Key Role for the Epsilon Isoform of Protein Kinase C in Painful Alcoholic Neuropathy in the Rat

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Chronic alcohol consumption produces a painful peripheral neuropathy for which there is no reliably successful therapy, attributable to, in great part, a lack of understanding of the underlying mechanisms. We tested the hypothesis that neuropathic pain associated with chronic alcohol consumption is a result of abnormal peripheral nociceptor function. In rats maintained on a diet to simulate chronic alcohol consumption in humans, mechanical hyperalgesia was present by the fourth week and maximal at 10 weeks. Thermal hyperalgesia and mechanical allodynia were also present. Mechanical threshold of C-fibers in ethanol fed rats was lowered, and the number of action potentials during sustained stimulation increased. The hyperalgesia was acutely attenuated by intradermal injection of nonselective protein kinase C (PKC) or selective PKCe inhibitors injected at the site of nociceptive testing. Western immunoblot analysis indicated a higher level of PKCe in dorsal root ganglia from alcohol-fed rats, supporting a role for enhanced PKCe second-messenger signaling in nociceptors contributing to alcohol-induced hyperalgesia.

Key words: protein kinase C ε; alcoholic peripheral neuropathy; pain; hyperalgesia; allodynia; primary afferent nociceptor

Ethanol consumption is the most common cause of peripheral nervous system, as well as CNS, neurotoxicity. Ethanol is thought to exert a direct neurotoxic action on the peripheral nervous system, resulting in a neuropathy that mostly involves small-diameter fibers (Diamond and Messing, 1994; Monforte et al., 1995; Kiellhorn, 1996; Ortiz-Plata et al., 1998; Tredici et al., 1999). The peripheral neuropathy is a potentially incapacitating complication of chronic consumption of ethanol, characterized by pain and dysesthesias, primarily in the lower extremities, and is poorly relieved by available therapies (Ratcliff, 1979; Monforte et al., 1995; Ortiz-Plata et al., 1998).

Whereas enhanced nociception and primary afferent nociceptor hypersensitivity have been demonstrated in animal models of other neuropathic pain states, such as those induced by diabetes (Ahlgren and Levine, 1994), chemotheraphy (Tanner et al., 1998; Authier et al., 1999), or trauma (Bennett and Xie, 1988; Campbell et al., 1988; Seltzer et al., 1990; Xie and Xiao, 1990; Kim and Chung, 1992; Kim et al., 1993; Sheen and Chung, 1993; Yoon et al., 1996; Pedersen and Kehlet, 1998; Zahn and Brennan, 1999), an animal model for alcohol-induced neuropathy does not exist, nor has it even been demonstrated that primary afferent nociceptor function is altered by chronic exposure to alcohol.

In animal models of other painful peripheral neuropathies, enhanced nociception involves alterations in intracellular signaling. Specifically, protein kinase C (PKC) (Ahlgren and Levine, 1994) [particularly the epsilon (ε) isoform (Gerstin et al., 1998; Khasar et al., 1999)] and protein kinase A (PKA) (Ahlgren and Levine, 1994) signaling pathways have been implicated in enhancing nociceptor function. Because alcohol has been shown to activate PKC and PKA (Coe et al., 1996; Pandey, 1996; Gerstin et al., 1998; Constantinescu et al., 1999; Sisson et al., 1999; Yoshimura and Tabakoff, 1999), we hypothesized that, in a well established model for chronic ethanol consumption in the rat, both enhanced nociception and nociceptor function occur and that PKC and PKA pathways contribute to the enhanced nociception.

MATERIALS AND METHODS

Chronic alcohol consumption. Male Sprague Dawley rats (200–450 g; Bantin and Kingman, Fremont, CA), individually caged and maintained under a 12 hr light/dark cycle, were used in these experiments. The experimental rats were fed Lieber–DeCarli liquid diet (Dyets Inc., Bethlehem, PA) (Lieber and DeCarli, 1982, 1989; Lieber et al., 1989) with ethanol (ED) (6.5% ethanol) for 12 weeks. The control diet (CD) rats were pair-fed (i.e., calorically matched to the ethanol-exposed rats) by giving a diet in which equal calories of maltose–dextrin or alcohol (Gold Shield Chem Co., Hayward, CA) was consumed (Lieber et al., 1989). Using a subset of a group of rats that had been maintained for 12 weeks on ethanol diet, an alcohol withdrawal study was performed by switching the rats to CD for a period of 5 weeks.

