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An orally active Epac inhibitor reverses mechanical allodynia and loss of intraepidermal nerve fibers in a mouse model of chemotherapy-induced peripheral neuropathy

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Abstract

Chemotherapy-induced peripheral neuropathy (CIPN) is a major side-effect of cancer treatment that significantly compromises quality of life of cancer patients and survivors. Identification of targets for pharmacological intervention to prevent or reverse CIPN is needed. We investigated Exchange protein regulated by cAMP (Epac) as a potential target. Epacs are cAMP-binding proteins known to play a pivotal role in mechanical allodynia induced by nerve injury and inflammation.

We demonstrate that global Epac1-knockout (Epac1^{-/-}) male and female mice are protected against paclitaxel-induced mechanical allodynia. In addition, spinal cord astrocyte activation and intra-epidermal nerve fiber (IENF) loss are significantly reduced in Epac1^{-/-} mice as compared to WT mice. Moreover, Epac1^{-/-} mice do not develop the paclitaxel-induced deficits in mitochondrial bioenergetics in the sciatic nerve that are a hallmark of CIPN.

Notably, mice with cell-specific deletion of Epac1 in Nav1.8-positive neurons (N-Epac1^{-/-}) also show reduced paclitaxel-induced mechanical allodynia, astrocyte activation and IENF loss indicating that CIPN develops downstream of Epac1 activation in nociceptors.

The Epac-inhibitor ESI-09 reversed established paclitaxel-induced mechanical allodynia in WT mice even when dosing started 10 days after completion of paclitaxel treatment. In addition, oral administration of ESI-09 suppressed spinal cord astrocyte activation in the spinal cord and protected against IENF loss. Ex vivo, ESI-09 blocked paclitaxel-induced abnormal spontaneous discharges in dorsal root ganglion neurons.

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Drs. Singhmar and Huo contributed equally to this study.

We have no conflicts of interests related to this study.

Collectively, these findings implicate Epac1 in nociceptors as a novel target for treatment of CIPN. This is clinically relevant as ESI-09 has the potential to reverse a debilitating and long lasting side effect of cancer treatment.

Introduction

Chemotherapy-induced peripheral neuropathy (CIPN) is a major dose-limiting side effect of standard anti-neoplastic treatments [48; 50; 64]. Approximately 25–75% of cancer patients treated with paclitaxel experience CIPN symptoms [5; 10; 11] including tingling, pain, hypersensitivity to pain, and numbness. These symptoms often persist long after completion of treatment and can be irreversible [12; 29; 38]. There is no FDA-approved treatment for CIPN. Identification of preventive or curative interventions and underlying mechanisms contributing to CIPN would be a significant step forward in improving cancer treatment adherence and the quality of life cancer survivors.

Paclitaxel is widely used for the treatment of solid tumors including breast, ovarian, lung, and head and neck cancer [50]. Paclitaxel acts by hyper-stabilizing microtubules to cause mitotic arrest and cell death. Pain sensing neurons (nociceptors) are vulnerable to chemotherapy drugs due to the presence of their ganglia outside the blood-brain barrier and lack of a restrictive vascular barrier in the periphery [64].

Cellular and molecular mechanisms associated with CIPN include mitochondrial dysfunction, oxidative stress, production of cytokines and chemokines in DRG and spinal cord, and altered expression of ion channels in DRG neurons [10; 66; 67; 69]. CIPN is associated with astrocyte activation in the spinal cord and loss of intra-epidermal nerve fibers (IENFs) [20; 37; 70]. Mitochondrial structure and functional abnormalities in the peripheral nerve axons is a key mechanism underlying CIPN [25; 37]. It is hypothesized that abnormal mitochondrial function creates energy deficits leading to an increase in spontaneous discharges by myelinated A-fibers (A β and A δ) and unmyelinated C-fibers, and IENF loss [8; 69].

