Original Article

The peripheral Epac1/p-Cav-1 pathway underlies the disruption of the vascular endothelial barrier following skin/muscle incision and retraction-induced chronic postsurgical pain

Hongsheng Chen[#], Qing She[#], Yanfang Liu[#], Junjie Chen, Yibin Qin, Cui'e Lu

Department of Anesthesiology, Affiliated Hospital of Nantong University, Nantong, China

Contributions: (I) Conception and design: C Lu; (II) Administrative support: Y Qin, Q She; (III) Provision of study materials or patients: H Chen; (IV) Collection and assembly of data: H Chen, J Chen, Q She; (V) Data analysis and interpretation: Y Liu, Y Qin; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Cui'e Lu. Department of Anesthesiology, Affiliated Hospital of Nantong University, 20 Xisi Road, Nantong 226001, China. Email: lucuie608@163.com.

Background: Vascular endothelial barrier disruption is pivotal in the development of acute and chronic pain. Here, we demonstrate a previously unidentified molecular mechanism in which activation of the peripheral Epac1/p-Cav-1 pathway accelerated the disruption of the vascular endothelial barrier, thereby promoting chronic postsurgical pain (CPSP).

Methods: We established a rat model of CPSP induced by skin/muscle incision and retraction (SMIR). Pain behaviors were assessed by the mechanical withdrawal threshold (MWT) at different times. Local muscle tissues around the incision were isolated to detect the vascular permeability and the expression of Epac1 and Cav-1. They were assessed by western blot and immunofluorescence staining.

Results: SMIR increased vascular endothelial permeability and the number of macrophages and endothelial cells in the muscle tissues around the incision. The peripheral upregulation of Epac1 was macrophage-derived, whereas that of p-Cav-1 was both macrophage and endothelial cell-derived in the SMIR model. Moreover, the Epac1 agonist 8-pCPT could induce mechanical sensitivity, increase the expression of p-Cav-1, and disrupt vascular endothelial barrier in normal rats. The Epac1 inhibitor CE3F4 attenuated established SMIR-induced mechanical hyperalgesia, the upregulation of p-Cav-1 and vascular endothelial barrier. Finally, we showed that intrathecal injection of Cav-1siRNA relieved SMIR-induced mechanical allodynia, but had no effects of the expression of Epac1.

Conclusions: Collectively, these results revealed a molecular mechanism for modulating CPSP through the peripheral Epac1/Cav-1 pathway. Importantly, targeting Epac1/Cav-1 signaling might be a potential treatment for CPSP.

Keywords: Epac1; Cav-1; vascular endothelial barrier; chronic postsurgical pain (CPSP)

Submitted Nov 02, 2022. Accepted for publication Dec 20, 2022.
doi: 10.21037/atm-22-6069
View this article at: https://dx.doi.org/10.21037/atm-22-6069

Introduction

Chronic postsurgical pain (CPSP) is a potential adverse outcome after surgery with an estimated incidence of up to 50% among postsurgical patients (1). This chronic pain is accompanied by depression, disability, and reduced quality of life (2,3). The development of chronic postsurgical pain is multifactorial, and its understanding remains scientifically basic and a clinical challenge (4,5). Peripheral sensitization and central sensitization induced by persistent changes of maladaptive neuroplasticity are involved in the process of

CPSP (6,7). However, the underlying cellular and molecular mechanisms are still unclear, and this is a major limitation for identification of novel and adequate treatments.

Peripheral nervous system (PNS) and central nervous system (CNS) lesions lead to neuropathic pain that is combined with remarkable immune responses, such as those induced by monocytes/macrophages and microglia. These immune cells contribute to chronic pain by producing inflammatory mediators and activating nociceptive neurons (8). However, the mechanisms are different between the PNS and CNS in regulating the immune cells (9). Blood-nerve barrier (BNB) disruption has been found to participate in many diseases including chronic pain (10-12). The neurovasculature of the BNB mainly comprises endothelial cells that are coupled to neurons via glial cells (13). This barrier can provide a selective transport system, which limits the access of substances into the blood. Peripheral nerve injuries increase the permeability of the BNB and induce neuropathic pain through the injured afferents (14). This chronic pain is maintained by continuous neuroinflammation (11,15). After PNS injury, the role of monocytes and macrophages are reported to remain peripheral. The soluble factors, produced by resident macrophages in dorsal root ganglia (DRG), initiate the recruitment of monocytes and neutrophils (16,17). Additionally, as a consequence of endothelial cell activation, monocytes permeate into peripheral nerves and promote the development of mechanical hypersensitivity (18,19).

Exchange proteins directly activated by cyclic adenosine monophosphate (cAMP) (Epac) have been reported to cAMP signaling pathway in a number of pathological and physiological processes, such as cell inflammation, metabolism, and neurological dysfunction (20-22). Recently, Epac1, a sensor of cAMP, was reported to display an increase in the DRG that could promote the transition toward chronic pain. This might be a potential pathway for treating chronic pain (4,23). However, the underlying mechanisms are still unclear. Some reports have shown that Epac1 regulates intercellular adhesion, links, and the vascular endothelial permeability after injury (24,25). Various effector proteins are the signals downstream from Epac1. Both Notch and vascular endothelial growth factor (VEGF) signaling, are two key pathways for angiogenesis regulation. It was reported that Epac1 contributes to pathological neovascularization by regulating these pathways (26). Recently, caveolae have emerged as mediators and targets of endothelial dysfunction (27). Caveolae are small invaginations of the plasma membranes in many mammalian cells. They constitute specific forms of lipid rafts (28,29). Caveolin-1 (Cav-1) is the signaling component and main structural of caveolae. It was mainly expressed in the inflammatory cells (30). More, Cav-1 was shown a notable role in different pain through modulation of central sensitization (31). But the effect of Cav-1 in peripheral sensitization is not clear. Here, we investigated the role of Epac1 in the disruption of the vascular endothelial barrier associated with CPSP and the role of Cav-1 in this process. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6069/rc).

Highlight box

Key findings

- The inhibition of peripheral Epac1/p-Cav-1 pathway might elicit the analgesic effects via the recovery of vascular endothelial barrier and the downregulation of macrophage and endothelial cells.

What is known and what is new?

- Blood-nerve barrier (BNB) disruption has been found to participate in many diseases including chronic pain. However, the underlying cellular and molecular mechanisms are still unclear, and this is a major limitation for identification of novel and adequate treatments.
- We firstly investigated the role of peripheral Epac1/p-Cav1 in the disruption of vascular endothelial barrier associated with CPSP.

What is the implication, and what should change now?

- Targeting Epac1/Cav-1 signaling might be a potential treatment for CPSP.

Methods

Animals

Male Sprague Dawley (SD) rats (200–250 g body weight, 8–10 weeks of age) were obtained from the Experimental Animal Center of Nantong University and maintained in a temperature-controlled room (23±1 ℃) with a 12-hour light/dark cycle. All procedures were approved by the Ethics Committee of Affiliated Hospital of Nantong University (No. 20170305-001), in compliance with national guidelines for the care and use of animals. Water and food were freely available in the Laboratory of Animal Behavior. A protocol was prepared before the study without registration.

Groups and drugs

Rats were divided randomly into 8 groups and anaesthetized with 10% chloral hydrate (300 mg/kg, i.p.). (I) The naive group received no treatment. (II) In the sham group, an incision was made on the skin of the medial thigh 3–4 mm medial to the saphenous vein. (III) In the skin/muscle incision and retraction (SMIR) group, that incision was bluntly separated 7–10 mm by exposing the superficial muscles, and the tip of the retractor was inserted into the superficial muscle to expand the incision to 2 cm. The incision was sutured 1 hour later with medical silk. (IV) In the 8-pCPT (Epac1 agonist) group, normal rats received an injection of 1 μg of the Epac1 agonist 8-pCPT (ABNUS, China, 1 μg) into the right hind paw. (V) In the saline group, the right hind paw of the rats was injected with the same dose of saline. (VI) In the SMIR + CE3F4 (Epac1 inhibitor) group, 1 μg of the Epac1 inhibitor CE3F4 (Tocris Bioscience, Bristol, UK, 1 μg) was injected into the right hind paw 7 day after SMIR modeling. (VII) In the SMIR+ Cav-1 siRNA (20 μL) group, intrathecal injections of Cav-1 siRNA (Ribobio, Guangzhou, China) were performed 1 day, 3 days, and 5 days after SMIR modeling. (VIII) In the solvent group, intrathecal injections of solvent were performed 1, 3, and 5 days after SMIR modeling. The Cav-1 siRNA sequence was 5'-CCAGAAGGGACACACAGTT-3', and the control siRNA sequence was 5'-GAGAAGCAGU GAUACGACG-3'.

