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Involvement of capsaicin-sensitive afferents and the Transient Receptor Potential Vanilloid 1 Receptor in xylene-induced nocifensive behaviour and inflammation in the mouse

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ABSTRACT

The inflammatory actions of xylene, an aromatic irritant and sensitizing agent, were described to be predominantly neurogenic in the rat, but the mechanism and the role of the Transient Receptor Potential Vanilloid 1 (TRPV1) capsaicin receptor localized on a subpopulation of sensory nerves has not been elucidated. This paper characterizes the involvement of capsaicin-sensitive afferents and the TRPV1 receptor in nociceptive and acute inflammatory effects of xylene in the mouse. Topical application of xylene on the paw induced a short, intensive nocifensive behaviour characterized by paw liftings and shakings, which was more intensive in Balb/c than in C57Bl/6 mice. Genetic deletion of the TRPV1 receptor as well as destroying capsaicin-sensitive nerve terminals with resiniferatoxin (RTX) pretreatment markedly reduced, but did not abolish nocifensive behaviours. In respect to the xylene-induced plasma protein extravasation detected by Evans blue leakage, significant difference was neither observed between the Balb/c and C57Bl/6 strains, nor the ear and the dorsal paw skin. These inflammatory responses were diminished in the RTX pretreated group, but not in the TRPV1 gene-deleted one. Injection of the antioxidant N-acetylcysteine 15 min prior to xylene smearing significantly reduced plasma protein extravasation at both sites. These results demonstrate that xylene-induced acute nocifensive behaviour is mediated by capsaicin-sensitive afferents via TRPV1 receptor activation in mice. Neurogenic inflammatory components play an important role in xylene-induced plasma protein extravasation, but independently of the TRPV1 ion channel. Reactive oxygen or carbonyl species participate in this process presumably via stimulation of the TRPA1 channel.

Xylene, which is generally the mixture of three isomeric forms (ortho, meta and para), is an aromatic irritant and sensitizing agent. It is found in jet fuels and also serves as an important organic solvent in a variety of applications such as histological processing of tissue samples as well as paint and printing industry. Exposures to xylene may occur during its production and in the manufacture of gasoline, protective metal coatings and petroleum products [15]. The mechanism of xylene-induced acute cutaneous inflammation was first studied more than 40 years ago, and it was described that after surgical denervation of a certain area or degeneration of sensory nerves after pretreatment with high doses of capsaicin, its topical application did not evoke plasma protein extravasation in the rat skin. Therefore, the xylene-evoked acute pro-inflammatory action was suggested to be purely or predominantly of neurogenic origin mediated by capsaicin-sensitive afferents [23]. Electrophysiological data showed that topical xylene application on the rabbit ear evoked a strong excitation of capsaicin-sensitive C-polymodal nociceptors, which lasted for minutes [40]. Later, xylene was commonly used as a test substance to induce experimental neurogenic inflammatory reactions in the skin [10,22,30], as well as inflammation [14,26,27], detrusor hyperreflexia [28] and visceral chemonociceptive responses [1] in the urinary bladder. These studies provided several lines of evidence for the involvement of pro-inflammatory sensory neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP) released from the activated capsaicin-sensitive fibres. SP induces plasma protein extravasation by activating tachykinin NK1 receptors on vascular endothelial cells, while CGRP evokes vasodilatation and potentiates SP-induced pro-inflammatory actions in the innervated area which is collectively called neurogenic inflammation [13,37]. Most experiments were done in rats, but the mechanism and the role of the Transient Receptor Potential Cation Channel, Subfamily V, Member 1 (TRPV1) capsaicin receptor in xylene-induced neural activation was not elucidated.

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TRPV1 is a non-selective cation channel expressed predominantly in a subset of primary sensory neurons and can be activated by a wide array of chemical as well as hot stimuli, e.g. capsaicin (8-methyl-N-vanillyl-6-nonenamide) or resiniferatoxin (RTX) [35,37,38], lipoxygenase products, noxious heat, protons, etc. [2,5,16,21]. It has been cloned in several species like the rat [5], guinea pig [34], man [17], rabbit [12] and mouse [6]. The TRPV1 receptor can be considered a pivotal molecular integrator to initiate responses to noxious chemical and thermal stimuli in the peripheral terminals of primary afferents involved in nociception and inflammation [11]. Beyond action potential generation and pain sensation, its activation induces the influx of Ca^{2+} and consequent release of sensory neuropeptides which are responsible for the development of neurogenic inflammation in the innervated area [19,25]. Other members of the TRP channel family are also targets for several irritants, inflammatory and nociceptive agents [31], such as the TRPA1 channel for formalin [28], 4-hydroxynonenal or iodoacetamide [24]. Although xylene-induced neurogenic actions were thoroughly studied in rats in the 1980s–1990s, the involvement of the TRPV1 receptors localized on these nerves has not been studied due to the lack of potent, selective and *in vivo* effective TRPV1 receptor antagonists. The generation of TRPV1 receptor gene-deleted mice [4,8] provided good novel opportunities for analysing the role of this receptor in pathophysiological mechanisms.

There are very few data available on the actions of xylene in mice [20] and the mechanism of nerve activation is not known, therefore the aim of the present experiments was to analyze the role of capsaicin-sensitive fibres as well as the TRPV1 capsaicin receptors in the acute nociceptive and inflammatory effects induced by this irritant with the help of resiniferatoxin pretreatment and TRPV1 receptor gene-deleted mice, respectively.