cAMP signaling has long been recognized as a major pathway in the sensitization of nociceptors [22; 24; 54; 55]. The cAMP effectors protein kinase A (PKA), exchange protein directly activated by cAMP (Epac, also known as RAP-GEF), and cyclic nucleotide-gated ion channels are involved in pain signaling [16; 27; 35; 58]. The cAMP/PKA pathway has been intensely studied for its role in inflammation-induced peripheral sensitization [19; 22; 31; 54; 55]. Research by our group and others has identified an important contribution of a cAMP-EPAC (Exchange proteins activated by cAMP) pathway to mechanical allodynia and thermal hyperalgesia in rodent models of inflammatory pain and in surgical models of neuropathic pain [23; 28; 31; 52; 59; 60]. For example, Epac1 knockout animals (Epac1 $^{-/-}$) are protected against mechanical allodynia in the complete Freund's adjuvant (CFA) model of chronic inflammatory pain and in the spinal nerve transection (SNT) model of neuropathic pain [21; 52; 60]. Inhibitors of Epac have been developed recently and the competitive inhibitor ESI-09 has emerged as an attractive therapeutic option for blocking Epac activity *in vivo* [1; 4; 26; 52].

Here we tested the hypothesis that Epac1 is a target for prevention or reversal of paclitaxel-induced peripheral neuropathy using pharmacological and genetic interventions. We identified ESI-09 as a novel inhibitor of chemotherapy induced pain. Administration of ESI-09 in mice reversed established paclitaxel-induced mechanical allodynia and associated hallmarks of CIPN i.e. astrocyte activation, IENF loss, and spontaneous neuronal discharges.

Methods

Animals

Male and female C57BL/6J mice aged 10–12 weeks and male and female mice homozygous for global [53] deletion of the Epac1 gene (Epac1^{-/-} mice) were used in this study. Nav1.8 Cre mice were used to drive cell specific deletion of Epac1 in nociceptors (N-Epac1^{-/-}). Mice were bred and maintained in the animal facility of the Institute of Biosciences and Technology of Texas A&M University and of UT M.D. Anderson Cancer Center, Houston, Texas. All experiments were performed in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and the Ethical issues of the International Association for the Study of Pain and were approved by the Institutional Animal Care and use Committees. Spared nerve injury (SNI) was performed in male C57BL/6J mice as previously described [17]. In brief, under 1.5% isoflurane anesthesia, the three terminal branches of the sciatic nerve were exposed carefully with minimal damage. Leaving the sural nerve intact, the tibial and the common peroneal nerves were tightly ligated with 6-0 silk surgical sutures and cut 1 to 2 mm distal to the ligation. Sham controls underwent anesthesia, incision, and exposure of the nerve only.

Drug

Paclitaxel (6 mg/ml) in 50% El Kolipher (Sigma Aldrich, St. Louis, MO) and 50% ethanol (Sigma Aldrich, St. Louis, MO) or vehicle alone was diluted in sterile saline to final concentration of 1 mg/ml and administered intraperitoneally i.p. at a dose of 10 mg/kg every other day for two weeks. This dosing schedule of paclitaxel was used in our previous studies to induce CIPN in mice [37; 44]. The Epac inhibitor ESI-09 [3; 68] was formulated with corn oil (Sigma Aldrich, St. Louis, MO), and administered daily by oral gavage at a dose of 20 mg/kg. CE3F4 (Tocris) was dissolved in DMSO and used at a concentration of 2.5 μ M.

Measurement of mechanical allodynia

Mechanical hyperalgesia was measured using von Frey hairs as previously described [44]. Briefly, mice were placed in a plastic cage (10×10×13 cm³) with a mesh floor for 30 min prior to testing. Animals were placed on a wire grid floor, through which the von Frey hairs (North Coast Medical Inc. CA, USA) were applied (bending force range from 0.02 to 1.4g, starting with 0.16g) by an investigator blinded to treatment. The hair force was increased or decreased according to the response. The 50% paw withdrawal threshold was calculated using the up-and-down method [13]. Clear paw withdrawal, shaking, or licking was considered as nociceptive-like responses. Ambulation was considered an ambiguous response, and in such cases, the stimulus was repeated.