Behavioral testing

Rats were adapted for 30 minutes on the metal screen in the organic glass box (22×12×22 cm^3) before experiments. The mechanical withdrawal threshold (MWT) was measured before surgery and on the 1, 3, 7, 14, and 28 days after surgery. In the resting state, the Von Frey filament cilia stimulator (North Coast Medical, Morgan Hill, CA, USA) with a stiffness of logarithmically shifted (1.4–26 g) was used to perform vertical stimulation to the hind paw. With Von Frey slightly bent as the total force standard, the duration was less than or equal to 4 seconds. The test was repeated 5 times with a 5-minute interval and the appearance of paw withdrawal, paw licking, and so on, was considered positive (at least 3 times out of five application), otherwise negative. The MWT was calculated according to the Chaplan up-down method (32).

Detection of local tissue vascular permeability

All operations were performed under10% chloral hydrate (300 mg/kg, i.p.) anesthesia. A 2% Evans blue solution (1 mL/kg) was slowly injected into the left femoral vein. After 60 minutes, normal saline (100–200 mL) was perfused through the ascending aorta of the left ventricle until clear fluid flowed from the auricula dextra. The operated muscle tissues were removed for further use. The tissues were soaked in the 2% carboxamide bath at a ratio of 100 mg: 1 mL, homogenized, and stored in a 37 ℃ incubator for 48 hours. Then, they were centrifuged at a high speed (11,000 rpm) for 15 minutes. The supernatant was carefully aspirated and divided into 3 sample tubes (50 μL/tube). The absorbance was measured at 620 nm by a microplate reader (BioTek, Winooski, VT, USA). Formamide blank tubes were used to make a standard curve for EB. The Evans blue contents of the samples were calculated based on the standard curve, and the mean value was used to explore the leakage outside the blood vessel.

Immunofluorescence staining

We anesthetized the animals with 10% chloral hydrate (300 mg/kg, i.p.). Then, rats were transcardially perfused with saline, and the tissues were fixed by perfusion fixative (4% paraformaldehyde). The tissues were immediately removed and dehydrated in 20% and 30% sucrose solutions successively. The submerged tissues were frozen at –20 ℃ and continuously sliced into 5 μm sections. After washing with phosphate-buffered saline (PBS) and blocking in 5% serum for 2 hours at room temperature, the sections were incubated with the respective primary antibodies Epac1 (1:50, sc-28366, Santa, USA), p-cav-1 (1:50, sc-373836, Santa, USA), CD34 (1:100, ab81289, Abcam, Cambridge, UK), and CD68 (1:50, ab125212, Abcam, UK) antibodies (overnight, 4 ℃). The tissue sections were then washed with PBS, and separately incubated with the secondary antibodies for 2 hours at room temperature. The secondary antibodies were Cy3-conjugated goat anti-rabbit IgG (1:1,000, Jackson ImmunoResearch, West Grove, PA, USA) and FITC-conjugated goat anti-mouse IgG (1:1,000,

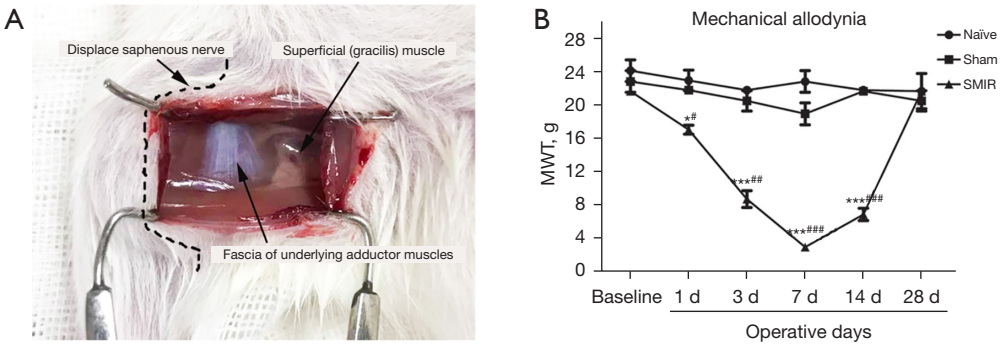

Figure 1 SMIR induced persistent mechanical allodynia (n=8 in each group). (A) Typical figure showed the operative field in rats under SMIR model. (B) Changes of pain sensitivity in different groups were determined by MWT on the day before operation (baseline) and on days 1, 3, 7, 14, and 28 after operation. *, P<0.05 *vs.* naive group, ***, P<0.001 *vs.* naive group; #, P<0.05 *vs.* sham group, ##, P<0.01 *vs.* sham group, ###, P<0.001 *vs.* sham group. SMIR, skin/muscle incision and retraction; MWT, mechanical withdrawal threshold.

Jackson, USA). Images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

Histopathology

The morphological changes of the local tissues were observed by hematoxylin and eosin (HE) staining.

The muscle tissues around the incision were fixed in 4% paraformaldehyde solution for 24 hours. After embedded in paraffin, the tissues were continuously sliced into 4 μm sections and stained with HE for histomorphological analysis. The HE staining clearly showed the external elastic lamina, the neointima, and the internal elastic lamina.

Western blotting

After rats were transcardially perfused with saline under deep anesthesia, the local muscle tissues around the incision were immediately removed and homogenized in the ice-chilled tissue protein extraction reagent. The tissues were prepared as reported previously (22). The protein samples were transferred onto polyvinylidene fluoride membranes. The membranes were subsequently blocked with the Tris-buffered saline solution containing 5.0% skim milk powder for 2 hours at room temperature and incubated with primary antibodies against Epac1, p-cav-1, and β-actin (1:5,000, Sigma, St. Louis, MO, USA) at 4 ℃ overnight. After washing with Tris-buffered saline/Tween 20 (TBST), the membranes were incubated with the appropriate secondary antibody (1:5,000, Jackson, USA) for 2 hours at room temperature. Immunoblots were detected by the Tanon 2500 gel imaging system (Yph-Bio Co. Ltd., Beijing, China) with an enhanced chemiluminescence (ECL) detection kit (Absin, Shanghai, China). The results were quantified and analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data are expressed as the mean ± SEM and were analyzed with SPSS 23.0 software (IBM Corp., Armonk, NY, USA). All statistical comparisons were done with 1-way or 2-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test. A value of P<0.05 was considered statistically significant.

Results

SMIR induced persistent mechanical allodynia

According to the literature (33), the SMIR model was established, and the right hind paw of the rats was stimulated with Von Frey filaments. Changes of nociceptive responses were determined by the MWT was on 1 day before operation (baseline) and on 1, 3, 7, 14, and 28 days after surgery. There were no differences of MWTs at baseline among 3 groups. Only in the SMIR group, the right hind limb of rats displayed a significant decrease of MWT on day 1 (P<0.05). It persisted until 14 days after SMIR. Moreover, the MWT recovered to the baseline on day 28. This showed that SMIR induced a profound and gradual decrease of MWT. No significant differences were observed in the naïve and sham group (*Figure 1*).

