INVOLVEMENT OF LPA$_1$ RECEPTOR SIGNALING IN CEREBRAL ISCHEMIA-INDUCED NEUROPATHIC PAIN

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Abstract—We demonstrated previously that the lysophosphatidic acid-1 (LPA$_1$) receptor plays a crucial role in the initiation of peripheral nerve injury-induced neuropathic pain through the alternation of pain-related genes/proteins expression and demyelination. The present study revealed that mild cerebral ischemia by left transient middle cerebral artery occlusion (tMCAO) for 15 min causes the hypersensitive responses (paw withdrawal) to the nociception by electrical stimuli to the paw by the use of Neurometer Current Perception Threshold/C (CPT/C). The hypersensitivity or neuropathic pain was only observed by the stimulation with 250 and 2000, but not 5 Hz, which are the characterized sine-wave frequencies of Aδ-, Aβ- or C-fibers, respectively. The significant neuropathic pain was observed from day 2 through week 2 on the right paw after tMCAO, while there was slight but significant pain sensitivity on the left paw at day 7. The neuropathic pain on the contralateral side at week 2 after tMCAO was completely abolished in LPA$_1{}^{-/-}$ mice. These results suggest that LPA$_1$ receptor signaling plays key roles in the development of central neuropathic pain following cerebral ischemia as well as the peripheral neuropathic pain following partial sciatic nerve injury. © 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: A-fibers, cerebral ischemia, LPA$_1$ receptor, neuropathic pain.

INTRODUCTION

Stroke, due to ischemia or hemorrhage, is one of the common medical emergencies leading to irreversible neurological damages in the brain with severe complications including the dysfunction of motor skills, memory and sensory perception, and even death (Feigin, 2005; Harukuni and Bhardwaj, 2006; Shuaib et al., 2007; Ueda, 2009; Sims and Muyderman, 2010; Truong et al., 2012). There are several types of pain disorders that occur after the onset of stroke/cerebral ischemia (Bowsher, 1996; Widar et al., 2002; Kong et al., 2004; Zorowitz et al., 2006; Klit et al., 2009). Among these, central post-stroke pain (CPSP) has been characterized as a long-lasting constant or intermittent pain syndrome arising after the stroke/cerebral ischemia-induced lesions at any level in the somatosensory pathways of brain including medulla, thalamus and cerebral cortex, with consequent deterioration of the quality of life by affecting mood, sleep, and social functioning (Boivie et al., 1989; Bowsher, 1995, 2001; Jensen and Lenz, 1995; Jonsson et al., 2006; Treede et al., 2008; Klit et al., 2009; Kumar et al., 2009; Brigo et al., 2012). The neuropathic or central pain has been estimated to occur approximately in 8% of patients after stroke (Hansson, 2004; Henry et al., 2008). In contrast, the development of stroke-induced pain is seen in more than 3/4 of CPSP patients with somatosensory deficits (Jensen and Lenz, 1995; Bowsher, 2005). Several classes of drugs including antidepressants, anticonvulsants, opioids, N-methyl-D-aspartate (NMDA) antagonists, as well as gabapentin and pregabalin that have multiple cellular effects including the inhibition of $\alpha_2\delta$ subunit of voltage-dependent calcium channel (Sills, 2006), are commonly used for the treatment of post-stroke pain (Bowsher, 1996; Kumar and Soni, 2009; Kim, 2009; Kim et al., 2011). However, the detailed cellular and molecular mechanisms of stroke or cerebral ischemia-induced development of neuropathic pain are largely unknown.

We previously demonstrated that lysophosphatidic acid-1 (LPA$_1$) receptor signaling initiates peripheral nerve injury-induced neuropathic pain by changing the expression of pain-related genes/proteins including voltage-gated calcium channel $\alpha_2\delta$-1 subunit in the dorsal root ganglion and protein kinase Cγ in the spinal dorsal horn (Inoue et al., 2004; Ueda, 2006, 2008; Ma et al., 2010). Furthermore, a single intrathecal (i.t.) injection with LPA induces neuropathic pain through the activation of LPA$_1$ receptor (Inoue et al., 2008; Ueda, 2008; Nagai et al., 2010). But, the involvement of LPA$_1$ receptor signaling in the development of stroke-induced pain syndromes is fully unknown. In the present study, we have attempted to find out the mechanisms of pain development upon cerebral ischemia in mice.