Immunohistochemistry

For IENFs, biopsies from the plantar surface of the hind paws were collected and processed as described [9; 44; 51]. Biopsies were immediately saved in Zamboni's fixative for 24 hours, cryo-protected in 20% sucrose, frozen in optimal cutting temperature compound, and sliced into 25 μ m sections. Sections were blocked in PBS containing 5% normal donkey serum/0.1% saponin and stained with antibodies against the pan neuronal marker PGP9.5 (Rabbit, AbD Serotec, Oxford, United Kingdom) and Collagen IV (Goat, Southern Biotech, Birmingham, AL) followed by Alexa-594 donkey anti-rabbit (Life Technologies, Grand Island, NY) and Alexa-488 donkey anti-goat (Invitrogen, Grand Island, NY). For negative control sections, primary antibody was omitted. Sections were visualized using a Leica fluorescence microscope and 3 to 4 randomly chosen sections from each paw were quantified. The number of nerve fibers crossing the collagen-stained dermal/epidermal junction into the epidermis was counted using the 40X objective in a blinded setup. The length of the epidermis within each field was measured using Leica software. Intraepidermal nerve fiber density is expressed as the total number of fibers/length of the epidermis (IENFs/mm). For GFAP staining, mice were perfused intracardially with PBS followed by 4% paraformaldehyde in PBS, and spinal cord was collected. Cryosections (6 μ m) of lumbar segments L2 to L5 were incubated with 1:200 rabbit anti-GFAP (Acris Antibodies GmbH, Hiddenhausen, Germany) followed by Alexa-488 goat anti-Rabbit (Invitrogen, Grand Island, NY). GFAP expression in dorsal horn was quantified in 6–8 sections per group (3–4 mice per group). The mean intensity of fluorescence and the percent area positive were calculated using ImageJ software.

Oxygen consumption rate

Oxygen consumption rate (OCR) was measured using the Seahorse XFe 24 (Seahorse Bioscience, North Billerica, MA) as previously described [36; 42]. Tibial nerves were dissected starting at their start point from sciatic nerve till the ankle of the foot and placed into islet capture XF24 microplate in XF media supplemented with 5.5 mM glucose, 0.5 mM sodium pyruvate, and 1 mM glutamine without desheathing. OCR was measured before (baseline respiration) and after addition of mitochondrial inhibitors. The following inhibitors were used during the assay- 12 μ M oligomycin, 20 μ M carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), and 20 μ M of rotenone and 20 μ M of antimycin A. An assay cycle of 3-minute mix, 3-minute wait, and 4-minute measure combination was repeated 4 times for baseline rates and after each drug injection. ATP-coupled respiration is the OCR used for mitochondrial ATP synthesis, and is reduction in OCR due to addition of oligomycin. Maximal mitochondrial respiration (MRC) is the reduction in OCR due to uncoupler FCCP. OCR values were normalized to the total protein content of each well.

Acute dissociation of DRG neurons for whole-cell patch clamp recordings

Rats were given paclitaxel as described previously [41]. Animals were deeply anesthetized with isoflurane, and the bilateral L4 and L5 ganglia were surgically exposed and removed. The ganglia were placed in a flask containing trypsin (0.0625 mg/mL, Hyclone) and type IA collagenase (1 mg/mL, Sigma-Aldrich) in Dulbecco's Minimal Essential Medium (DMEM)

and shaken for 50 min in a heated (37°C) bath. After the cells were mechanically dispersed, they were plated on poly-L-lysine-coated glass sheets and held in culture dishes with DMEM (10% FBS) until use; the cells were used for patch clamp within 6 hours after plating [39]. Whole-cell patch recording was performed as previously described, the cells were transferred to a recording chamber and perfused with oxygenated ACSF (2 ml/min) at room temperature. Only neurons with a stable resting membrane potential of at least -40 mV and evoked spikes that overshoot 0 mV were used. Series resistance was compensated to above 70%.

Statistical analysis

Data are expressed as Mean $\pm$ SEM. Statistical analysis was performed using one-way or two-way ANOVA followed by Turkey analysis. $P < 0.05$ was considered statistically significant.

Results

Effect of the Epac inhibitor ESI-09 on paclitaxel-induced mechanical allodynia

Male mice were treated with paclitaxel every other day for two weeks (10 mg/kg, three injections per week with two days' rest in between) to induce peripheral neuropathy [37]. Paclitaxel-treated mice developed mechanical allodynia as tested using von Frey hairs (Figure 1A, open circles). To test whether Epac inhibition reverses paclitaxel-induced peripheral neuropathy, we administered the Epac-inhibitor ESI-09 (20 mg/kg daily for six days) starting at day 1 after completion of paclitaxel treatment. Treatment with ESI-09 strongly inhibited mechanical allodynia (Figure 1A, filled circles). ESI-09 was still effective in inhibiting mechanical allodynia even when we delayed administration of ESI-09 until 10 days after the last dose of paclitaxel (Figure 1B). ESI-09 also inhibits paclitaxel-induced mechanical allodynia in female mice (Supplementary Figure 1A). In mice treated with ESI-09 alone mechanical sensitivity was not changed [52].