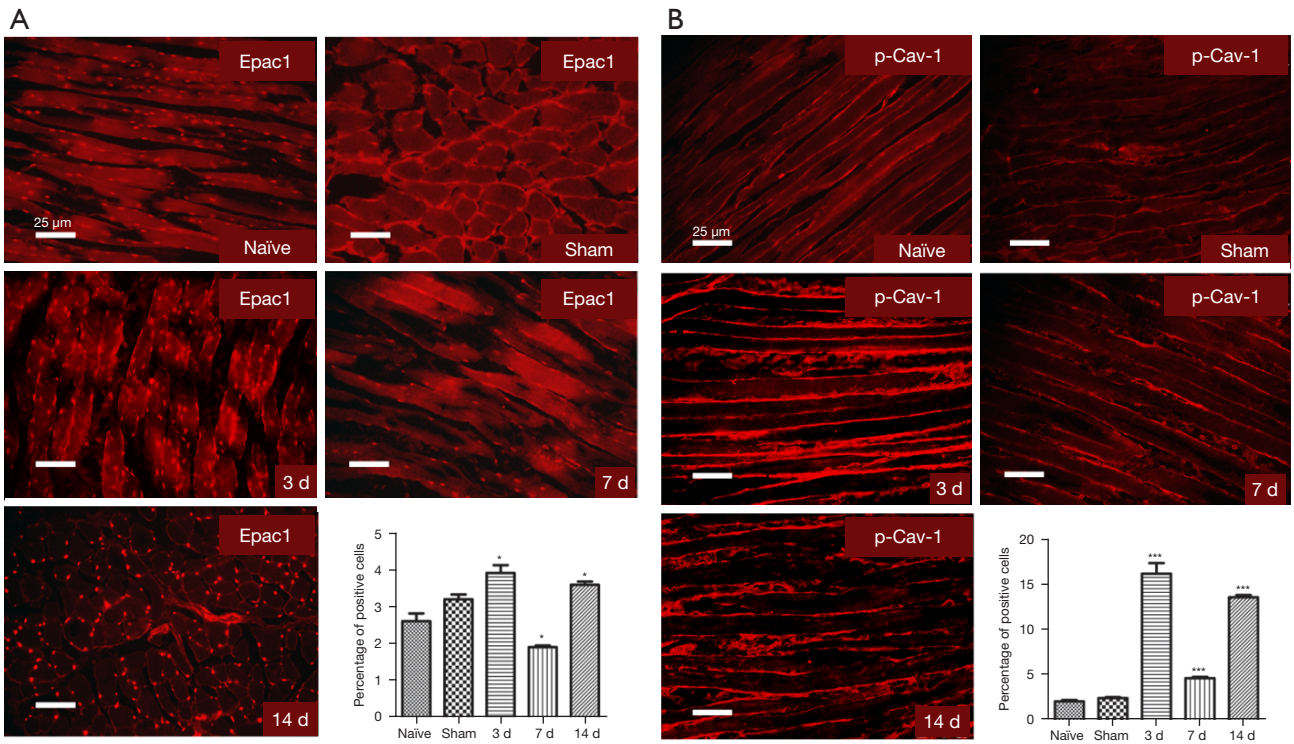

Figure 2 Expression of Epac1 and p-Cav-1 in muscle tissue surrounding the incision (n=6 in each group). (A) Immunostaining of red reaction product for Epac1 on 1, 3, 7, and 14 days after operation was shown in the left panel. (B) That of p-Cav-1was shown in the right panel. SMIR induced a long-lasting modification of Epac1 and p-Cav-1 expression in the muscle tissue surrounding the incision. No corresponding changes were observed in the sham group. *, P<0.05 *vs.* naive group, ***, P<0.001 *vs.* naive group. SMIR, skin/muscle incision and retraction.

These results indicated that SMIR could induce persistent mechanical allodynia.

SMIR increased the accumulation of Epac1 and p-Cav-1 in muscle tissues surrounding the incision

To elucidate the mechanism underlying chronic postsurgical pain, immunofluorescence was used to analyze the expressions of Epac1 and p-Cav-1 in peripheral muscle tissues. *Figure 2* illustrates the representative micrographs of immunostaining for Epac1 and p-Cav-1 at different time. Compared with the naive group, the rats had a long-lasting modification of Epac1 and p-Cav-1 expression in the SMIR group. The accumulation of Epac1 and p-Cav-1was first evident on day 3, and it reached a peak on that same day (P<0.05). However, the expression of Epac1 and p-Cav-1 decreased and reached the lowest point on day 7 (P<0.05). On day 14, SMIR rats presented significantly higher levels of Epac1 and p-Cav-1 (P<0.05). In contrast, no differences were observed in the sham group (*Figure 2*). These results suggested that the ongoing pain-dependent changes of Epac1 and p-Cav-1 expressions in local muscle tissues might be components in the development of chronic postsurgical pain.

SMIR induced a long-term disruption of vascular endothelial barrier in the muscle tissue around the incision

In order to investigate the possible implication of SMIR in the increased vascular endothelial permeability, we firstly explored morphological changes of the muscle tissue around the incision by HE staining. *Figure 3A* illustrates the representative graphs of HE staining on day 7. As can be seen in *Figure 3A*, the SMIR group showed the incomplete small vessel wall. Compared with the naive group, SMIR group presented a lower density of red blood cells in vessels. However, the distribution of red blood cells was increased in the interstitial space (*Figure 3A*). Then,

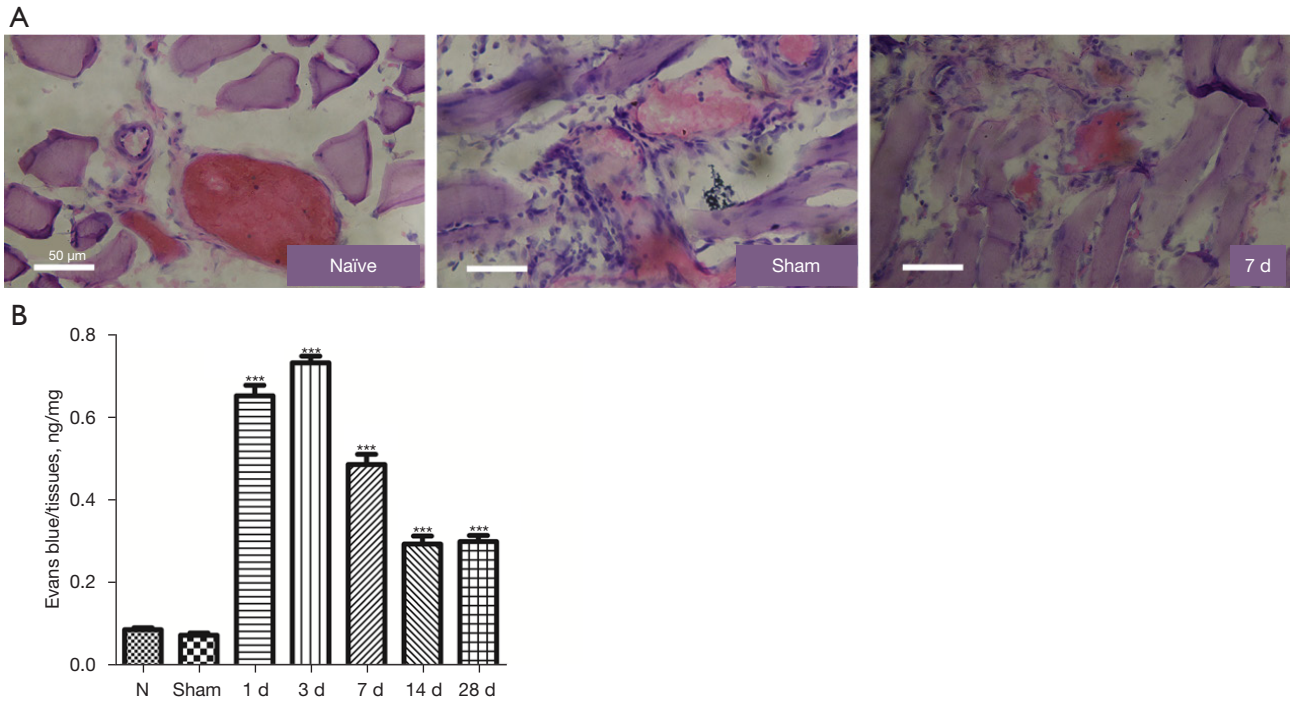

Figure 3 SMIR induced a long-term dysfunction of vascular endothelial barrier in the muscle tissue around the incision (n=5 in each group). (A) This panel showed HE staining of local muscle tissues around the incision. The tissues were taken from the naive, sham, and SMIR group. (B) The intravascular Evan extravasation of different group was measured on days 1, 3, 7, 14, and 28. ***, P<0.001 *vs.* naive group. SMIR, skin/muscle incision and retraction.

we injected Evans blue into the femoral vein to test local vascular permeability, and measured the Evans blue leakage on days 1, 3, 7, 14, and 28 after SMIR. The results showed that increases of the intravascular Evans blue extravasation were first evident on day 1 after SMIR and peaked on day 7 (P<0.05). More, the increases could continue to day 28 after SMIR (*Figure 3B*). In contrast, no changes were observed in the sham group. These results suggested that SMIR induced vascular endothelial barrier dysfunction and a long-term hyperpermeability.

SMIR increased the number of macrophage and endothelial cells in the muscle tissues around the incision

Since we had found the morphological changes of the muscle tissue around the incision via HE staining, we hypothesized that SMIR could also change the number of macrophage and endothelial cells. Then, we chose CD34 as the specific maker of endothelial cells and CD68 as the specific maker of macrophages. Immunofluorescence staining confirmed our prediction and showed a significantly higher number of macrophages and endothelial cells in SMIR group. This change could continue to day 14 (*Figure 4*). There was also a significant increase of both macrophages and endothelial cells in sham group on day 1 after operation (P<0.05). These results indicated that SMIR might promote the transformation of monocytes into macrophages and the continuous proliferation of endothelial cells at the injury site.

