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ORIGINAL ARTICLE

Intrathecal glutamate release during hindlimb tourniquet inflation and femoral artery occlusion in rats

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Background/Purpose: A tourniquet is commonly used in limb surgery. Tourniquet inflation after a period of time may produce painful sensation. While the mechanisms of tourniquet-induced pain are still unknown, two components, pressure and ischemia, have been proposed. In this study, *in vivo* microdialysis was used to detect changes in intrathecal glutamate, an excitatory amino acid highly relevant to pain transmission, following hindlimb tourniquet application and femoral artery occlusion in the rat.

Methods: Male Wistar rats were used. For the tourniquet study, 6 rats of the study group received 30 minutes right hindlimb tourniquet inflation and another 6 rats as the control group received only tourniquet application without inflation. In the femoral artery occlusion study, 6 rats of the study group received 30 minutes right femoral artery occlusion and another 6 rats as the control group received only sham operation without femoral artery occlusion. Cerebrospinal fluid dialysates were collected prior to, during, and after tourniquet application or femoral artery occlusion. Glutamate was measured by HPLC.

Results: A significant increase in intrathecal glutamate release was found during the tourniquet inflation period, and it returned to baseline after tourniquet deflation. No change of glutamate release was noted during femoral artery occlusion or after femoral artery reperfusion.

Conclusion: The intrathecal glutamate release was increased by the hindlimb tourniquet inflation, but not influenced by femoral artery occlusion in the rat.

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Introduction

A pneumatic tourniquet used on an extremity for operation may induce a painful sensation during the maintenance of inflation. The mechanism of this sensation, termed tourniquet pain, is not completely understood. Nerve compression and arterial occlusion may produce clinical pain problems, and both of them could be induced by tourniquet inflation. Two components, pressure pain and ischemic pain, have been proposed.[1] Pressure pain is caused by mechanic pressure of the cuff and felt at the site of cuff. Ischemic pain is felt distally to the cuff and is proposed to be caused by the accumulated metabolites being produced in the ischemic tissue. Glutamate, an excitatory amino acid, is a major neurotransmitter in the central nervous system and plays an important role in both normal and pathophysiological nociception.[2] Glutamate is released from the central terminals in the spinal cord upon noxious stimulation. There are no previous reports investigating the relationship between tourniquet pain and glutamate release in the spinal cord. In this study, we used the *in vivo* microdialysis method to examine the glutamate release in the cerebrospinal fluid under two situations in the rat: hindlimb tourniquet inflation and femoral artery occlusion.

Methods and materials

Animal preparation

Male Wistar rats (350—400 g) from the National Laboratory Animal Center, Taiwan were used. The investigations were performed using a protocol approved by the Institutional Animal Care Committee of the National Defense Medical Center, Taiwan. The rats were implanted with a microdialysis probe that inserted via the cisternal membrane of the C-spine and advanced caudally to the lumbar enlargement of the spinal cord. The ends of the microdialysis probe were externalized and fixed to the cranial aspect of the head. The rats then returned to their home cages for a 3-day recovery period. The animals were maintained on a 12-hour light/dark cycle with food and water freely available. Rats showing any neurologic deficits after surgery were excluded.

Construction of the spinal cord microdialysis probe

The intrathecal dialysis loop is constructed using two 7-cm pressure-equalizing tubes (200-μm inner diameter, 356-μm outer diameter) and a 4-cm cuprophan hollow fiber (300-μm outer diameter, 200-μm inner diameter, 11-kDa molecular weight cutoff; Filtral, AN 69-HF; Eicom Co., Kyoto, Japan). A Nochrome-Formvar wire and the cuprophan hollow fiber are connected to a PE-10 catheter with epoxy glue. The middle portion of the cuprophan hollow fiber is bent to form a U-shaped loop and both ends of the dialysis loop, which consisted of silastic tubes, are sealed with silicone sealant. During the *in vivo* measurements, the recovery rate of the dialysis probe was 40% at an infusion rate of 5 μl/min.

Tourniquet study

There were 2 groups allocated: the control group ($n = 6$) with no inflation of tourniquet and tourniquet group ($n = 6$) with tourniquet inflated by 300 mmHg. The animals were anesthetized intraperitoneally with pentobarbital (50 mg/kg). An inflatable pneumatic tourniquet (1 cm in width, made by our medical engineering department) was applied on the animal's right hindlimb thigh, and was connected to a sphygmomanometer. First, the baseline dialysates of both groups were collected three times with 15-minute intervals, then the tourniquet was inflated by 300 mmHg in the tourniquet group and no tourniquet inflation was performed in the control group. The dialysates of both groups were collected during the following 30 minutes with tourniquet inflation or not. After tourniquet deflation, the dialysates of both groups were collected every 15 minutes for 30 minutes.