EXPERIMENTAL PROCEDURES

Animals

Male wild-type (WT) C57/BLJ and LPA$_1$ receptor knockout (LPA$_1^{-/-}$) mice weighing 20–30 g were purchased from Tagawa Experimental Animals (Nagasaki, Japan) and used for all the experiments. Mice were kept in a room maintained at constant temperature (21 ± 2 °C) and relative humidity (55 ± 5%) with an automatic 12-h light/dark cycle with free access to standard laboratory diet and tap water. Animal care and all experimental procedures were formally approved by the Nagasaki University Animal Care and Use Committee (Animal Experiments Approval Number: 1104190914).

Middle cerebral artery occlusion

The transient middle cerebral artery occlusion (tMCAO) model was induced following the method as described previously (Halder et al., 2012) Briefly, mice were anesthetized by 2% isoflurane (Mylan, Tokyo, Japan), and the body temperature was monitored and maintained at 37 °C during surgery. After a midline neck incision, the middle cerebral artery was occluded transiently using 8–0 in size monofilament nylon surgical suture (Natsume Co., Ltd., Tokyo, Japan) coated with silicon (Xantopren; Bayer dental, Osaka, Japan) that was inserted through the left common carotid artery and advanced into the left internal carotid artery. Following 60-min tMCAO, the animals were briefly re-anesthetized with isoflurane and the monofilament was withdrawn for reperfusion studies. As the silicon-coated nylon suture also plugs the branch from the middle cerebral artery to supply blood to the hippocampus in mice, due to small brain size, the ischemia-induced brain damages are also observed in the hippocampus. Following the similar protocol, 15-min tMCAO mice were prepared by the occlusion of middle cerebral artery for 15 min. Cerebral blood flow was monitored by laser Doppler flowmeter (ALF21, Advance Co., Tokyo, Japan) using a probe (diameter 0.5 mm) of a laser Doppler flowmeter (ALF2100, Advance Co., Tokyo, Japan) inserted into the left striatum (anterior: −0.5 mm; lateral: 1.8 mm from bregma; depth: 4.2 mm from the skull surface) through a guide cannula.

Nociception tests

The electrical stimulation-induced paw withdrawal (EPW) test was performed following the protocol as described previously (Koga et al., 2005; Matsumoto et al., 2008; Ueda, 2008). Briefly, the mouse was held in the investigator's hand and stimulated through the electrodes (Neurotron Inc., Baltimore, MD, USA), which were fastened to the right plantar surfaces and the insteps of mice. Transcutaneous nerve stimuli with each of the three sine wave pulses (5, 250, and 2000 Hz) were applied using the Neurometer CPT/C (Neurotron Inc.). The minimum intensity (microampere) at which each mouse withdrew its paw was defined as the current stimulus threshold. Stimuli were applied at 10-min intervals.

In the thermal paw withdrawal test, nociception was measured as the latency to paw withdrawal evoked by exposure to a thermal stimulus (Hargreaves et al., 1988; Inoue et al., 2004). Unanesthetized animals were placed in Plexiglas cages on top of a glass sheet, and an adaptation period of 1 h was allowed. The thermal stimulator (IITC Inc., Woodland Hills, CA) was positioned under the glass sheet, and the focus of the projection bulb was aimed exactly on the middle of the plantar surface of the animal. A mirror attached to the stimulator permitted visualization of the plantar surface. A cut-off time of 20 s was set to prevent the tissue damage. The paw pressure test was performed as described previously (Rashid et al.,

2003; Inoue et al., 2004). In brief, mice were placed into a Plexiglas chamber on a 6- × 6-mm wire mesh grid floor and were allowed to acclimatize for a period of 1 h. The mechanical stimulus was then delivered onto the middle of the plantar surface of the right hind paw using a transducer indicator (model 1601; IITC Inc.). The pressure needed to induce a flexor response was defined as the pain threshold. All pain-related behavioral experiments were carried out after cerebral ischemia (15 min tMCAO) and sham operation using at least 5 mice for each group.