SMIR-induced upregulation of Epac1 was macrophage-derived, while that of p-Cav-1 was both macrophage and endothelial cell-derived

In our study, we had found that SMIR induced long-term modifications of peripheral Epac1 and p-Cav-1 expression. Then, we wanted to explore the cell types expressing Epac1 and p-Cav-1 in muscle tissue around the incision. We performed double immunofluorescence experiments using cell type-specific antibodies against CD34 (an endothelial cell marker), CD68 (a macrophage marker), and specific antibodies to Epac1 and p-Cav-1. The present results

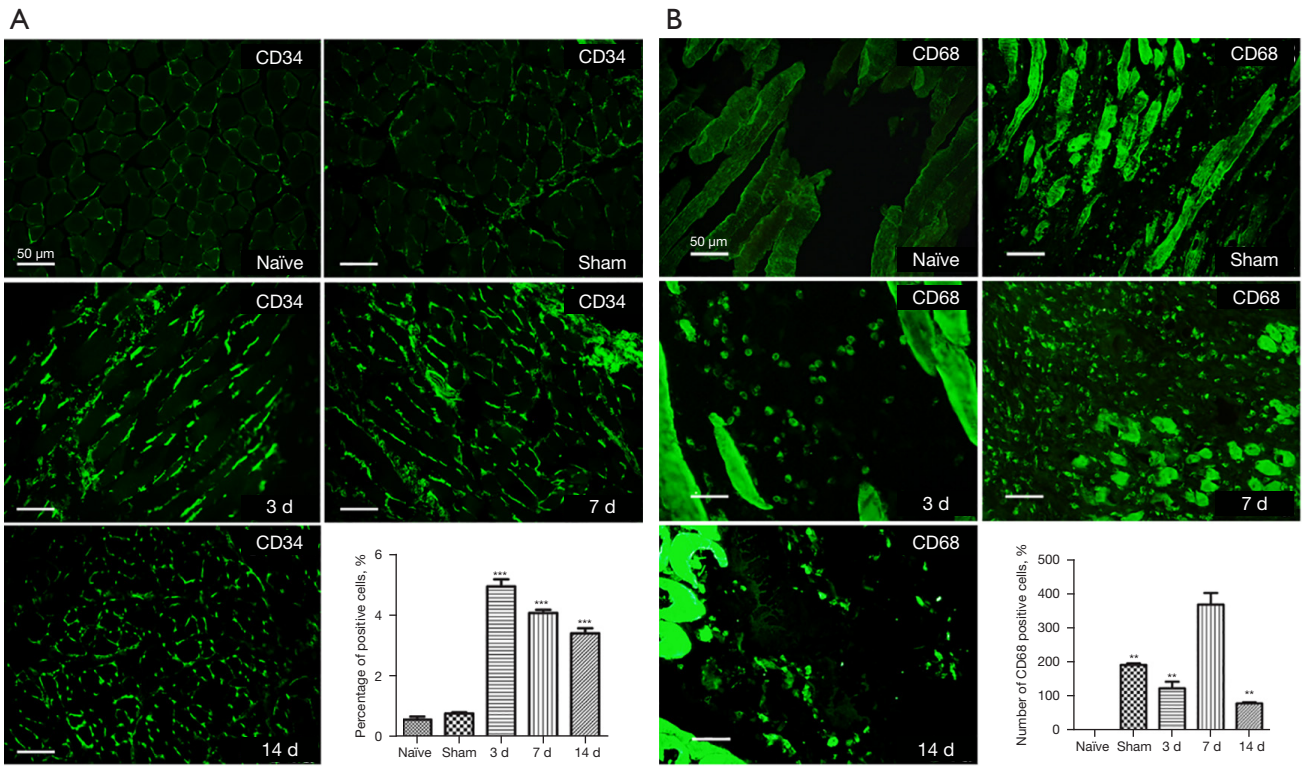

Figure 4 Expression and distribution of macrophages and endothelial cells in muscle tissue around the incision (n=5 in each group). (A) Immunostaining of green reaction products for endothelial cell marker CD34 and statistical analyses were shown in the left panel. (B) Immunostaining of green reaction products for macrophage marker CD68 and statistical analyses were shown in the right panel. The tissues taken from naive group and sham group were on day 1 after surgery. While those from SMIR group were on days 3, 7, and 14 after operation. **, P<0.01 *vs.* Naive group, ***, P<0.001 *vs.* Naive group. SMIR, skin/muscle incision and retraction.

showed that the increased expression of Epac1 by SMIR was mainly localized in macrophage and was not found in endothelial cells (*Figure 5A*). The increased expression of p-Cav-1 by SMIR was localized in both macrophage and endothelial cells (*Figure 5B*). It indicated that Epac1 and p-Cav-1 were involved in the development of CPSP in different cell types.

Effects of the Epac1 agonist 8-pCPT on pain behaviors, the expression of p-Cav-1, and vascular endothelial barrier in rats.

Does the increase of peripheral Epac1 contribute to pain behavior modulation? To answer this question, we injected the Epac1 agonist 8-pCPT (1 μg) into the right hind paw of normal rats. As controls, rats were injected with normal saline. Injection of 8-pCPT into the right hind paw decreased MWT in a time-dependent manner. This effect was exclusively observed at 1, 3, 6, and 12 hours after injection (P<0.05). However, the MWT returned to the baseline at 24 hours after injection of 8-pCPT (*Figure 6A*). To elucidate the regulatory mechanism of Epac1on CPSP, we explored the expression of p-Cav-1 and the changes of vascular endothelial barrier at 3 and 6 h after the injection of 8-pCPT. We found that injection of 8-pCPT significantly increased the expression of p-Cav-1 and increased the number of macrophage and endothelial cells in local muscle tissues (*Figure 6B-6D*). Additionally, the intravascular Evans blue extravasation was significantly increased after injection of 8-pCPT (*Figure 6E*).

Effects of the Epac1 inhibitor CE3F4 on pain behaviors, the expression of p-Cav-1, and vascular endothelial barrier in SMIR rats