The beneficial effect of ESI-09 is not limited to chemotherapy-induced neuropathic pain; the results in Figure 1C and 1D demonstrate that ESI-09 also reverses mechanical allodynia induced by spared nerve injury when the treatment is started early (3 days) or late (8 days) post injury. ESI-09 did not affect mechanical sensitivity in the contralateral paw (Supplementary Figure 1B and 1C).

Effect of Epac1 deletion on paclitaxel-induced mechanical allodynia

In vitro, ESI-09 inhibits activation of both Epac1 and Epac2 [56; 74]. To investigate the contribution of Epac1 we tested mice lacking the Epac1 gene. The results in Figure 2 demonstrate that Epac1^{-/-} mice are protected from paclitaxel-induced mechanical allodynia indicating that only deleting the Epac1 gene is enough to protect against taxol-induced mechanical allodynia. We did not detect differences between male and female Epac1^{-/-} mice and therefore pooled data for the two sexes. In line with previous studies [21; 52], we did not observe any effect of genetic deletion of Epac1 on mechanical sensitivity at baseline (Figure 2; bar graph). Using in situ hybridization analysis, we showed previously that Epac1 mRNA is expressed in all cells in the DRG [61]. To determine whether nociceptor Epac1 is

required for the development of mechanical allodynia in response to paclitaxel treatment, we used male and female mice with cell specific deletion of Epac1 in Nav1.8+ neurons (N-Epac1^{-/-} mice). N-Epac1^{-/-} male and female mice were protected against paclitaxel-induced mechanical allodynia (Figure 2). Consistent with what we observed in mice with global deletion of Epac1 (Epac1^{-/-} mice), cell specific deletion of Epac1 from nociceptors did not affect baseline mechanical sensitivity (Figure 2).

Collectively, these findings indicate that the beneficial effects of ESI-09 in the paclitaxel model of neuropathy are mediated via inhibition of nociceptor Epac1, although we cannot fully exclude a partial contribution of Epac2 or other potential cAMP targets to the observed effects of the pharmacological intervention.

Impact of Epac inhibition or Epac1 deletion on paclitaxel-induced astrocyte activation

Paclitaxel-induced peripheral neuropathy is associated with activation of astrocytes in the spinal cord [47; 70]. Therefore, we determined whether the inhibition of Epac signaling by oral administration of ESI-09 or genetic deletion of Epac1 affected astrocyte activation. Consistent with earlier studies [47; 70], paclitaxel increased GFAP staining indicating astrocyte activation in the dorsal horn of the spinal cord of WT mice (Figure 3A). Treatment with ESI-09 starting at 10 days after completion of paclitaxel normalized the expression of GFAP (Figure 3A and B). Moreover, in Epac1^{-/-} mice paclitaxel did not increase spinal cord GFAP expression (Figure 3C). Cell specific deletion of Epac1 from Nav1.8+ neurons also prevented astrocyte activation in response to paclitaxel (Figure 3C and D). These findings indicate that paclitaxel-induced astrocyte activation occurs downstream of Epac1 signaling in sensitized nociceptors. In line with previous studies [43; 49; 71] we did not observe changes in microglia activation as assessed by staining for Iba-1 in spinal cord of paclitaxel-treated mice (not shown).

Impact of Epac inhibition or deletion on paclitaxel-induced IENF loss

CIPN is known to be associated with a reduction in density of intra-epidermal nerve fibers (IENFs) in the plantar surface of the hind paws and this phenomenon is thought to be related to the impaired mitochondrial function in the peripheral nerve [33]. The data in Figure 4 show that also in our hands paclitaxel treatment reduces IENF density as assessed at day 18 after completion of paclitaxel treatment. ESI-09 was administered daily 7 days starting 10 days after completion of paclitaxel treatment. The results show that this schedule of ESI-09 administration significantly reduced the loss in IENF density (Figure 4A and B). In addition, mice with either global or nociceptor specific deletion of Epac1 are protected from the paclitaxel-induced reduction in IENF density (Figure 4C and D).

