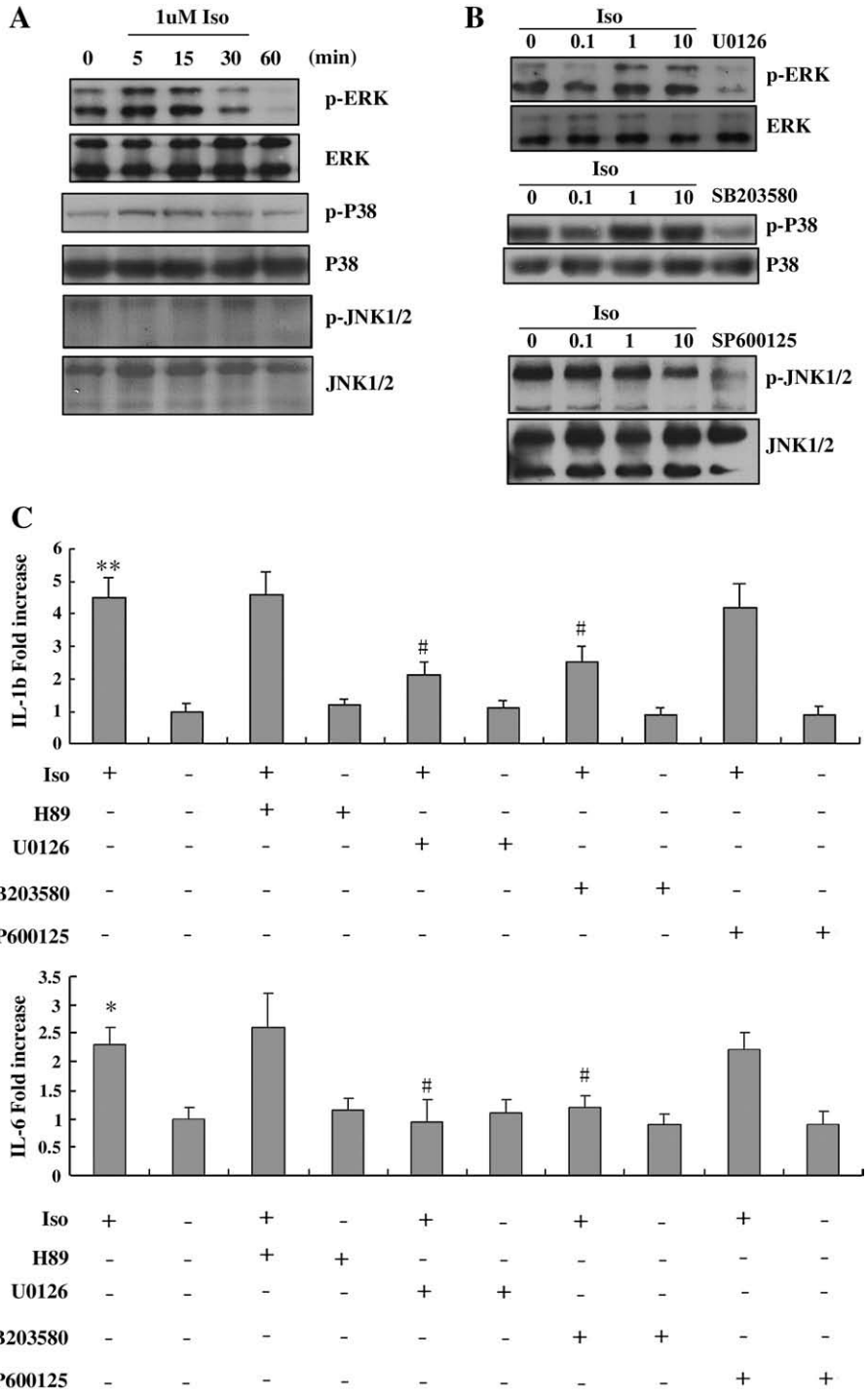

Fig. 5. Signaling pathway involved in the β-AR activation induced IL-1β and IL-6 mRNA expression. (A) Effect of β-AR agonist Iso on ERK1/2, p38MAPK and JNK phosphorylation. Microglia cells were incubated with 1 μM Iso for the indicated time point. The cell lysates were subjected to western blot analysis. (B) Microglia cells were treated with different doses of Iso. (C) Microglia cells were pretreated with H89 (10 μM), U0126 (10 μM), SB203580 (10 μM) or SP600125 (10 μM) for 30 min and then stimulated by 1 μM Iso for 2 h. The Iso-mediated up-regulation of IL-1β and IL-6 transcripts was antagonized by U0126 or SB203580 but not by H89 and SP600125. Experiments were carried out in three independent times. Data were expressed as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, vs control, $\#P<0.05$, vs Iso group.

thus suggests a slower, more sustained signal possibly leading to prolonged cytokine production by microglia cells.

Some of the patient that underwent major surgery will develop post-surgery depression and anxiety, which is associated with increased pro-inflammatory cytokine expression of the brain (Minor et al., 2006). Thus, understanding the mechanisms that regulate pro-inflammatory cytokine response during times of major surgery may have clinical importance in the treatment of numerous post-surgery disorders. The results present here provide further rational application for administrating β-blockade before certain major operations.

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