To confirm the role of Epac1 on SMIR, we injected the

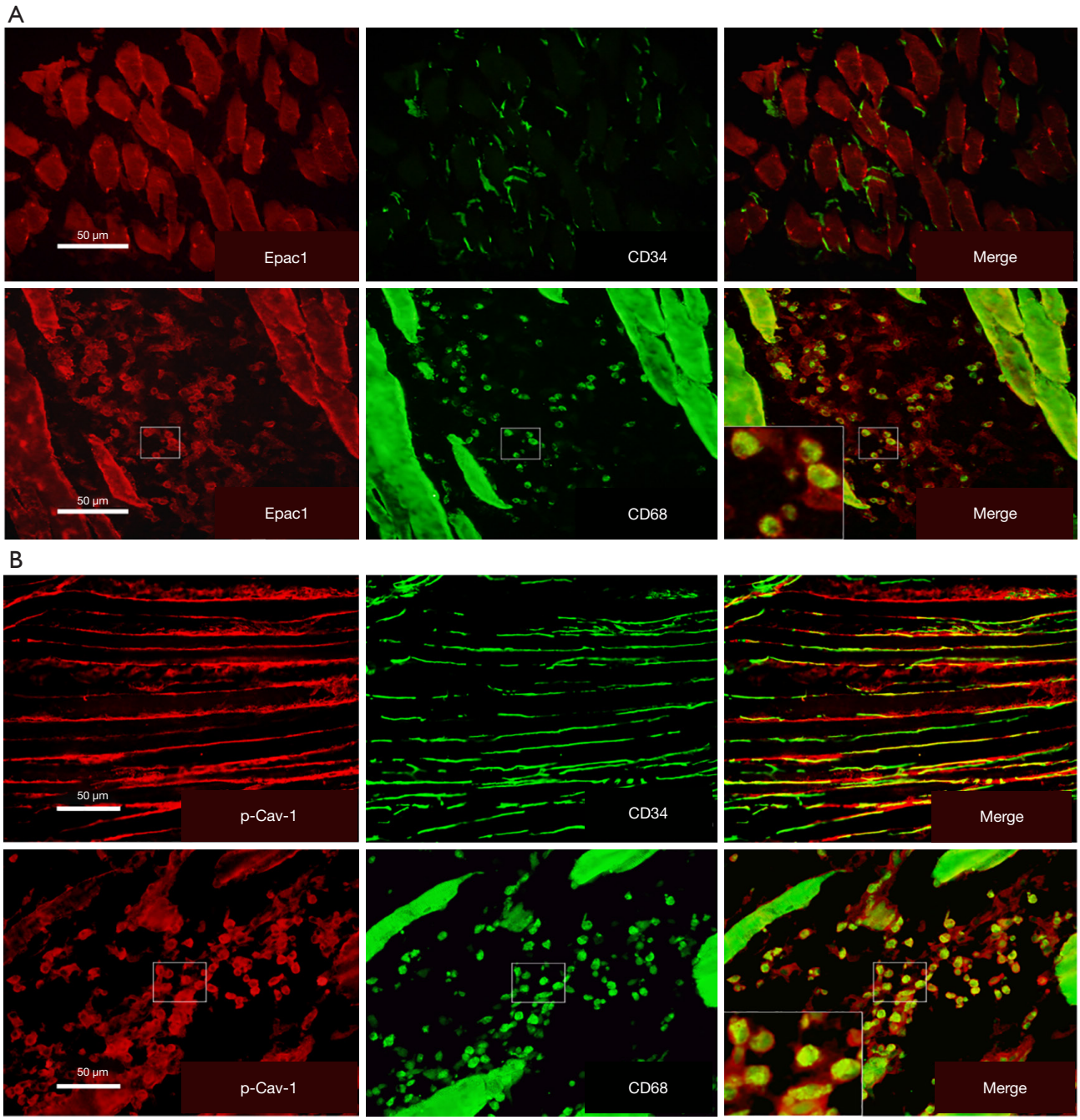

Figure 5 Immunofluorescence staining presented the locations of Epac1 and p-Cav-1 (n=5 in each group). (A) Double immunofluorescence showed that Epac1 was colocalized with macrophage marker CD68, but not colocalized with the endothelial cell marker CD34. (B) Double immunofluorescence showed that p-Cav-1 was colocalized with macrophage marker CD68 and endothelial cell marker CD34.

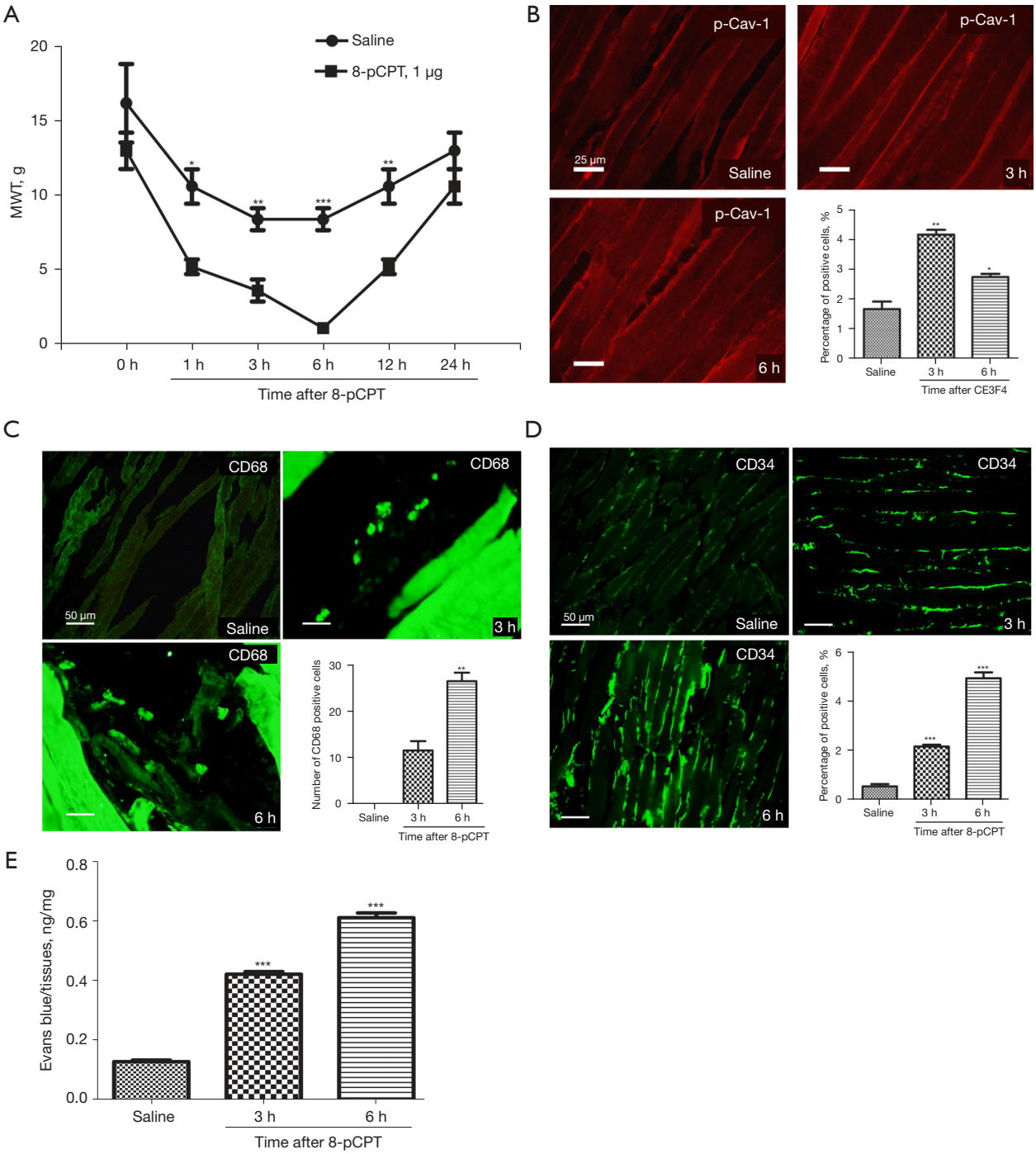

Figure 6 Effects of peripheric injection of 8-pCPT on pain behaviors, the expression of p-Cav-1, the number of macrophage and endothelial cells in local muscle tissues, and vascular endothelial permeability in rats (n=5 in each group). (A) The MWT in response to von Frey filaments was analyzed at 1, 3, 6, 12 and 24 hours after administration of 8-pCPT or normal saline. (B) The expression of p-Cav-1 was analyzed at 3 and 6 hours after administration. (C) Immunostaining of green reaction products for macrophage marker CD68 and statistical analyses were shown at 3 and 6 hours after administration. (D) Immunostaining of green reaction products for endothelial cell marker CD34 and statistical analyses were shown at 3 and 6 hours after administration. (E) The intravascular Evan extravasation was measured at 3 and 6 h after administration of 8-pCPT. *, P<0.05 *vs.* Saline group, **, P<0.01 *vs.* Saline group, ***, P<0.001 *vs.* Saline group. MWT, mechanical withdrawal threshold.

Epac1 inhibitor CE3F4 into the right hind paw of SMIR rats on day 7 after operation. Control rats were injected with normal saline on day 7. We found that injection of CE3F4 could alleviate CPSP in a time-dependent manner. This was manifested by a significant increase of MWT 1, 3, 6, 12, and 24 hours after administration of the CE3F4 (*Figure 7A*). Then we analyzed the expression of p-Cav-1 and the function of vascular endothelial barrier 3 and 6 hours after the administration of CE3F4 (*Figure 7B-7E*). CE3F4-treated group displayed a down-regulation of p-Cav-1 and the number of macrophage and endothelial cells in local muscle tissues induced by SMIR ($P<0.05$). Furthermore, the intravascular Evans blue extravasation was significantly decreased after injection of CE3F4 ($P<0.05$). These results suggested that Epac1 could make the disruption of vascular endothelial barrier and lead to the development and maintenance of CPSP.

Inhibition of p-Cav-1 relieved SMIR-induced mechanical allodynia, but have no effects of the expression of Epac1

To further clarify the implication of Epac1 in the increased vascular permeability following SMIR, we explored whether Epac1 signaling could modulate the disfunction of vascular endothelial barrier through Cav-1 that led to CPSP. Rats received intrathecal injection of Cav-1 siRNA on days 1, 3, and 5 after SMIR. As controls, the rats were injected with negative small interfering RNA (siRNA). Treatment with Cav-1 siRNA strongly relieve SMIR-induced mechanical allodynia ($P<0.05$). The beneficial effect continued to day 14 even after the last dose of Cav-1 siRNA on day 7 ($P<0.05$). The mechanical sensitivity was not changed in rats treated with negative siRNA (*Figure 8A*). Additionally, Western blot confirmed that intrathecal injection of Cav-1 siRNA had no effects on the expression of Epac1 (*Figure 8B*). Conversely, the expression of p-Cav-1 was significantly inhibited after Cav-1 siRNA adminication on day 7 ($P<0.05$). No differences were observed in the negative siRNA-treated group (*Figure 8C*). It suggested that inhibition of Cav-1 could not prevent the upregulation of Epac1 resulting from SMIR.

