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Kv1.1 EXPRESSION IN MICROGLIA REGULATES PRODUCTION AND RELEASE OF PROINFLAMMATORY CYTOKINES, ENDOTHELINS AND NITRIC OXIDE

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Abstract—Potassium channels play an important role in microglial activation but their involvement in main functions of microglia including secretion of proinflammatory cytokines has remained uncertain. This study has revealed the specific expression of Kv1.1 in microglia both *in vivo* and *in vitro*. Kv1.1 immunoreactivity was localized in the amoeboid microglia in the rat brain between postnatal (P) day 1 (P1) and day 10 (P10); it was, however, progressively reduced with age and was hardly detected at P14 and P21 in ramified microglia, a derivative cell of amoeboid microglia. Following hypoxic exposure, Kv1.1 expression in amoeboid microglia was enhanced or induced in ramified microglia in more mature brain at P21 when compared with their matching controls. RT-PCR and Western blot analysis confirmed Kv1.1 mRNA and protein expression in murine BV-2 cells which was up-regulated by hypoxia or lipopolysaccharide (LPS) treatment; it was reduced significantly by dexamethasone. Neutralization with Kv1.1 antibody suppressed the expression and release of tumor necrosis factor-α, interleukin-1β, endothelins and nitric oxide (NO) in LPS-activated BV-2 cells. It is concluded that Kv1.1, constitutively expressed by microglia, is elicited by hypoxia and LPS and this may be linked to production of proinflammatory cytokines, endothelins and NO.

Key words: microglia, Kv1.1, proinflammatory cytokines, nitric oxide, endothelins, hypoxia.

It is well documented that microglial activation is a hallmark of brain pathology (Dheen et al., 2007). The cells are responsive to various stimuli including infections and ischemia (Ling et al., 2001). In this connection, both lipopolysaccharide (LPS) and hypoxia have been used to induce microglial activation both *in vivo* and in *vitro* (Zhou et al., 2007; Li et al., 2008). The reactive changes in microglial activation include release of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), nitric oxide (NO) and reactive oxygen species (ROS) (Cao et al., 2008; Deng et al., 2008). Along with the above, it has been reported that under pathological conditions, microglial cells show diverse physiological characteristics including expression of potassium channels that regulate the membrane potential. The expression of outward potassium currents through delayed rectifying channels appears to be highly correlated with the activation of microglial cells. The current is normally absent in resting microglia in culture and it usually requires a stimulating agent for expression. It has been reported that microglia express Kv1.3 (Nörenberg et al., 1993; Schilling et al., 2000), although the existence of Kv1.5-like current in the hippocampus microglia shortly after tissue prints prepared from the rat brain slices has also been reported (Kotecha and Schlichter, 1999). Western blot analysis confirmed the presence of Kv1.3 and Kv1.5 proteins in primary microglia culture, but no Kv1.5-like current was detected (Khanna et al., 2001). It would appear that Kv1.3 is the main voltage-gated potassium channel in the microglia and that it can be up-regulated during activation (Walz and Bekar, 2001). The correlation between expression of potassium channels and microglial activation has remained to be fully explored. We reported recently (Li et al., 2008) expression of Kv1.2 in amoeboid microglial cells (AMC) in the postnatal rat brain. Additionally, using BV-2 cells *in vitro*, it was demonstrated that Kv1.2 was linked to microglial activation and that it modulated release of proinflammatory cytokines TNF-α and IL-1β as well as ROS. Our study has also provided evidence that intracellular potassium concentration is associated with the microglial secretory function. Schmidt et al. (1999) first reported expression of all Kv1 channels in the oligodendrocyte progenitors at the mRNA level. Recent study by Fordyce et al. (2005) has revealed Kv1.2 expression along with Kv1.3 and Kv1.5 in the cultured microglia at the RNA and protein levels, but its role, and indeed also for other potassium channels has remained to be fully clarified.