2,3,5-Triphenyltetrazolium chloride (TTC) staining

To perform TTC staining, the brain was quickly removed at 24 h after the cerebral ischemia (60- and 15-min tMCAO) and sham operation, sectioned coronally with 1-mm thickness and washed with K$^+$-free phosphate-buffered saline (PBS). Brain slices were incubated in 2% TTC (Sigma–Aldrich, St. Louis, MO, USA) in 0.9% NaCl in a dark place for 15–20 min at room temperature and transferred into 4% paraformaldehyde (PFA) overnight. Slices were then scanned by scanner (EPSON GT-9700F) and the infract areas were calculated by image analysis software (Image J; NIH, MD, USA).

Behavioral assessments

WT and LPA$_1^{-/-}$ mice were prepared to perform the behavioral study. Following cerebral ischemia (60- and 15-min tMCAO; $n = 10$ and $n = 7$, respectively) and sham operation, neurological scores were assessed for 14 days. The clinical score including motor dysfunction was evaluated from day 1 after tMCAO in the following way: 0, no observable deficits; 1, failure to extend the forepaw fully; 2, circling to the ipsilateral or contralateral way; 3, falling to one side; 4, no spontaneous movement; 5, death. In this study, 0.5 point was added to each score when the motor dysfunction was severe for scores between 1 and 4. The survival rate was evaluated from day 1 after tMCAO and calculated by the percentage of mice that were alive after ischemia.

Statistical analysis

Data were analyzed using Student's t-test. The differences between multiple groups were analyzed using a one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison post hoc analysis. The criterion of significance was set at $p < 0.05$. All results are shown as means ± standard error of the mean (S.E.M.).