Discussion

In chronic inflammatory pain model, Epac1 in the DRG neurons is upregulated. It plays an important role in sensitization after activating prostaglandin E2 (PGE2) (34). In our experiment, a rat CPSP model was successfully established and utilized. We found peripheral Epac1 was highly expressed on days 3 and 14 after SMIR in the muscle tissue around the incision. Subcutaneous injection of the Epac1 agonist 8-pCPT could induce mechanical sensitivity, whereas the Epac1 inhibitor CE3F4 attenuated established SMIR-induced mechanical hyperalgesia. Interestingly, there was a reversal in the high expression of Epac1 on the 7 d after SMIR. It was reported that a selective Epac1 inhibitor inhibited the persistent mechanical hypersensitivity induced by PGE2 but had no effects on trauma-induced acute hypersensitivity (35). In consideration of these above findings, we proposed that a reactivation of Epac1 after acute injury played an important role in chronic pain. Therefore, understanding the role of reactivate Epac1 has vital significance for the mechanism of the development of acute postoperative pain into CPSP.

Historically, Epac1 is highly expressed during endothelial barrier dysfunction, ischemia/reperfusion (I/R) injury and hemodynamic overload (36-38). Moreover, high expression of Epac1 after vascular mechanical injury promotes vascular reconstruction and vasodilation (39). Therefore, we investigated the function of endothelial barrier in CPSP. We found that SMIR induced a long-term disruption of vascular endothelial barrier and increased vascular permeability in the muscle tissue around the incision. Subcutaneous injection of the Epac1 agonist 8-pCPT could disrupt the vascular endothelial barrier in normal rats. Conversely, subcutaneous injection of the Epac1 inhibitor CE3F4 attenuated the disruption of vascular endothelial barrier induced by SMIR. Continuous high vascular endothelial permeability promotes inflammatory reactions and pain in local tissues (40-42). In addition, the high vascular endothelial permeability can increase the interstitial tension in local tissues and further aggravate ischemia and hypoxia in local tissues (43). Circulating hypovolemia induces changes in hemodynamic and vascular tension. All of these factors may contribute to the transformation acute postoperative pain into CPSP.

Cav-1 plays a fundamental role in the mechanisms of I/R injury, vascular tension, endothelial barrier function, and inflammatory reactions (44-47). In this CPSP model, p-Cav-1 was continuously and highly expressed on days 3, 7, and 14 after SMIR. This upregulation was reversed by subcutaneous injection of the Epac1 inhibitor CE3F4. However, intrathecal injection of Cav-1siRNA relieved SMIR-induced mechanical allodynia, but had no effects of the expression of Epac1. These findings suggested that p-Cav-1 was the downstream effector of Epac1 in CPSP.

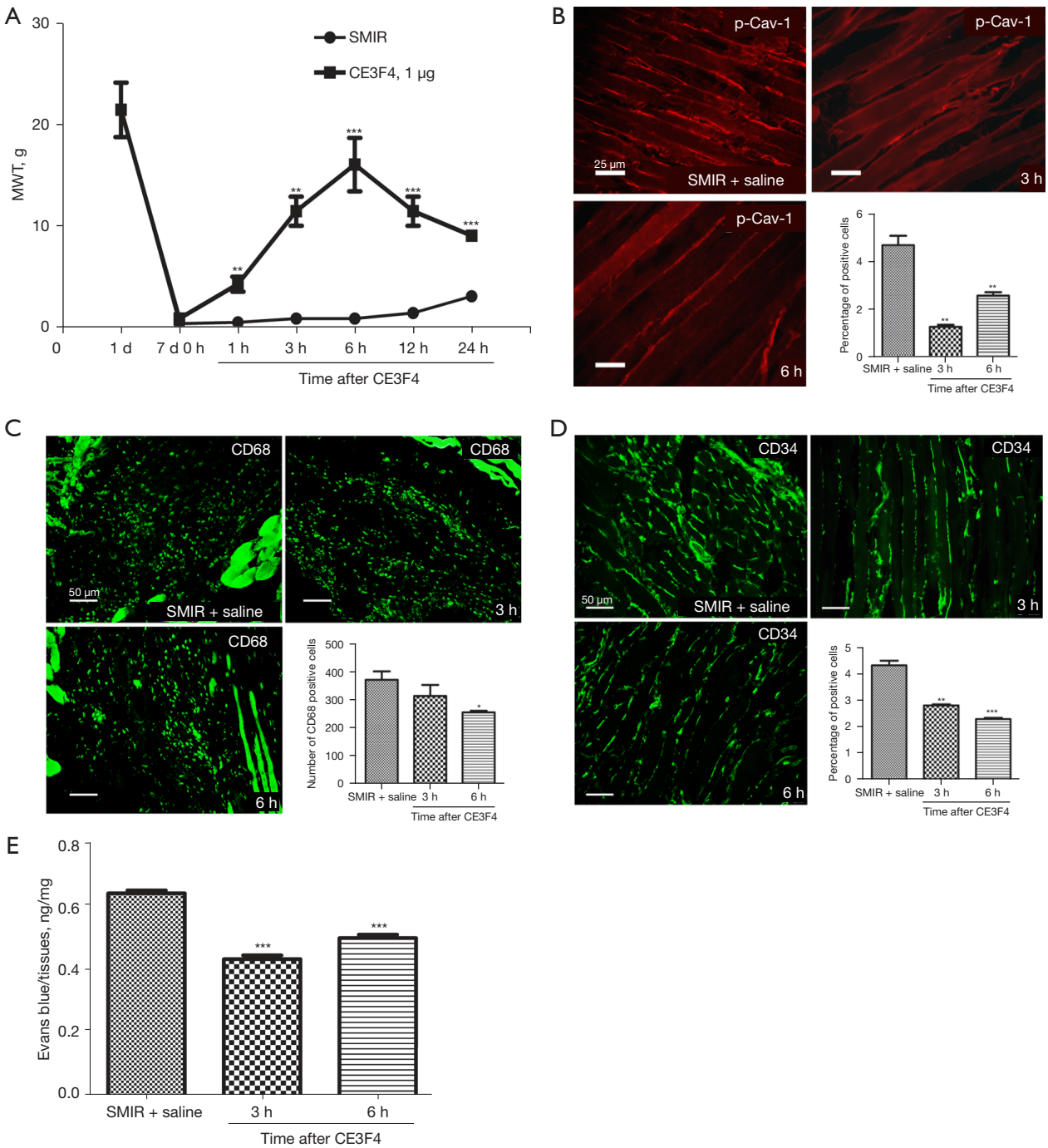

Figure 7 Effects of peripheric injection of Epac1 inhibitor CE3F4 on pain behaviors, the expression of p-Cav-1, the number of macrophage and endothelial cells in local muscle tissues, and vascular endothelial permeability in rats (n=5 in each group). (A) The MWT in response to von Frey filaments was analyzed at 1, 3, 6 hours after administration of CE3F4 or normal saline. (B) The expression of p-Cav-1 was analyzed at 3 and 6 hours after administration. (C) Immunostaining of green reaction products for macrophage marker CD68 and statistical analyses were shown at 3 and 6 hours after administration. (D) Immunostaining of green reaction products for endothelial cell marker CD34 and statistical analyses were shown at 3 and 6 hours after administration. (E) The intravascular Evan extravasation was measured at 3 and 6 h after administration of CE3F4. *, P<0.05 *vs.* Saline group, **, P<0.01 *vs.* Saline group, ***, P<0.001 *vs.* Saline group. MWT, mechanical withdrawal threshold.