It is obvious from different studies that microglia express Kv1.2, Kv1.3 and Kv1.5 (Khanna et al., 2001; Walz and Bekar, 2001; Fordyce et al., 2005; Li et al., 2008). Our study (Li et al., 2008) in particular has shown that Kv1.2 plays an important role in microglial functions as demonstrated both *in vivo* and *in vitro* under altered conditions. The present study was aimed to extend this investigation as a step to further the understanding of the varied roles of other potassium channels in microglial cells. In this connection, a putative candidate potassium channel that may

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Abbreviations: AMC, amoeboid microglial cells; Dex, dexamethasone; DMEM, Dulbecco's modified Eagle's medium; EIA, enzyme immunoassay; ET, endothelin; IL-1β, interleukin-1β; IR, immunoreactive; LPS, lipopolysaccharide; MAP, mitogen-activated protein; NO, nitric oxide; P, postnatal; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

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also play an important role in microglial function would be Kv1.1. This is because Kv1.1 is closely related to Kv1.2 in its localization and distribution (Wang et al., 1994; Chung et al., 2001; Yang et al., 2007). Furthermore, Kv1.1, a Shaker-like voltage-gated potassium channel is expressed transiently in non-neuronal cells during embryonic development and that it may play a role in several developmental processes, including proliferation or cell–cell adhesion (Hallows and Tempel, 1998). Since the AMC in the postnatal brain proliferate, migrate and release endothelins (ETs) (Wu et al., 2006) and proinflammatory cytokines (Deng et al., 2008) especially during activation, it was therefore surmised that Kv1.1, in view of its close association with Kv1.2 (Li et al., 2008), may also be expressed by these cells. This information would provide further insights into the specific roles of potassium channels as well as the molecular mechanism of microglial activation in neuroinflammation such as hypoxic periventricular white matter injury in postnatal brain that has been shown to be mediated by AMC (Deng et al., 2008).

EXPERIMENTAL PROCEDURES

Animals and Kv1.1 immunoexpression in the developing brain

Postnatal Wistar rats at days 1 (P1), 3 (P3), 5 (P5), 10 (P10), 14 (P14) and 21(P21) (n=6 for each age group) were used. At the respective age groups, the rats were anesthetized with 6% sodium pentobarbital (100 mg/kg) injected intraperitoneally and sacrificed by perfusion transcardially first with Ringer's solution, followed by fixation with 2% paraformaldehyde. The brain was removed, post-fixed for 2 h in the same fixative, and then cryoprotected in 15% sucrose for 24 h. Frozen sections at 40 μm were cut coronally through the forebrain with a cryostat (Model CM3050, Leica, Bensheim, Germany) and mounted onto gelatin-coated slides and stored at −20 °C until use. All experiments were approved by Institutional Animal Care and Use Committee, National University of Singapore. All efforts were made to minimize the number and suffering of animals used.

Immunohistochemistry for Kv1.1 was carried out using the avidin-biotinylated peroxidase complex method. Briefly, the tissue sections were first washed 3×10 min in 0.1% Triton X-100 in 0.1 M phosphate-buffered saline (PBS) pH 7.4. They were then preincubated with 5% normal goat serum and 0.1% Triton X-100 in 0.1 M PBS for 1 h at room temperature (22–24°C), followed by incubation overnight with rabbit anti-Kv1.1 polyclonal antibody (1:200, Chemicon International, Temecula, CA, USA) at room temperature. Incubation in the secondary antibody (biotin-conjugated anti-rabbit IgG, 1:200, Vector Laboratories, Burlingame, CA, USA) was for 1 h at room temperature. The reaction product was revealed with 3–3′ diaminobenzidine tetrahydrochloride and nickel sulfate. The tissue sections were counterstained with 0.25% Methyl Green in 0.1 M acetate buffer pH 4.8 and coverslipped with Permount after dehydration and clearing. For controls, the tissue sections were incubated in the incubation medium without the primary antibody or the medium was replaced with normal goat serum.