Contribution of Epac1 to paclitaxel-induced mitochondrial dysfunction

Multiple studies have reported mitochondrial abnormalities in the peripheral neurons in rodent models of CIPN [37; 67]. We examined whether the protection against paclitaxel-induced mechanical allodynia in Epac1-deficient mice was associated with preservation of mitochondrial function in the peripheral nerves. Ex vivo mitochondrial respiration of tibial nerve samples from WT and Epac1^{-/-} mice was compared using Seahorse extracellular flux analyzer. In WT mice, paclitaxel treatment resulted in reduced basal, ATP-coupled, and

maximal (MRC) mitochondrial respiration. Proton leak was not affected by paclitaxel treatment. Epac1^{-/-} mice were protected against all of the paclitaxel-induced defects in mitochondrial bioenergetics. (Figure 5A and B).

Impact of Epac inhibition on paclitaxel-induced spontaneous discharges by DRG neurons

Next, we investigated the effect of ESI-09 on the paclitaxel-induced spontaneous discharges in DRG neurons. Similar to previous studies, paclitaxel treatment led to high incidence of abnormal spontaneous activity in DRG neurons. The data in Figure 6A shows that treatment with ESI-09 reversed these spontaneous discharges in DRG neurons, returning it to normal activity. We also used Epac1-selective inhibitor, CE3F4, and found it inhibited spontaneous discharges as well. These results indicate that Epac1 is involved in the development of abnormal spontaneous activity in the DRG neurons.

Discussion

We demonstrate for the first time that pharmacological inhibition of Epac using the orally active compound ESI-09 inhibits already established mechanical allodynia in mice treated with paclitaxel (Figure 1A and B). ESI-09 inhibits the activation of astrocytes in the spinal cord and loss of pain sensory nerve endings in the periphery, two well established hall marks of CIPN (Figure 3A and 4A). Moreover, ESI-09 significantly reduced the incidence of abnormal spontaneous discharges in DRG neurons from paclitaxel-treated rats (Figure 6). We show that genetic deletion of Epac1 recapitulates the effect of the inhibitor ESI-09. Epac1 knockout animals show reduced paclitaxel-induced mechanical allodynia, spinal cord astrocyte activation, and loss of IENF density compared to WT mice. Moreover, Epac1^{-/-} knockout animals do not develop the paclitaxel-induced energy deficits in mitochondria in the peripheral nerve. We further show that Epac1 in nociceptors is critical for development of CIPN symptoms. Mice with a selective Epac1- deficiency in Nav1.8-positive primary sensory neurons do not show spinal cord astrocyte activation and IENF loss in response to paclitaxel treatment (Figure 3C and 4C). The protective effect of Epac1 inhibition is not limited to CIPN induced by paclitaxel but extends to mechanical allodynia in response spared nerve injury-induced neuropathy (Figure 1C) and cisplatin (unpublished). The finding that ESI-09 and genetic deletion of Epac1 both inhibit chemotherapy-induced mechanical allodynia identifies Epac1 as a novel potential therapeutic target for the treatment and prevention of CIPN. This is important because CIPN is a major adverse side effect of chemotherapy treatment and there are currently no FDA-approved drugs available to prevent or alleviate CIPN.

There have been many studies which investigated the effects of opioids, antiepileptic's, and antidepressants on CIPN, but clinical results have been disappointing [46; 65]. As far as pre-clinical studies aimed at identifying targets to treat CIPN is concerned, most drugs have been shown to only prevent the CIPN. Our present data clearly show that ESI-09 is effective even when treatment is initiated up to 10 days after completion of paclitaxel treatment in both males and females (Figure 1). Not all patients treated for cancer develop CIPN and therefore, the potential to target Epac signaling to alleviate already existing CIPN is promising. Before clinical translation can be considered, further studies are needed to

examine the effect of Epac inhibition on pain and IENF density in additional rodent studies in which paclitaxel is given i.v. in different dosing schedules. It should be noted, however, that the cumulative dose of paclitaxel used here is in the range of what is used to treat tumors in mice [14; 32; 34]. In addition to general analysis of potential toxic side effects, the potential interference of ESI-09 treatment with the anti-cancer effect of treatment needs to be examined. It is encouraging that ESI-09 inhibits invasion and metastasis of pancreatic cancer cells [2; 4]. These findings indicate that Epac1 inhibition during or after chemotherapeutic treatment may serve as a two pronged approach, on the one hand reducing CIPN and on the other hand reducing tumor cell metastasis.