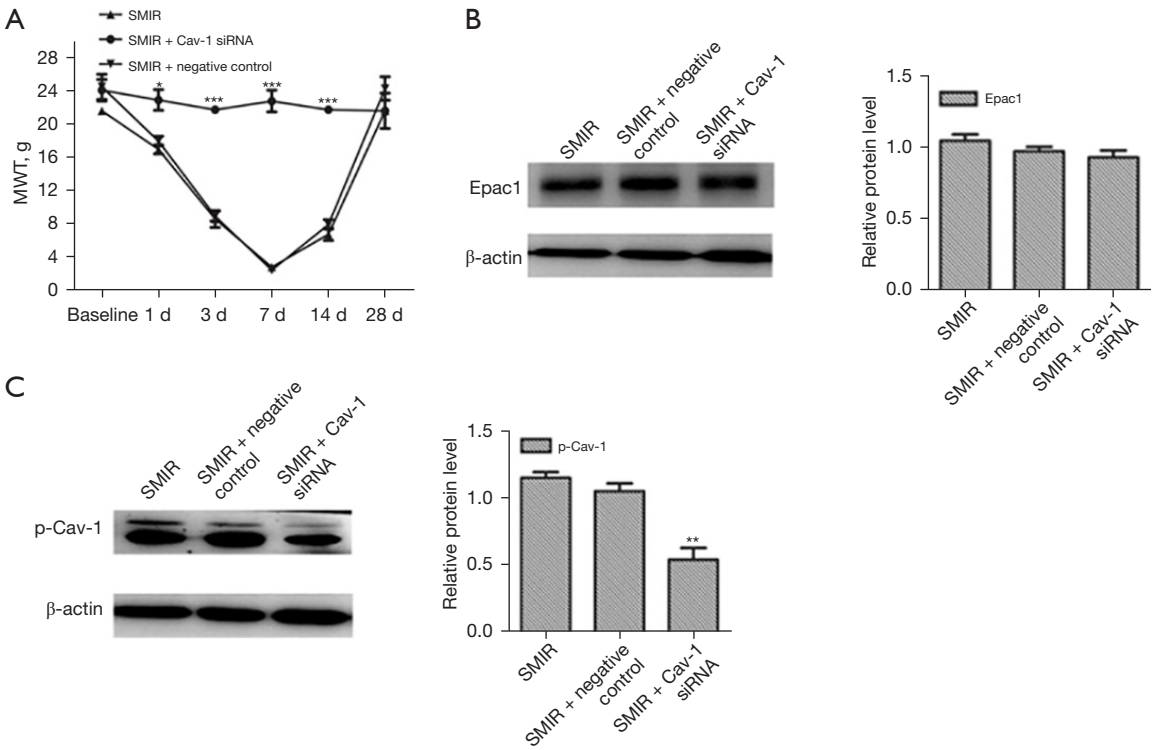

Figure 8 Effects of p-Cav-1 inhibition on pain behaviors and the expression of Epac1 (n=5 in each group). Cav-1 siRNA or negative siRNA was given by intrathecal injection on 1 d, 3 d and 5 d after SMIR. (A) The MWT in response to von Frey filaments was observed 1 d on the day before operation (baseline) and on days 1, 3, 7, 14, and 28 after operation. (B) Western blot and statistical analysis of Epac1 were shown on day 7 after different treatments. (C) Western blot and statistical analysis of p-Cav-1 were shown on day 7 after different treatments. *, P<0.05 *vs.* SMIR + negative control group, **, P<0.01 *vs.* SMIR + negative control group, ***, P<0.001 *vs.* SMIR + negative control group. siRNA, small interfering RNA; SMIR, skin/muscle incision and retraction; MWT, mechanical withdrawal threshold.

Additionally, the low expression of p-Cav-1 was similar to that of Epac1 on 7 d after SMIR. Previously, we found a secondary inflammatory injury caused by I/R at the injury site (48). It seemed that the local microenvironment protected the body after acute injury. Under the sustained damage, the local microenvironment was destroyed eventually. This might be associated with the reactivation of Epac1 and p-Cav-1 on 14 d after SMIR.

Macrophages and endothelial cells have a significant impact on the regulation of immunity and inflammation (49). The secretory activity of macrophages regulates angiogenesis and supports vascular anastomosis (50,51). The differentiation of monocytes into macrophages is helpful for the formation of the neointima (52). Under a high concentration of tumor necrosis factor α (TNF-α) stimulation, endothelial cells produce inflammatory reactions (53). The inflammatory reaction is characterized by the promotion of leukocyte binding and migration through adhesion molecules (54), the proliferation and migration of endothelial cells stimulate the body to secrete a variety of cytokines, and also accelerate wound healing (55,56). We observed that peripheral upregulation of Epac1 was macrophage-derived, whereas that of p-Cav-1 was both macrophage and endothelial cell-derived in the SMIR model. The proliferation of endothelial cells and macrophages was significantly and continuously increased after SMIR. Subcutaneous injection of the Epac1 agonist 8-pCPT increased the number of macrophage and endothelial cells in local muscle tissues. Conversely, subcutaneous injection of the Epac1 inhibitor CE3F4 inhibited the proliferation of macrophage and endothelial cells. It has been reported that Epac1 is involved in the megakaryocyte phenotype (36). Cav-1 promotes macrophage recruitment to human umbilical vein

endothelial cells (57), which is vital for the permeability of the Transwell transport pathway (58,59).

Conclusions

In conclusion, our study demonstrated an important role of peripheral Epac1/p-Cav-1 pathway in the development of CPSP. And the inhibition of peripheral Epac1/p-Cav-1 pathway might elicit the analgesic effects via the recovery of vascular endothelial barrier and the downregulation of macrophage and endothelial cells.

Acknowledgments

Funding: This research was supported by National Natural Science Foundation of China (No. 81701106).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6069/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6069/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6069/coif). All authors report that this research was supported by National Natural Science Foundation of China (No. 81701106). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The protocols were approved by the Ethics Committee of Affiliated Hospital of Nantong University (No. 20170305-001), in compliance with national guidelines for the care and use of animals.

References

1. Lopes A, Seligman Menezes M, Antonio Moreira de Barros G. Chronic postoperative pain: ubiquitous and scarcely appraised: narrative review. Braz J Anesthesiol 2021;71:649-55.
2. van Helden EV, van Uitert A, Albers KI, et al. Chronic postsurgical pain after minimally invasive adrenalectomy: prevalence and impact on quality of life. BMC Anesthesiol 2022;22:153.
3. Toscano A, Barbero C, Capuano P, et al. Chronic postsurgical pain and quality of life after right minithoracotomy mitral valve operations. J Card Surg 2022;37:1585-90.
4. Benlolo S, Hanlon JG, Shirreff L, et al. Predictors of Persistent Postsurgical Pain After Hysterectomy-A Prospective Cohort Study. J Minim Invasive Gynecol 2021;28:2036-46.e1.
5. López-Muñoz E, Mejía-Terrazas GE. Epigenetics and Postsurgical Pain: A Scoping Review. Pain Med 2022;23:246-62.
6. Zeilhofer HU, Benke D, Yevenes GE. Chronic pain states: pharmacological strategies to restore diminished inhibitory spinal pain control. Annu Rev Pharmacol Toxicol 2012;52:111-33.
7. Chapman CR, Vierck CJ. The Transition of Acute Postoperative Pain to Chronic Pain: An Integrative Overview of Research on Mechanisms. J Pain 2017;18:359.e1-359.e38.
8. Costigan M, Scholz J, Woolf CJ. Neuropathic pain: a maladaptive response of the nervous system to damage. Annu Rev Neurosci 2009;32:1-32.
9. Montague-Cardoso K, Pitcher T, Chisolm K, et al. Changes in vascular permeability in the spinal cord contribute to chemotherapy-induced neuropathic pain. Brain Behav Immun 2020;83:248-59.
10. Reinhold AK, Salvador E, Förster CY, et al. Microvascular Barrier Protection by microRNA-183 via FoxO1 Repression: A Pathway Disturbed in Neuropathy and Complex Regional Pain Syndrome. J Pain 2022;23:967-80.
11. Fujino K, Yokota A, Ohno K, et al. Impairment and restoration of the blood-nerve barrier and its correlation with pain following gradual nerve elongation of the rat sciatic nerve. Int J Neurosci 2021;131:254-63.
12. Ubogu EE. Biology of the human blood-nerve barrier in