For Kv1.1 immunofluorescence and double labeling with lectin, frozen coronal sections of the brain at 40 μm thickness containing the corpus callosum were cut and rinsed in PBS. For blocking of non-specific binding proteins, tissue sections were incubated in 5% normal goat serum diluted in PBS followed by incubation for 1 h at room temperature (22~24 °C). After discarding serum, the sections were incubated with primary antibody Kv1.1 (1:200, Chemicon International) at room temperature (22~24 °C). Subsequent antibody detection was carried out with Cy3-conjugated goat anti-rabbit IgG (1:100, Sigma-Aldrich, St. Louis, MO, USA). After washing with PBS, the sections were incubated with FITC-labeled lectin from *Lycopersicon esculentum* (1:100; Sigma-Aldrich, MO, USA), a marker for blood vessels and microglia. The sections were then washed in PBS and mounted in fluorescent mounting medium (DAKO Cytomation, Denmark). Cellular colocalization was then examined in a confocal microscope (FV 1000, Olympus Company Pte. Ltd., Tokyo, Japan). Some sections were treated simultaneously without the primary antibody to confirm the specificity of immunoreactivities.

Hypoxia exposure of postnatal rats

P1 postnatal rats (n=8) were kept in a chamber (Model MCO 18M, Sanyo Electrical Co. Ltd., Tokyo, Japan) filled with a gas mixture of 5% oxygen and 95% nitrogen for 2 h. The rats were allowed to recover under normoxic conditions and killed at 3 (n=4; denoted as HPE1) and 24 h (n=4; denoted as HPE2) after hypoxia. In addition to the above, P21 rats (n=4; denoted as P21HPE) were subjected to similar hypoxic treatment and sacrificed 24 h later. This was aimed to determine if ramified microglia in mature brain also respond to hypoxia. For normoxic matching control, the rats (n=4 for each time point) were kept outside of the chamber. Coronal brain sections were then processed for Kv1.1 immunofluorescence labeling as described above.

BV-2 cell culture and treatment

BV-2 murine cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (FCS) and penicillin (100 U/ml) at 37 °C in a humidified incubator under 5% CO_2. To study the effects of hypoxia and LPS, one group of cells was exposed to hypoxia by placing them in a chamber (Model: MCO 18M; Sanyo Electrical) filled with a gas mixture of 3% O_2, 5% CO_2 and 92% N_2 at 37 °C for 4 h. The other group of cells was incubated with LPS (*Escherichia coli*; 1 μg/ml, Sigma-Aldrich) for 6 h. In addition, cells were exposed to dexamethasone (Dex; 1 μg/ml, Sigma-Aldrich) in the presence of LPS for 6 h to investigate its suppressive effect on Kv1.1 expression in activated cells. The culture medium was replaced with basic DMEM (without penicillin) before treatment with Dex. For controls, the medium was replaced with basic DMEM (without penicillin) incubated in a chamber 95% air/5% CO_2. Finally total RNA and protein were extracted for RT-PCR and Western blotting analysis.

Neutralization test

To study the function of Kv1.1, BV-2 cells were exposed to Kv1.1 antibody at different concentrations: 10, 15 and 20 μg/ ml 20 μg/ml (Chemicon International) in the presence of LPS (1 μg/ml) for 6 h to analyze its effects on expression of inflammatory cytokines, TNF-α and IL-1β, and ETs, including ET-1 and ET-3 in activated BV-2 cells.

Real time RT-PCR

Total RNA was extracted from the control, hypoxia, LPS, LPS and Dex/Kv1.1 antibody BV-2-treated cells using RNeasy mini kit (Qiagen, Valencia, CA, USA). The amount of total RNA was quantified with a Biophotometer (Eppendorf, Westbury, NY, USA). Quantitative RT-PCR was carried out on a Light Cycler 2 instrument using a FastStart DNA Master plus SYBR Green I kit (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Forward and reverse primer sequence for each gene and their corresponding amplicon size are provided in Table 1. The rat β-actin, as an internal control, was also amplified for specific