ESI-09 acts as a competitive inhibitor of both Epac isoforms (Epac1 and Epac2), but has especially emerged as an attractive therapeutic option for blocking Epac1 cellular functioning [1; 4; 52]. ESI-09 inhibits cAMP-mediated Epac1 GEF activity and inhibits GTP transfer to Rap1 in response to the specific Epac1 agonist 007-AM [1; 4]. In vivo treatment of ESI-09 recapitulates the phenotype of Epac1 knockout animals in multiple model systems. For example, ESI-09 administration and genetic deletion of Epac1 both inhibit pancreatic cancer metastasis [1], bacterial invasion in a model of rickettsiosis [26], CFA-induced pain [52], and neointima formation in a mouse carotid artery ligation model [62]. In the present study, we demonstrate that genetic deletion of Epac1 recapitulates the effect of ESI-09 at different levels of CIPN *i.e.* at the level of inhibition of paclitaxel induced-mechanical allodynia, spinal cord glia activation, and IENF loss. This indicates Epac1 rather than Epac2 or other potential targets of the cAMP analogue ESI-09 is critical for CIPN. In addition, ex vivo, the competitive Epac inhibitor ESI-09 and the non-competitive Epac1 inhibitor CE3F4 both suppress the paclitaxel-induced spontaneous discharges of DRG neurons.

Our findings showing that oral administration of the Epac inhibitor ESI-09 attenuates mechanical allodynia in paclitaxel-treated mice indicate that the persistent sensitization is dependent on persistent activation of a cAMP-to-Epac1 signaling pathway. In this respect it is of interest that there is evidence for persistent activation of nociceptor membrane adenylyl cyclase [7] in a model of neuropathic pain induced by spinal cord injury, and it is possible that the same is happening in response to paclitaxel. Moreover, in both the spinal cord injury model and the model of CIPN we use here, abnormal spontaneous activity is detected in the DRG and we show here that this is blocked by ESI-09. In addition, it is known that cAMP can be produced locally in mitochondria by soluble adenylyl cyclase which is activated by bicarbonate ions and regulated by cellular ATP and calcium levels [18; 57; 63]. It would be interesting to explore whether the mitochondrial abnormalities that develop in response to paclitaxel treatment activate this cAMP-inducing pathway.

Epac1 signaling can also be activated independently of cAMP and we recently showed that cAMP-independent activation of Epac1 regulates neurite outgrowth in vitro [6]. Specifically, we showed that a decrease in levels of the protein Importin- β 1 promotes translocation of Epac1 to the plasma membrane to inhibit neurite outgrowth. It would be interesting to investigate whether paclitaxel treatment affects the level of importin- β 1 in IENFs with potential consequences for Epac1 regulation and IENF density.

It is known that cAMP-mediated activation of Epac1 leads to translocation of Epac1 to the plasma membrane and activation of Rap1 and other downstream effectors including PKC ϵ [15; 31]. Activated PKC ϵ can translocate to different subcellular compartments, including to the mitochondria where it can reduce complex-I respiration and Na⁺-K⁺-ATPase activity [45], potentially leading to mitochondrial insufficiency. Levine and group originally proposed a role for PKC ϵ in paclitaxel-induced neuropathy and showed that paclitaxel-induced hyperalgesia was attenuated by an antagonist of PKC ϵ [19]. More recently, Wang and colleagues reported that paclitaxel induces long term activation of PKC ϵ , PKC β II, and PKC δ as monitored by their plasma membrane translocation in DRG neurons of paclitaxel-treated mice. Intrathecal administration of a PKC ϵ inhibitor attenuated paclitaxel-induced mechanical allodynia. As a mechanism of action, these authors proposed that PKC ϵ activate of TRP and TRPV channels [30].

Mitochondrial structural and functional abnormalities in peripheral sensory neurons have been documented in various animal models of CIPN [25; 36; 37; 73]. In paclitaxel- and oxaliplatin-treated rats, sciatic nerves showed decreased Complex I- and Complex II-mediated respiration and ATP production [72]. We show here that in WT mice, paclitaxel treatment results in an overall decrease in mitochondrial oxygen consumption rate; basal, ATP coupled and maximal respiration in tibial nerve were all reduced indicating an overall reduction in mitochondrial content or mitochondrial health in the distal axons. Notably, we recently showed that prevention of mitochondrial damage using an inhibitor of mitochondrial p53 accumulation also prevented CIPN induced by either paclitaxel [37] or cisplatin [42]. These earlier findings indicate a causal relation between chemotherapy-induced mitochondrial deficits in sensory neurons and CIPN. Our present findings show that protection against CIPN by genetic deletion of Epac1 was associated with prevention of the paclitaxel-induced deficits in mitochondrial bioenergetics in tibial nerves in mice (Figure 5), supporting the causal relation between mitochondrial damage and signs of CIPN. It remains to be determined how Epac1 activation contributes to mitochondrial deficiencies in models of CIPN and if this is a cAMP-dependent mechanism.