Experiments were performed on male Balb/c and C57Bl/6 mice (Charles Rivers Hungary Ltd., Budapest, Hungary), as well as TRPV1 receptor gene-deleted mice backcrossed with C57Bl/6 wildtypes for 10 generations (TRPV1 knockout, $TRPV1^{-/-}$; Jackson Laboratories via Charles Rivers Hungary Ltd., Budapest, Hungary). The weight of all animals were 20–25 g, they were kept in the Laboratory Animal Centre of the University of Pécs at 24–25 °C and provided with standard chow and water *ad libitum*. Xylene (mixture of the three isomeric forms) and formamide were purchased from Szkarabeusz Ltd. (Hungary), in the nociception experiment xylene was diluted with 96% ethanol (Central University Pharmacy of Pécs, Hungary). Resiniferatoxin (RTX) and Evans blue were obtained from Sigma (St. Louis, MO, USA), RTX was dissolved in 96% ethanol to make a 1 mg/ml stock solution and further diluted with saline. Ketamine was purchased from Richter Gedeon Plc. (Hungary), xylazine from Lavet Ltd. (Hungary), N-acetylcysteine (NAC, 100 mg/ml injection) from Zambon (Hungary).

For studying xylene-induced acute somatic chemonociception, 100% or 50% xylene (50 μl) diluted with ethanol was topically applied on the skin of the right hindpaw and the number of paw liftings and shakings, as nocifensive behaviours were counted during 10 min. This is a rapid chemonociceptive test, which is painful, but the effect of the applied irritant lasts only for a short period of time.

To analyze the xylene-induced acute neurogenic inflammatory response, the Evans blue leakage technique was used [32]. Mice were anaesthesized with ketamine (5 mg/kg i.p.) and xylazine (100 mg/kg i.m.) and Evans blue (2.5% 0.1 ml/10 g; 250 mg/kg i.v.) was injected into the tail vein. Xylene (50 μl) was smeared simultaneously on the dorsal skin of the right paw and on the right ear 5 min later. Since Evans blue binds to plasma albumin, leakage can be detected at the site of acute plasma extravasation, therefore, the amount of the accumulated dye in the skin pieces quantitatively correlates with the extent of the inflammation. Animals were exsanguinated 20 min after the induction of the inflammation, the ears and the paw skin samples were cut, weighed and put into 1 ml formamide for the extraction of their dye content over a 3-day period. Evans blue concentration was then determined by spectrophotometry at 620 nm using a microplate reader (Labsystems Multiskan RC) and expressed as μg dye/g wet tissue. The minimal Evans blue content measured in the respective samples of the contralateral side (due to the remaining blood in the small vessels after exsanguination) was taken off the results of the xylene-treated ones.

In the control groups 96% ethanol was smeared on the paw skin. For inactivation of capsaicin-sensitive afferents, some groups of both Balb/c and C57Bl/6 mice were repeatedly pretreated with high doses of the potent TRPV1 receptor agonist RTX (30, 70 and 100 μg/kg s.c. on 3 consecutive days) one week before the experiment [9,18,23]. Systemic RTX pretreatment destroys the whole capsaicin-sensitive sensory nerve endings by permanent opening of the TRPV1 non-selective cation channel. Increased intracellular Ca^{2+} concentration and Ca^{2+} accumulation in the mitochondria causes ultrastructural changes (swelling) and metabolic damage of the cells which is accompanied by the loss of further responsiveness of these neurones [36,39]. The success of RTX pretreatment was confirmed by the lack of wiping movements in response to 20 μl of 0.1% capsaicin administration into the eye. To study the role of reactive oxygen and carbonyl species in the xylene-induced acute inflammatory reaction, C57Bl/6 mice were pretreated with the antioxidant N-acetylcysteine (300 mg/kg i.p.) 15 min prior to the local administration of the irritant.

All experimental procedures were carried out in accordance with the Declaration of Helsinki and the Animals (Scientific Procedures) Act 1998 (Hungary), complied with the recommendations of the International Association for the Study of Pain. The studies were approved by the Ethics Committee on Animal Research of the University of Pécs and permission was given (license No.: BA 02/2000-11-2006). Results are expressed as means ± S.E.M. of $n = 8–19$ mice per group. Statistical analysis was performed by ANOVA followed by Bonferroni's modified *t*-test; $p < 0.05$ was regarded as significant.

Topical application of xylene on the paw induced a short, but intensive nocifensive behaviour characterized by liftings and shakings of the affected limb. Although the number of these reactions was counted during 10 min, the most prominent responses were observed in the first 3 min. It was striking, that Balb/c mice produced a significantly greater number of nocifensive reactions than the C57Bl/6 ones. This might suggest that the Balb/c strain is more sensitive to the nociceptive action of xylene or that they have a greater behavioural response to the same level of perceived nociception. However, there was no significant difference between the nociceptive actions of 100% and 50% diluted xylene. In mice lacking the TRPV1 receptor as well as in both Balb/c and C57Bl/6 animals pretreated with RTX to impair the function of capsaicin-sensitive sensory nerves, the number of paw liftings and shakings was markedly reduced, but not abolished (Fig. 1). Topical application of ethanol on the paw induced no nocifensive behaviours (zero events over the 10-min period; data not shown).

In respect to the xylene-induced Evans blue accumulation in the skin, as an indicator of acute plasma protein extravasation, difference was neither observed between Balb/c and C57Bl/6 mice, nor the ear and the dorsal paw skin. Similarly to the nocifensive reactions, the acute inflammatory response was also markedly diminished in both mouse strains pretreated with RTX. The extent of the inhibition after destroying the capsaicin-sensitive fibres was about 50% in the ear of Balb/c as well as C57Bl/6 mice. However, in the paw skin of the latter strain the inhibition was much greater, about 85% showing an organ difference between the importance of these afferents in the xylene-induced inflammatory action. On