Femoral artery occlusion study

The two groups allocated were: the sham group ($n = 6$) with no femoral artery occlusion; and the ischemic group ($n = 6$) with femoral artery occlusion. The rats were anesthetized intraperitoneally with pentobarbital (50 mg/kg) and placed on a heated mat during the operation. Under an operating microscope, after cutaneous incision of the right inguinal region, the right femoral artery was exposed and dissected free from the femoral vein and nerve. The baseline dialysates of both groups were collected three times with 15-minute intervals, then the femoral artery was occluded by a vessel clip in the ischemic group and no femoral artery occlusion was done in the sham group. In the ischemic group complete arterial occlusion post vessel clip application was checked by microscope. The dialysates of both groups were collected during the following 30 minutes with femoral artery occlusion or not. After this, the vessel clip was removed and the dialysates of both groups were collected every 15 minutes for 30 minutes. Reappearance of femoral blood flow after removing the vessel clip was checked by microscope in the ischemic group.

Microdialysis procedure and measurement of glutamate

One of the dialysis tubes was connected to a syringe pump (CMA-100) using a 30 cm length of PE-10 tubing (inflow), and the other were connected to a 30 cm PE-10 tube (outflow) for sample collection. Samples were collected in polypropylene tubes on ice and frozen at −80°C until analysis. The concentrations of glutamate were measured by high-performance liquid chromatography (HPLC; Agilent 1100; Agilent Technologies, Wilmington, DE, USA) using a fluorescence detector (Gilson model 121, set at 428 nm; Gilson, Inc., Middleton, WI, USA), as described previously.[3] In brief, amino acids were assayed by recolumn derivatization with o-phthalaldehyde/t-butylthiol (OPA) reagent and iodoacetamide/methanol scavenger. Derivatization was performed by adding 4 ml of OPA reagent to 40 μl of sample, shaking the mixture, then allowing it to react for 2 minutes. A 4 ml aliquot of reagent B (185 mg of iodoacetamide/ml of

methanol) was added and the mixture allowed to react for another 2 minutes. The derivatized sample was then injected onto a C18 reversed phase column and eluted at a flow rate of 0.45 ml/min. A linear gradient from 100% eluent A [0.1 M sodium acetate buffer, pH 6.8/acetonitrile (80:20)] to 100% eluent B [acetonitrile/double-distilled water (80:20)] was used to separate the aminoacids. All solvents were vacuum filtered through a 0.22-Am membrane (Millipore, Billerica, MA, USA) and degassed by sonication before use. External standard solutions, containing 0 M, 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M standard amino acids, were run before and after each sample group.

Data analysis

Because of the wide variation of the baseline concentration of glutamate, we chose to represent our results in percentage of the mean baseline for data analysis. The data are presented as mean ± standard error of the mean. An unpaired two-tailed *t* test was used for statistical analysis. A *p*-value <0.05 was considered statistically significant.

Results

The intrathecal glutamate release was significantly increased during the tourniquet inflation period, and it returned to baseline following tourniquet deflation (Fig. 1). However, there was no change of the glutamate release during femoral artery occlusion (Fig. 2).

Discussion

The results demonstrated an increase of glutamate release in cerebrospinal fluid during hindlimb tourniquet inflation in rats, and no change of glutamate release during femoral arterial occlusion. According to the nociresponsive role of glutamate, we hypothesized that tourniquet-induced painful sensation might be mainly caused by compression of the nerves or the tissues with sensory innervation beneath tourniquet cuff rather than the tourniquet-induced tissue ischemia, especially in the initial phase.

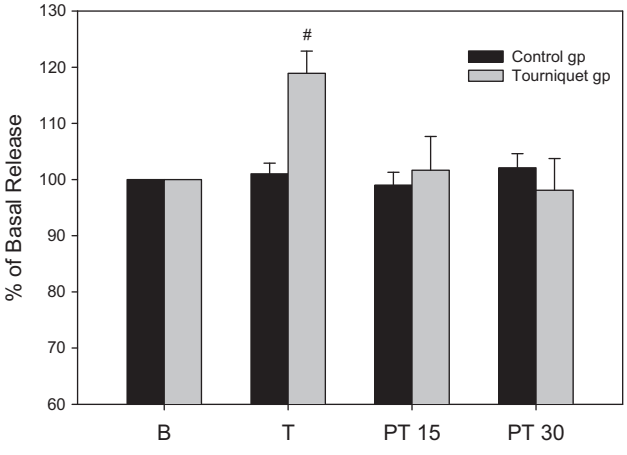

Figure 1 The glutamate release in the cerebrospinal fluid is significantly increased during the period of tourniquet inflation, and it returned to basal level after tourniquet deflation. Data presented as mean ± SEM. $^{\#}p < 0.05$ compared with control group. B = baseline; T = tourniquet inflation; PT 15 = post tourniquet deflation 15 minutes; PT 30 = post tourniquet deflation 30 minutes.