RESULTS

Mild cerebral ischemia

Four categories of behavioral rating are mainly used between normal (score 0) and death (score 5) in the clinical score, which is widely used for the evaluation of motor dysfunction following cerebral ischemia (Murakami et al., 1998; Zhang et al., 2002; Ohsawa et al., 2007). Following cerebral ischemia by left tMCAO for 60 min in mice and subsequent assessment of clinical scores for 14 days revealed that most of mice died through day 4–6 after ischemic stress (Fig. 1A). The TTC staining data showed that the infarct volume was significantly increased in the ipsilateral side of WT brain at 24 h after 60-min tMCAO, whereas no infarct volume was observed in sham-operated mice (Fig. 1B, C). As the higher lethality was observed in 60-min
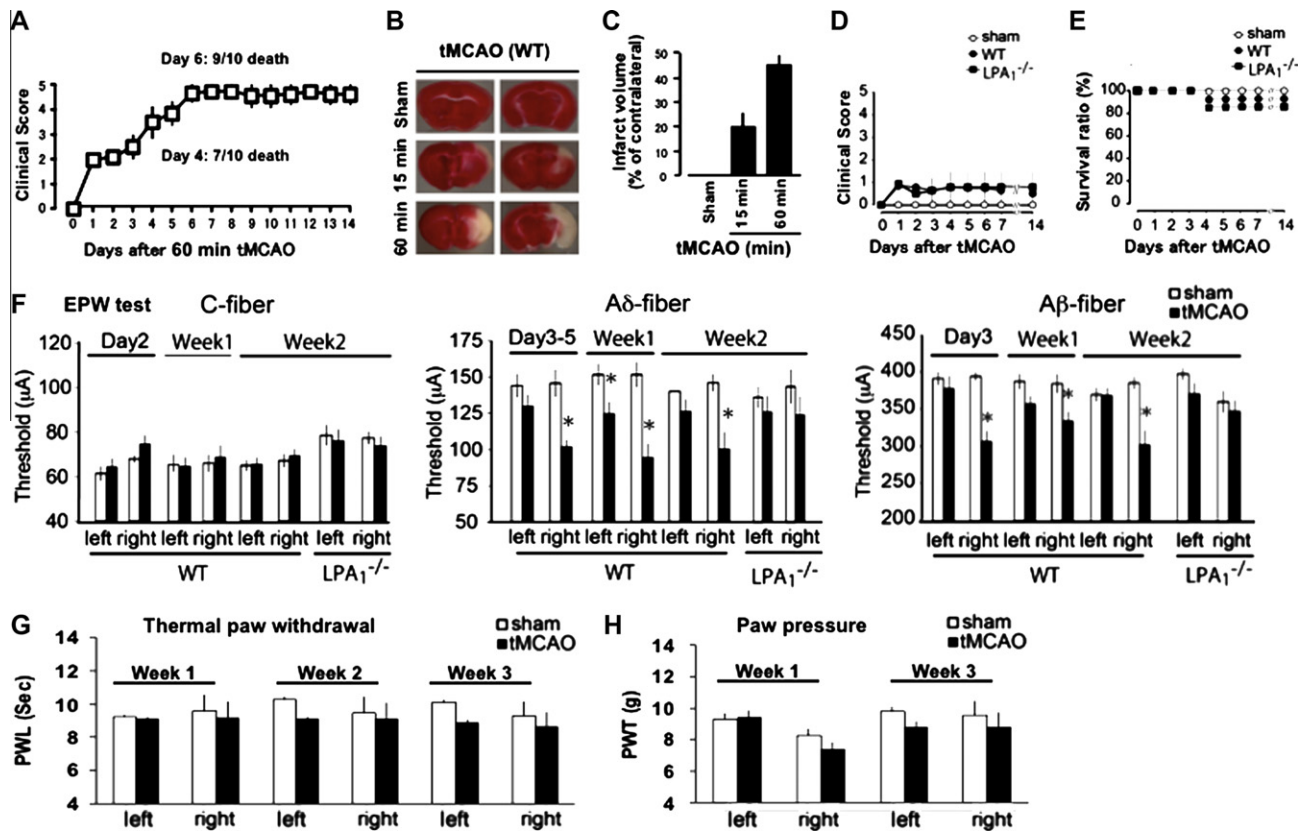
Fig. 1. LPA$_1$ receptor signaling is involved in the development of cerebral ischemia-induced neuropathic pain through the stimulation of A-fibers. (A) Measurement of clinical scores through 14 days after 1-h tMCAO using WT mice (n = 10). Following 1-h tMCAO, 7 and 9 mice died at day 4 and day 6 after ischemia, respectively. (B, C) TTC staining (B) and measurement of infarct volume (C) are performed at 24 h after 15- and 60-min tMCAO as well as sham-operation in WT mice. (D, E) Mild cerebral ischemic mice (15 min tMCAO) show no significant motor dysfunctions. Measurement of clinical score (D) and survival ratio (E) are performed through 14 days after 15-min tMCAO in WT and LPA knockout (LPA$_1^{-/-}$) as well as in sham-operated mice. (F) Following 15-min tMCAO and sham operation, the threshold levels are measured at day 2–5, week 1 and week 2 after ischemia in WT mice as well as week 2 in LPA$_1^{-/-}$ ischemic mice by EPW test, which represents the minimum intensity (µA) required to produce a paw withdrawal response to electrical stimulation with 5 Hz for C-fiber (left panel), 250 Hz for Aδ-fiber (middle panel), and 2000 Hz for Aβ-fiber (right panel). (G) Following mild cerebral ischemia (15 min tMCAO), thermal paw withdrawal test is done at week 1, 2 and 3 after ischemic stress and sham operation. (H) Paw pressure test is performed at week 1 and 3 after 15 min tMCAO and sham operation. Data are mean ± S.E.M. using at least 5 mice for each group ($^*P$ < 0.05, vs. Sham).

tMCAO mice, we attempted to develop the mild ischemic stroke model, in which cerebral ischemia was performed by left tMCAO for 15 min using WT and LPA$_1$ receptor knockout (LPA$_1^{-/-}$) mice, and behavioral study was carried out through 14 days after ischemic stress. The findings with TTC staining showed that limited infarction in the ipsilateral brain was observed at 24 h after 15 min tMCAO in WT mice, compared to 60-min tMCAO (Fig. 1B, C). The neurological assessment revealed that there is no significant change in clinical score and survival activity observed through 14 days after 15 min tMCAO in WT and LPA$_1^{-/-}$ mice, an indication of mild cerebral ischemia without showing significant behavioral dysfunctions (Fig. 1D, E).