The Institutional Animal Care Committee of the University of California, San Francisco, approved the experimental protocol.

Blood ethanol determination. Blood ethanol concentration was measured by drawing 100 μl blood samples from the tail vein of ED rats into heparinized Eppendorf tubes. The blood samples, collected 3 hr after commencement of feeding, were centrifuged to separate plasma. The samples were analyzed for alcohol by gas chromatography (Erickson, 1973; Tabakoff et al., 1976). The blood samples were taken on the last day of the eighth week of ED to a separate group of rats, at which time behavioral testing had demonstrated a significant hyperalgesia.

Mechanical nociceptive threshold. The nociceptive flexion reflex was quantified using the Randall-Selitto paw pressure device (Stoelting, Chicago, IL), which applies a linearly increasing mechanical force to the dorsum of the rat’s hindpaw (Taiwo et al., 1989b). The mechanical nociceptive threshold was defined as the force in grams at which the rat withdrw paw. Rats were familiarized in the test apparatus at 5 min intervals for a period of 1 hr per day for 3 d in the week preceding the experiment to decrease nociceptive thresholds (Taiwo et al., 1989b). The rat was allowed to crawl into a cotton sock, which is then placed on a Perspex block and covered with an elastic blanket that is attached to the block, on two sides, by Velcro strips. The rat was allowed to acclimatize in the restrainer as described for a period of 15–20 min, after which it was exposed to the test stimulus (Taiwo et al., 1989b). Briefly, the training procedure consisted of repeated paw-withdrawal tests at 5 min intervals for 1 hr per day. The stimulus was applied using a Basile alganesy meter, an instrument that applied a linearly increasing mechanical force onto the dorsum of the rat’s paw at the same site at which test agents were to be injected. Baseline paw-withdrawal threshold was defined as the mean of the last six readings before test agents were injected. Behavioral testing was done on both ED and CD rats once per week, and the two groups were tested the same day. After 8 weeks of ethanol diet feeding, the decrease in...
paw-withdrawal threshold had plateaued, and test agents were injected intradermally into the dorsum of the hindpaws. **Stimulation with von Frey hair.** Mechanical allodynia was assessed with von Frey hair (VFH) using the up–down method (Chaplan et al., 1994; Kinman and Levine, 1995; Aley et al., 1996). Calibrated VFH (1.32, 3.63, 10.0, and 27.5 mN; Ainsworth, London, UK) were applied to the planter skin of each hindpaw in these studies.

**RESULTS**

**Effect on weight of chronic exposure to ethanol compared with an isocaloric diet**

Just before initiation of the Lieber–DeCarli liquid diets, rats assigned to the CD group weighed 211.0 ± 1.0 gm compared with 223.8 ± 1.7 gm in rats assigned to the ED group (Fig. 1A). Animals in the two groups gained weight comparably throughout the period of study. After 12 weeks of feeding with the Lieber–DeCarli diets, CD and ED rats weighed 409.3 ± 4.7 gm and 405.0 ± 9.3 gm, respectively (p < 0.05) (Fig. 1A). Blood ethanol concentration was determined as 66.8 ± 7.0 mmol/l (mean ± SEM; range, 46.4–92.6 mmol/l; n = 6 ethanol-treated rats).

**Effect of ethanol on mechanical nociceptive threshold (1B)**

Before the administration of the liquid diets, the mean baseline paw-withdrawal threshold of ED rats (104.9 ± 1.1 gm; n = 34) (Fig. 1B) was not significantly different (p > 0.05) from that in CD rats (109.3 ± 2.8 gm; n = 24). A significant decrease in mechanical nociceptive threshold (i.e., hyperalgesia) was produced in the ED rats after 4 weeks (p < 0.0001; F = 234.1) (Fig. 1B). Further reduction (p < 0.05) in the paw-withdrawal threshold of ED rats to 81.6 ± 1.3 gm occurred at 10 weeks, after which hyperalgesia was maintained in the range of 81.6–84.1 gm until the conclusion of the study, at 12 weeks. There was no significant change (p > 0.05) in the mean paw-withdrawal threshold of CD rats over the same time period.
Role of PKC/PKC\(\varepsilon\) and PKA in ethanol-induced mechanical hyperalgesia