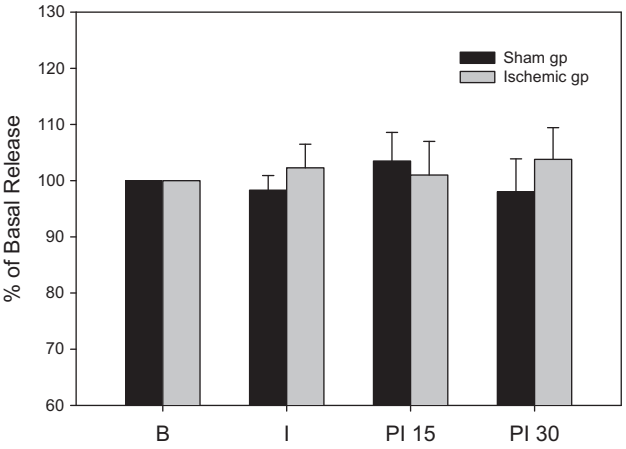

Figure 2 The glutamate in the cerebrospinal fluid has no change during femoral artery occlusion and after the femoral artery reperfusion. Data presented as mean ± SEM. B = baseline; I = ischemia by femoral artery occlusion; PI 15 = postischemia 15 minutes (after vessel clip removal); PI 30 = postischemia 30 minutes (after vessel clip removal).

Regarding the possible mechanisms of tourniquet-induced pain, the unmyelinated, slow-conducting C-fibers have been reported to be involved.[4–6] Chabel et al[4] recorded microfilaments firing in an animal model of tourniquet-induced ischemia and demonstrated that a group of spontaneously active fibers with slow firing rates and conduction velocities in the C-fiber range were not found prior to tourniquet inflation. MacIver et al[5] used an *in vitro* study under metabolic perturbations associated tourniquet ischemia (hypoxia, hypoglycemia, lactic acid, and decreased pH) and showed that neither hypoxia nor hypoglycemia alone or in combination caused A-δ fibers to become spontaneously active, but these conditions could increase the C-fibers' action potential frequency. Crews et al[6] used neurophysiologic recording of the spinal cord neurons and found that compression and ischemia during the maintenance of tourniquet inflation resulted in a reduction inactivity of the low threshold mechanoreceptors of dorsal horn neurons and marked increasing of the spontaneous firing rate of the high threshold nociresponsive neurons. Activation of nociceptive afferents will produce excitatory amino acids release at the first synapses within the spinal cord dorsal horn.[2,7] Prolonged firing of C-fiber nociceptors may cause the release of glutamate, which acts on *N*-methyl-D-aspartic acid (NMDA) receptors in the spinal cord.[8] Takada et al[9] found that preadministration of low-dose ketamine (0.1 mg/kg), an NMDA receptor antagonist, attenuates tourniquet pain and prolongs tourniquet time during high-pressure tourniquet application in healthy volunteers. Segerdahl et al[10] also demonstrated a decrease of tourniquet-associated pain by pretreatment of low-dose ketamine in healthy volunteers. In a laboratory study, Lee

et al[11] reported that intravenous administration of ketamine before tourniquet inflation suppressed spinal c-*fos* mRNA expression in rats. It is thought that the expression of Fos protein in spinal dorsal horn neurons could be induced by afferent nociceptive stimulations.[12]

It has been proposed that the tourniquet pain includes two separate components, pressure pain and ischemic pain.[1] Pressure pain is induced by the compression of nerve fibers beneath the tourniquet cuff, and ischemic pain is associated with the tissue ischemia produced by tourniquet-induced arterial occlusion. Currently, femoral arterial ligation represents a common model used to investigate peripheral ischemic pain.[13] Mostly, these studies were to examine the possible mechanisms of chronic ischemic pain. The acute painful phenomenon just following arterial occlusion was seldom researched. According to the result of the current study that femoral artery occlusion did not influence the spinal glutamate release, we hypothesized that the painful sensation induced by tourniquet, especially in the initial phase, is mainly caused by compression of the tissues with sensory innervation beneath cuff such as nerve itself, muscle, and periosteum. Ischemia alone is unlikely to generate pain in the beginning of tourniquet inflation. Ludwig et al[14] reported that acute ischemia-reperfusion injury induced by femoral artery ligation in Wistar rats did not produce painful symptoms. Clinically, pain sensation is one of the symptoms of peripheral artery occlusion disease; however, the pain does not occur at rest, but with exercise or on critical limb ischemia.[15] In the presents study, the rats were immobilized by intraperitoneal pentobarbital anesthesia during the experimental course. This situation is similar to the clinical resting condition.

In this study, the animals were anesthetized during experimental period, to allow smooth collection of dialysates, thus no pain behavior measurement was done. Therefore, it is difficult to conclude whether the increase or no change of intrathecal glutamate release during hindlimb tourniquet inflation or femoral artery occlusion is related to tourniquet pain. However, the result that intrathecal glutamate release was increased during hindlimb tourniquet inflation but not during femoral artery occlusion, may provide a new direction for future tourniquet pain-related studies.

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