Long-lasting neuropathic pain

To examine whether mild cerebral ischemia develops neuropathic pain, transcutaneous nerve stimuli, specifically three sine-wave pulses with frequencies of 5, 250 and 2000 Hz that stimulate C-, Aδ- and

Aβ-fibers, respectively, were applied to the hind paws of WT mice through 14 days after 15-min tMCAO. The EPW test revealed that there is no change in withdrawal threshold through 2 weeks following the application of 5 Hz for C-fibers at day 2, week 1 and week 2 after cerebral ischemia, compared to the sham operation (Fig. 1F). On the other hand, the threshold at 250 Hz (Aδ-fibers) was significantly decreased in the contralateral (right) paw at day 3–5, week 1 and week 2 after the ischemic stress, while the decrease in threshold in the ipsilateral (left) paw was only observed at week 1, but not at week 2 (Fig. 1F). The significant decrease in threshold at 2000 Hz for Aβ-fibers was also observed only in the contralateral (but not ipsilateral) paws at day 3, week 1 and week 2 after 15-min tMCAO, an indication of mild cerebral ischemia-induced contralateral and long-lasting pain development through A-fibers stimulation (Fig. 1F).

However, cerebral ischemia failed to decrease the ipsilateral or contralateral nociceptive thresholds in

thermal paw withdrawal or paw pressure tests through 1–3 week after tMCAO (15 min) in WT mice (Fig. 1G, H, respectively).

Involvement of LPA$_1$ receptor signaling

To investigate whether LPA$_1$ receptor is involved in the development of cerebral ischemia-induced neuropathic pain, the pain behavioral study by EPW test was performed through 2 weeks after mild left cerebral ischemia (15 min tMCAO) in LPA$_1^{-/-}$ mice. We observed that LPA$_1^{-/-}$ ischemic mice show no change in threshold level at 5 Hz (C-fibers) through 2 weeks after 15 min tMCAO (Fig. 1F). Interestingly, the ischemia-induced decrease in contralateral thresholds at both 250 (Aδ-fibers) and 2000 Hz (Aβ-fibers) in the WT mice was completely rescued to the basal levels at week 2 after left cerebral ischemic stress in LPA$_1^{-/-}$ mice (Fig. 1F), a novel observation of LPA$_1$ receptor-mediated stimulation of A-fibers in the development of contralateral-predominant and long-lasting neuropathic pain upon mild cerebral ischemia.

DISCUSSION

Stroke/cerebral ischemia is a cerebrovascular accident that causes the permanent functional and cellular damages in the brain, along with the development of several types of long-lasting central pain syndromes such CPSP, shoulder pain, painful spasticity and tension-type headache (Widar et al., 2002; Zorowitz et al., 2006; Shuaib et al., 2007; Kumar et al., 2009; Klit et al., 2011; Truong et al., 2012). Ischemic and hemorrhagic stroke-induced chronic pain has been estimated to occur in 11–55% of patients (Jonsson et al., 2006; Klit et al., 2009). Among these stroke patients, the prevalence of shoulder pain is between 11% and 14% and for CPSP between 8% and 35% (Kumar et al., 2009). On the basis of clinical investigations, CPSP is a chronic neuropathic pain syndrome arising in parts of the body as a consequence of cerebrovascular lesions after stroke/cerebral ischemia associated with sensory abnormalities (Kumar and soni, 2009; Klit et al., 2009; Siniscalchi et al., 2012). In addition, the development of contralateral-dominant central pain syndromes through sensory abnormalities after a minor stroke or mild cerebral infarction has also been reported (Yoshita and Yamada, 2006; Kim et al., 2007). Although several drugs including antidepressants, opioids, and calcium channel blockers are used for the management of post-stroke pain (Klit et al., 2009; Kim et al., 2011), the detailed pathophysiology and mechanisms underlying the stroke/cerebral ischemia-induced development of pain disorders are not clear.

In the present study, we firstly attempted to establish a mild brain ischemic animal model upon minor cerebral ischemia to perform pain-related experiments. The clinical score has been frequently used for the assessment of behavioral dysfunctions in animal models (Murakami et al., 1998; Ohsawa et al., 2007). Following 60-min tMCAO in WT mice, the findings with TTC staining revealed the significant increase in infarct volume at 24 h, but evaluating by behavioral study suggested that most of the mice died through day 4–6 after ischemic stress. For this reason, we prepared ischemic mice by 15-min tMCAO, in which the limited cerebral infarction was observed at the same time point. Following 15-min tMCAO in WT and LPA$_1^{-/-}$ mice, the neurological assessment in terms of clinical score and survival activity suggested that mice showed no significant behavioral dysfunctions through 14 days after the ischemic stress, an indication of the novel model of mild cerebral ischemia.