After 8 weeks of ethanol administration, the intradermal injection of BIMM or PKC\(\varepsilon\)-I (each administered at 1 \(\mu\)g) resulted in a significant reduction in mechanical hyperalgesia in ED rats (each \(p < 0.0001; F = 14.8\)) (Fig. 2A,B). There was no significant difference \((p > 0.05)\) in the effect of these two inhibitors on paw-withdrawal threshold in the hyperalgesic ED rats. WIPTIDE did not significantly \((p = 0.07)\) affect nociceptive threshold in hyperalgesic ED or nonhyperalgesic CD rats, although there was a trend toward an effect.

von Frey hair stimulation (mechanical allodynia)

To determine whether the mechanical hyperalgesia induced by alcohol was associated with mechanical allodynia, we examined responses to von Frey hair stimulation in ED and CD rats. ED rats demonstrated a significantly \((p < 0.05)\) increased response frequency to 27.5 mN VFH stimulation (Fig. 3A) when compared with the response in CD rats.

Thermal hyperalgesia

Because thermal hyperalgesia also occurs in patients with neuropathic pain, we compared thermal responses in ED and CD rats using the method of Hargreaves et al. (1988). ED rats demonstrated a significantly \((p < 0.05)\) lower \((6.8 \pm 0.4\) sec) paw-withdrawal latency in response to thermal stimulation when compared with CD rats \((8.6 \pm 0.5\) sec) (Fig. 3B). Thus, ethanol treatment evoked thermal hyperalgesia.

Effect of ethanol withdrawal on ethanol-induced hyperalgesia

After 12 weeks of ethanol diet, a subset, comprised of three rats, from the ED rats were placed on the control diet to determine whether ethanol-induced hyperalgesia was reversible. In ED rats placed on a CD for 2 weeks, the paw-withdrawal threshold was not significantly different \((p > 0.05)\) than it had been after 12 weeks on the ethanol diet (Fig. 4). Interestingly, 5 weeks after being placed on the CD, the nociceptive threshold in this group was even more reduced than during the last week of ethanol treatment \((p < 0.0002)\), indicating that hyperalgesia had increased during the 5 week period on CD (Fig. 4).

Effect of ethanol on C-fiber threshold and responsivity

Study of C-fibers from ED and CD rats revealed no spontaneous activity in most fibers. In a few fibers, spontaneous activity, although present, was \(< 0.01\) Hz. The conduction velocities for C-fibers on the dorsum of the hindpaw from CD and ED rats was also similar. However, the mean mechanical threshold for C-fibers from the ED rats was lower than that from CD rats \((p < 0.05)\) (Fig. 5A). The location of receptive fields of C-fibers on the dorsum of the hindpaw from CD and ED rats was similar. However, the mean mechanical threshold for C-fibers from the ED rats was lower than that from CD rats \((p < 0.05)\) (Fig. 5A).
Figure 5. Conduction velocity and mechanical threshold of C-fibers in CD and ED rats. A, There was no difference in conduction velocity between C-fibers from ED (filled bar; n = 10) and CD (hatched bar; n = 14) rats. Unpaired t test, p > 0.05. B, The mechanical threshold of C-fibers from ED rats (filled bar; n = 10) was significantly lower than that of C-fibers from control rats (hatched bar; n = 14) using nonparametric Mann–Whitney U test; * p < 0.05. C, The number of action potentials evoked by sustained (60 sec) threshold stimulation was significantly greater in C-fibers in ED rats (filled bar; n = 10) compared with controls (hatched bar; n = 14). D, The number of action potentials evoked by sustained suprathreshold (10 gm) stimulation was significantly greater in C-fibers in ethanol-fed rats (filled bar; n = 10) compared with controls (hatched bar; n = 14). Unpaired (one-tailed) t test; *p < 0.05 (Student’s t test).

5B). Also, the mean number of action potentials evoked by a sustained 60 sec threshold or suprathreshold (10 gm) stimulus was significantly higher for C-fibers from ED rats compared with those from CD rats (p < 0.05) (Fig. SC,D).