Interestingly, the protective effect of genetic deletion of Epac1 from all cells or from nociceptors only on mechanical allodynia was associated with prevention of the paclitaxel-induced loss of IENFs. Moreover, inhibition of Epac1 by oral administration of ESI-09 restored IENF density (Figure 4). It is not known how the loss of IENFs in models of CIPN is regulated. The prevailing idea is that the reduction of IENF density is not due to neuronal death, but rather that reduced mitochondrial function leading to energy deficiency promotes retraction of distal endings. In line with this model, we showed earlier in the paclitaxel as well as the cisplatin model of CIPN that prevention of mitochondrial damage by the small compound PFT- μ not only protected against mitochondrial deficits but also against the reduction in IENF density [37; 42]. Our present findings support a model in which mitochondrial deficits in the distal axons are related to IENF loss.

CIPN in rodent models is known to be associated with spontaneous firing by DRG neurons [40; 66; 69]. Limited availability of ATP in the sensory axons is believed to generate spontaneous and irregular discharges [8; 66; 73]. Here we show that ex vivo ESI-09 treatment inhibits the abnormal spontaneous discharges in DRG neurons from paclitaxel-

treated animals (Figure 6). Similar results were obtained with a different Epac1 inhibitor, indicating that the effects are indeed mediated by inhibition of Epac1. We propose that at least part of the beneficial effect of ESI-09 on mechanical allodynia is mediated via the suppression of spontaneous activity in nociceptive neurons. Notably, the suppressive effect of ESI-09 on spontaneous discharges is rapid and reversible, indicating that persistent Epac activation is required for these spontaneous discharges. This observation is consistent with our observation that mechanical allodynia is inhibited but does not resolve in response to ESI-09 treatment. It would be interesting to know whether and how mitochondrial dysfunction in nociceptive neurons contributes to the spontaneous discharges.

In summary, we demonstrate that Epac1 is required for paclitaxel-induced mechanical allodynia and ESI-09 can be a potentially effective treatment to prevent and treat already existing paclitaxel-induced neuropathy. We propose that Epac1 inhibition could develop into an effective strategy to improve quality of life of cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Effect of the Epac inhibitor ESI-09 on paclitaxel-induced mechanical allodynia and SNI-induced neuropathic pain

Male WT mice were treated with paclitaxel for two weeks (3 doses of 10 mg/kg i.p. per week). (A) ESI-09 (20 mg/kg) or vehicle was given by oral gavage for 6 days starting on the first day (n=5/group) or (B) 10 days after completion of paclitaxel treatment (n=11/group). Mechanical allodynia was measured over time. (C) and (D) Spared nerve injury (SNI) or sham surgery was performed on male WT mice. (C) ESI-09 (50 mg/kg) or vehicle was given by oral gavage for 6 days starting on the 3rd day (n=7/group) after SNI or (D) 8 days after SNI (n=6/group). Mechanical allodynia was measured over time in ipsilateral and contralateral (Supplementary Figure 1) hind paw at 24 hours after ESI-09 treatment. In all panels, mechanical allodynia was quantified with von Frey Hairs using the up and down method. Data are expressed as 50% withdrawal threshold in grams and represent Mean $\pm$ SEM. Repeated measures two-way analysis of variance (ANOVA) showed a significant effect after ESI-09 administration vs vehicle group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. bsl, baseline.

Figure 2. Effect of Epac1 deletion on paclitaxel-induced mechanical allodynia

Male and female WT, Epac1^{-/-}, and N-Epac1^{-/-} mice were treated with paclitaxel as in Figure 1B and mechanical allodynia was measured over time. WT, n=14, Epac1^{-/-} n=9, N-Epac1^{-/-} n=12 mice. Data are expressed as mean $\pm$ SEM. ***p<0.001 for WT vs. N-Epac1^{-/-} and ###p<0.001 for WT vs. Epac1^{-/-}. bsl, baseline.