health and disease. Exp Neurol 2020;328:113272.
13. Pelz J, Härtig W, Weise C, et al. Endothelial barrier antigen-immunoreactivity is conversely associated with blood-brain barrier dysfunction after embolic stroke in rats. Eur J Histochem 2013;57:e38.
14. Martínez-Lavín M. Dorsal root ganglia: fibromyalgia pain factory? Clin Rheumatol 2021;40:783-7.
15. Tuckey B, Srbely J, Rigney G, et al. Impaired Lymphatic Drainage and Interstitial Inflammatory Stasis in Chronic Musculoskeletal and Idiopathic Pain Syndromes: Exploring a Novel Mechanism. Front Pain Res (Lausanne) 2021;2:691740.
16. Santa-Cecília FV, Ferreira DW, Guimaraes RM, et al. The NOD2 signaling in peripheral macrophages contributes to neuropathic pain development. Pain 2019;160:102-16.
17. Liu JA, Yu J, Cheung CW. Immune Actions on the Peripheral Nervous System in Pain. Int J Mol Sci 2021;22:1448.
18. Domoto R, Sekiguchi F, Tsubota M, et al. Macrophage as a Peripheral Pain Regulator. Cells 2021;10:1881.
19. Merriwether EN, Agalave NM, Dailey DL, et al. IL-5 mediates monocyte phenotype and pain outcomes in fibromyalgia. Pain 2021;162:1468-82.
20. Lee K. Epac: new emerging cAMP-binding protein. BMB Rep 2021;54:149-56.
21. Gündüz D, Troidl C, Tanislav C, et al. Role of PI3K/Akt and MEK/ERK Signalling in cAMP/Epac-Mediated Endothelial Barrier Stabilisation. Front Physiol 2019;10:1387.
22. Wehbe N, Slika H, Mesmar J, et al. The Role of Epac in Cancer Progression. Int J Mol Sci 2020;21:6489.
23. Qian J, Lin X, Zhou Z. Skin/muscle incision and retraction regulates the persistent postoperative pain in rats by the Epac1/PKC-βII pathway. BMC Anesthesiol 2022;22:230.
24. Liu L, Jiang Y, Steinle JJ. Prohibitin 1 Regulates Inflammatory Mediators and Reactive Oxygen Species in Retinal Endothelial Cells. J Clin Med 2022;11:1915.
25. Pan P, Huang SS, Shen SR, et al. Role of p120 Catenin in Epac1-Induced Chronic Postsurgical Pain in Rats. Pain Res Manag 2019;2019:9017931.
26. Liu H, Mei FC, Yang W, et al. Epac1 inhibition ameliorates pathological angiogenesis through coordinated activation of Notch and suppression of VEGF signaling. Sci Adv 2020;6:eaay3566.
27. Luchetti F, Crinelli R, Nasoni MG, et al. LDL receptors, caveolae and cholesterol in endothelial dysfunction: oxLDLs accomplices or victims? Br J Pharmacol 2021;178:3104-14.
28. Parton RG, McMahon KA, Wu Y. Caveolae: Formation, dynamics, and function. Curr Opin Cell Biol 2020;65:8-16.
29. Tiruppathi C, Regmi SC, Wang DM, et al. EphB1 interaction with caveolin-1 in endothelial cells modulates caveolae biogenesis. Mol Biol Cell 2020;31:1167-82.
30. Jia GL, Huang Q, Cao YN, et al. Cav-1 participates in the development of diabetic neuropathy pain through the TLR4 signaling pathway. J Cell Physiol 2020;235:2060-70.
31. Chen JL, Lu JH, Xie CS, et al. Caveolin-1 in spinal cord modulates type-2 diabetic neuropathic pain through the Rac1/NOX2/NR2B signaling pathway. Am J Transl Res 2020;12:1714-27.
32. Dixon WJ. Staircase bioassay: the up-and-down method. Neurosci Biobehav Rev 1991;15:47-50.
33. Flatters SJ. Characterization of a model of persistent postoperative pain evoked by skin/muscle incision and retraction (SMIR). Pain 2008;135:119-30.
34. Wang H, Heijnen CJ, van Velthoven CT, et al. Balancing GRK2 and EPAC1 levels prevents and relieves chronic pain. J Clin Invest 2013;123:5023-34.
35. Matsuda M, Oh-Hashi K, Yokota I, et al. Acquired Exchange Protein Directly Activated by Cyclic Adenosine Monophosphate Activity Induced by p38 Mitogen-activated Protein Kinase in Primary Afferent Neurons Contributes to Sustaining Postincisional Nociception. Anesthesiology 2017;126:150-62.
36. Nygaard G, Herfindal L, Asrud KS, et al. Epac1-deficient mice have bleeding phenotype and thrombocytes with decreased GPIbβ expression. Sci Rep 2017;7:8725.
37. Korayem AH, Mujica PE, Aramoto H, et al. Endothelial cAMP deactivates ischemia-reperfusion-induced microvascular hyperpermeability via Rap1-mediated mechanisms. Am J Physiol Heart Circ Physiol 2017;313:H179-89.
38. Laudette M, Coluccia A, Sainte-Marie Y, et al. Identification of a pharmacological inhibitor of Epac1 that protects the heart against acute and chronic models of cardiac stress. Cardiovasc Res 2019;115:1766-77.
39. Kato Y, Yokoyama U, Yanai C, et al. Epac1 Deficiency Attenuated Vascular Smooth Muscle Cell Migration and Neointimal Formation. Arterioscler Thromb Vasc Biol 2015;35:2617-25.
40. Aouey B, Samet AM, Fetoui H, et al. Anti-oxidant, anti-inflammatory, analgesic and antipyretic activities of grapevine leaf extract (Vitis vinifera) in mice and identification of its active constituents by LC-MS/MS analyses. Biomed Pharmacother 2016;84:1088-98.
41. Xing W, Carvalho PA, Polfus JM, et al. Thermochemically

stable ceramic composite membranes based on Bi2O3 for oxygen separation with high permeability. Chem Commun (Camb) 2019;55:3493-6.
42. Gong LL, Yang S, Liu H, et al. Anti-nociceptive and anti-inflammatory potentials of Akebia saponin D. Eur J Pharmacol 2019;845:85-90.
43. Bátori R, Kumar S, Bordán Z, et al. Differential mechanisms of adenosine- and ATP S-induced microvascular endothelial barrier strengthening. J Cell Physiol 2019;234:5863-79.
44. Shang D, Peng T, Gou S, et al. High Mobility Group Box Protein 1 Boosts Endothelial Albumin Transcytosis through the RAGE/Src/Caveolin-1 Pathway. Sci Rep 2016;6:32180.
45. Huang Q, Zhong W, Hu Z, et al. A review of the role of cav-1 in neuropathology and neural recovery after ischemic stroke. J Neuroinflammation 2018;15:348.
46. Mu YP, Lin DC, Yan FR, et al. Alterations in Caveolin-1 Expression and Receptor-Operated Ca2+ Entry in the Aortas of Rats with Pulmonary Hypertension. Cell Physiol Biochem 2016;39:438-52.
47. Gao L, Chen X, Peng T, et al. Caveolin-1 protects against hepatic ischemia/reperfusion injury through ameliorating peroxynitrite-mediated cell death. Free Radic Biol Med 2016;95:209-15.
48. Cao S, Qin Y, Chen J, et al. Effects of pinacidil on changes to the microenvironment around the incision site, of a skin/muscle incision and retraction, in a rat model of postoperative pain. Mol Med Rep 2015;12:829-36.
49. Castro-Dopico T, Dennison TW, Ferdinand JR, et al. Anti-commensal IgG Drives Intestinal Inflammation and Type 17 Immunity in Ulcerative Colitis. Immunity 2019;50:1099-1114.e10.
50. de Araújo Pimenta L, de Almeida MES, Bretones ML, et al. Crotoxin promotes macrophage reprogramming towards an antiangiogenic phenotype. Sci Rep 2019;9:4281.
51. Munro DAD, Wineberg Y, Tarnick J, et al. Macrophages restrict the nephrogenic field and promote endothelial connections during kidney development. Elife 2019;8:43271.
52. Liang M, Guo Q, Huang F, et al. Notch signaling in bone marrow-derived FSP-1 cells initiates neointima formation in arteriovenous fistulas. Kidney Int 2019;95:1347-58.
53. King D, Glynn M, Cindric S, et al. Label-Free Multi Parameter Optical Interrogation of Endothelial Activation in Single Cells using a Lab on a Disc Platform. Sci Rep 2019;9:4157.
54. Mussbacher M, Salzmann M, Brostjan C, et al. Cell Type-Specific Roles of NF-κB Linking Inflammation and Thrombosis. Front Immunol 2019;10:85.
55. Herrero D, Cañón S, Albericio G, et al. Age-related oxidative stress confines damage-responsive Bmi1+ cells to perivascular regions in the murine adult heart. Redox Biol 2019;22:101156.
56. Kong D, Zhang D, Cui Q, et al. Sapylin (OK-432) alters inflammation and angiogenesis in vivo and vitro. Biomed Pharmacother 2019;113:108706.
57. Lin F, Pei L, Zhang Q, et al. Ox-LDL induces endothelial cell apoptosis and macrophage migration by regulating caveolin-1 phosphorylation. J Cell Physiol 2018;233:6683-92.
58. Yang ZH, Liu LB, Zhao LN, et al. Permeability of the blood-tumor barrier is enhanced by combining vascular endothelial growth factor with papaverine. J Neurosci Res 2014;92:703-13.
59. Wang N, Zhang D, Sun G, et al. Lipopolysaccharide-induced caveolin-1 phosphorylation-dependent increase in transcellular permeability precedes the increase in paracellular permeability. Drug Des Devel Ther 2015;9:4965-77.

Cite this article as: Chen H, She Q, Liu Y, Chen J, Qin Y, Lu C. The peripheral Epac1/p-Cav-1 pathway underlies the disruption of the vascular endothelial barrier following skin/muscle incision and retraction-induced chronic postsurgical pain. Ann Transl Med 2022;10(24):1377. doi: 10.21037/atm-22-6069