PKCε level
To determine whether the PKCε-dependent decrease in nociceptive threshold observed in ED rats is associated with a higher level of expression of PKCε in primary afferents, a Western analysis of DRG protein samples from CD and ED rats was performed. There was a higher level of PKCε in dorsal root ganglia in ED rats (Fig. 6). When the PKCε-immunoreactive bands observed were quantified, the average PKCε level in alcohol-fed (n = 11) rats was found to be 50% higher (p < 0.001) (Fig. 6A,B) than that in the control rats (n = 10).

DISCUSSION
We have developed the first animal model for alcohol-induced painful peripheral neuropathy and describe alterations in primary afferent nociceptor function and in specific second-messenger signaling that contributes to the enhanced nociception. Rats chronically fed ethanol exhibited mechanical and thermal hyperalgesia and tactile allodynia, all of which are symptoms frequently occurring in patients with painful peripheral neuropathy (Scadding, 1992). This model is highly relevant to painful alcoholic neuropathy in humans because the blood alcohol level that results in neuropathic changes is similar (Bosch et al., 1979; Lieber and DeCarli, 1989; Lieber et al., 1989). Also, the fact that hyperalgesia is stable for weeks renders this model very useful for study of underlying mechanism.

Ethanol-induced hyperalgesia did not resolve and, in fact, continued to increase at 5 weeks after cessation of alcohol intake. This is consistent with reports of persisting or even worsening peripheral neuropathy in patients who have withdrawn from chronic ethanol use (Gadner, 1972; Weise et al., 1985; Yokoyama et al., 1991; Spahn et al., 1995).

We tested whether two intracellular second-messenger pathways contribute to ethanol-induced hyperalgesia. PKC and PKA are both known to be important in nociceptor function (Taiwo et al., 1989a; Ahlgren and Levine, 1994; Khasar et al., 1999) and in mediating other effects of alcohol (Diamond et al., 1987; Hoffman et al., 1987; Hoek et al., 1988, 1992; Messing et al., 1991; Lovinger and Zhou, 1994; Gordon et al., 1997). PKC was found to contribute to the enhanced nociception, whereas cAMP/PKA, if it does contribute, appears to be less important. This second-messenger dependence differs from that for the enhanced nociception produced by hyperalgesic inflammatory mediators, to which both PKA and PKC contribute (Taiwo et al., 1989a; Taiwo and Levine, 1991; Khasar et al., 1999). Because the PKCε antagonist (PKCε-I) alone was able to reverse the hyperalgesia to the same extent as the nonspecific PKC antagonist BIMM, the epsilon isoform of PKC accounts for the role of PKC in ethanol-induced enhanced nociception (Khasar et al., 1999). Because the PKCε antagonist was able to attenuate established ethanol-induced hyperalgesia, ongoing PKCε activity must be required to maintain the hyperalgesia. A role for the ε isoform of PKC is consistent with previous demonstrations that chronic ethanol exposure increases levels of PKCε in...
develops during chronic ethanol exposure. PKC, but not of PKCβ or PKCζ, in cardiac tissue (Miyamae et al., 1999). PKC activity also contributes to neuropathic pain induced by cancer chemotherapy (vincristine) (K. O. Aley and J. D. Levine, unpublished observation) and diabetes (Ahlgren and Levine, 1994). The fact that neither PKC antagonist had an effect on mechanical threshold in control animals strongly supports the suggestion that the contribution of PKCε to enhanced nociception develops during chronic ethanol exposure. It appears, for several reasons, that the PKCε activity that maintains ethanol-induced hyperalgesia occurs in the peripheral nociceptor terminal. First, we observed hyper-responsiveness (i.e., decrease in threshold and enhanced responsivity to mechanical stimuli) in C-fiber nociceptors. Second, intradermal injections of extremely small amounts of inhibitors attenuated the enhanced nociception. Finally, at the site of study, the skin of the hindpaw, PKCε is believed to be expressed only in primary afferent terminals (Khasar et al., 1999).

In summary, we have demonstrated that hyperalgesia is present in an established model in the rat for chronic alcohol consumption in humans and that PKCε signaling plays a critical role in the enhanced nociception produced by chronic alcohol. The findings suggest that PKCε might be an excellent therapeutic target for this common and, at present, primarily untreatable chronic pain syndrome.

REFERENCES