Figure 3. Impact of Epac1 inhibition or deletion on paclitaxel-induced astrocyte activation

(A) Effect of ESI-09 on paclitaxel-induced GFAP activation in lumbar spinal cord dorsal horn. WT mice (n=4/group) were treated with paclitaxel $\pm$ ESI-09 as in Figure 1B. 24 hours after the last dose of ESI-09, lumbar spinal cord was collected and immuno stained with GFAP (green). A representative example is shown from each group, vehicle alone (veh +veh), paclitaxel-treated (Taxol+veh), ESI-09 + vehicle-treated (veh+ESI-09), and paclitaxel + ESI-09 treated (Taxol+ESI-09). (B) Bar graph shows the quantitation in approximately 15 to 20 dorsal horn sections. The positive percentage of naïve wild-type mice was set as 100%. (C) Spinal cord dorsal horn GFAP activation in $\pm$ paclitaxel treated WT, Epac1^{-/-}, and N-Epac1^{-/-}. n=3–4 mice/group. Mice were treated with paclitaxel as in Figure 1B and 18 days after the treatment completion lumbar spinal cord were stained with GFAP as in panel (A). A representative image is shown for each group. (D) Bar graph shows the quantitation in approximately 15 to 20 dorsal horn sections. The positive percentage of naïve wild-type

mice was set as 100%. 2-way-ANOVA, Data are expressed as means $\pm$ SEM. **p<0.01.
Scale bar indicates 50 μ m.

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Figure 4. Impact of Epac1 inhibition or deletion on paclitaxel-induced IENF loss

(A) Effect of ESI-09 on paclitaxel-induced IENFs retraction. Mice (n=4 per group) were treated with paclitaxel $\pm$ ESI-09 as in Figure 1B. Hind paw biopsies were obtained a day after the last dose of ESI-09, fixed, and stained with pan-neuronal marker PGP9.5 for IENFs (red) and collagen (green). Representative images from each group are showed, white arrows denote IENF (red) crossing the basement membrane (in green). Vehicle alone (veh+veh), ESI-09 + vehicle-treated (veh+ ESI-09), paclitaxel-treated (Taxol+veh), and paclitaxel + ESI-09 treated (Taxol + ESI-09). (B) Bar graph showing quantitation of data from all paws. IENF density is number of nerve fibers crossing the basement membrane/length of the basement membrane (mm). * $p < 0.05$, ** $p < 0.01$. (C) Paclitaxel-induced IENFs retraction in WT (n=4), (Epac1^{-/-}) (n=3), (N-Epac1) (n=4) and their naïve mice (n=3/group). Mice were

treated with paclitaxel as in Figure 1B. Hind paw biopsies were obtained 18 days after the completion of the paclitaxel treatment and stained for IENFs as in panel (A). A representative image from each group is shown. (D) Bar graph showing quantitation of data from all paws. ** $p < 0.01$.

Figure 5. Epac1 knockout protects mitochondrial bioenergetics and contents in the tibial nerve of taxol-treated mice

WT and Epac1^{-/-} mice were treated with paclitaxel for two weeks (3 doses of 10 mg/kg i.p. per week) and tibial nerves were obtained 18 days after the completion of the paclitaxel treatment. Oxygen consumption rate (OCR) was measured in the tibial nerves using the Seahorse XFe 24. Two-way ANOVA revealed a significant interaction ($P < 0.05$) for baseline respiration, ATP-coupled respiration, and maximal respiratory capacity (MRC). Tukey post-hoc analysis revealed significant differences between groups: $*P < 0.05$. $n = 12$ mice/group.

Figure 6. Impact of Epac inhibition on paclitaxel-induced spontaneous discharges by DRG neurons in rats

DRG neuron with spontaneous action potential (SA) isolated from segments at day 7 after paclitaxel treatment show frequency of SA was both reduced by bath application of ESI-09 (1 μ M) and CE3F4 (2.5 μ M). Panel 6A shows a representative recording of trace baseline, after administration of ESI-09 (1 μ M) and after washout. Panel 6B shows a representative recording of trace baseline, after administration of CE3F4 (2.5 μ M) and after washout. Bar graph C summarizes ESI-09 results obtained in 9 cells from 6 paclitaxel-treated animals. ***p < 0.001. Bar graph D summarizes CE3F4 results obtained in 8 cells from 7 paclitaxel-treated animals. **p < 0.01.