Next, we investigated the possible mechanism of brain ischemia-induced central pain production using a mild cerebral ischemic mice model. In the present study, we used a EPW nociception test using Neurometer CPT/C, in which applying electrical stimuli to the hind paws (Matsumoto et al., 2008; Ueda, 2008), significant neuropathic pain was observed through the contralateral stimulation of Aδ-fibers at 250 Hz and Aβ-fibers at 2000 Hz, but not C-fibers at 5 Hz at day 2–5, week 1 and week 2 after 15 min tMCAO, whereas, only Aδ-fiber-stimulated hypersensitivity in the ipsilateral paw was observed at week 1, but not day 3–5 or week 2 after 15-min tMCAO. Neurometer is a clinically used device for measuring pain thresholds. Several reports mentioned the usefulness of this device in the quantification of nerve dysfunctions in patients. Furthermore, the neurometer was established as a method for the measurement of three subsets of nerve fibers (innocuous Aβ fiber, noxious Aδ- and C-fibers) in animals (Kiso et al., 2001), in which the neurometer has been demonstrated in electrophysiological (Koga et al., 2005) and pharmacological experiments (Matsumoto et al., 2008; Ueda, 2008).

The present study also revealed that cerebral ischemia failed to show significant decreases in ipsilateral or contralateral nociceptive threshold levels for thermal hyperalgesia and mechanical allodynia in the thermal paw withdrawal and paw pressure tests, respectively. Thus, it is suggested that the EPW test may detect the fiber-specific nociception free from the influence by non-specific mechanical perception from the plate or floor mesh, which is observed in thermal paw withdrawal or paw pressure tests. In the present EPW test, however, the investigator holds mouse in hand for the stimulation through electrodes in paws so that it is free from the non-specific mechanical perception, which may suppress the nociceptive inputs, as known in so-called "gate-control theory" (Melzack and Wall, 1965). In addition, possible imbalanced movement due to ischemia-induced motor dysfunction may also affect the sensitivity of measurements in the thermal paw withdrawal and paw pressure tests. Thus, our findings suggest that cerebral ischemia-induced neuropathic pain is more eminent when Aβ- and Aδ-, but not C-fibers are stimulated. The details of the differential effects of cerebral ischemia on the Aδ- and C-fiber nociceptive responses remain to be determined. However, it is interesting to investigate whether the ischemia-induced damages of cerebral cortex may differentially affect on the pain-related inputs derived

from Aδ- or C-fibers to the somatosensory cortical neurons, or may differentially affect the indirect influence on the descending pain-inhibitory/facilitatory pathways to spinal dorsal horn neurons, which are innervated by Aδ- or C-fibers.

Interestingly, cerebral ischemia-induced contralateral neuropathic pain through A-fibers stimulation was completely abolished at week 2 after 15 min tMCAO in $LPA_1^{-/-}$ mice. We previously described that the LPA_1 receptor is involved in the development of peripheral nerve injury-induced neuropathic pain (Inoue et al., 2004; Ueda, 2006, 2008). Therefore, we can suggest that LPA_1 receptor signaling might be implicated in the induction of neuropathic pain upon cerebral ischemia. However, in the present study, the mild ischemia caused a decrease in the threshold of response to sensory input, despite that the residual motor dysfunction, if any, should have effected an increase to it. Thus, it is suggested that mild ischemia-induced neuropathic pain is not related to potential motor dysfunction. Taken together, this is the first demonstration of a mild brain ischemic mouse model that indicates the involvement of LPA_1 receptor in the development of cerebral ischemia-induced long-lasting neuropathic pain.

CONCLUSION

LPA_1 receptor signaling plays a crucial role in the development of cerebral ischemia-induced contralateral-predominant neuropathic pain through the stimulation of primary afferent Aδ- and Aβ-fibers. Therefore, detailed investigations of LPA_1 receptor-mediated induction of central pain upon cerebral ischemia may provide novel information for the discovery of new drugs in the treatment of post-stroke pain syndromes.

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