

Role of Transient Receptor Potential Vanilloid 1 Receptors in Adjuvant-Induced Chronic Arthritis: In Vivo Study Using Gene-Deficient Mice

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ABSTRACT

The transient receptor potential vanilloid 1 (TRPV1) receptor is a nonselective cation channel localized on a subset of primary sensory neurons and can be activated by a wide range of stimuli. The present study investigated the role of this receptor in chronic arthritis evoked by complete Freund's adjuvant (CFA) using TRPV1 receptor gene-deleted (TRPV1^{-/-}) mice and wild-type counterparts (TRPV1^{+/+}). In TRPV1^{+/+} mice, CFA injected intraplantarly into the left hindpaw and the root of the tail induced swelling of the injected and contralateral paws up to 130 and 28%, respectively, measured by plethysmometry throughout 18 days. Mechanonociceptive threshold measured with dynamic plantar aesthesiometry was decreased by 50 and 18% on the injected and contralateral paws, respectively. Histological examination and scoring of the tibiotarsal joints revealed marked arthritic changes in wild-type mice. In TRPV1^{-/-} animals edema, histological score and mechanical allodynia were significantly smaller. Daily treatment with the lipoxygenase inhibitor nordihydroguarectic acid (NDGA), the cyclooxygen-

ase inhibitor indomethacin, the bradykinin B2 receptor antagonist HOE-140 [D-arginyl-L-arginyl-L-prolyl-*trans*-4-hydroxy-L-prolylglycyl-3-(2-thyenylyl)-L-alanyl-L-seryl-D-1,2,2,4-tetrahydro-3-isoquinolinecarbonyl-L-(2*a*,3*b*,7*ab*)-octahydro-1*H*-indole-2-carbonyl-L-arginine], or the B1 receptor antagonist desArgHOE-140 [D-arginyl-L-arginyl-L-prolyl-*trans*-4-hydroxy-L-prolylglycyl-3-(2-thyenylyl)-L-alanyl-L-seryl-D-1,2,2,4-tetrahydro-3-isoquinolinecarbonyl-L-(2*a*,3*b*,7*ab*)-octahydro-1*H*-indole-2-carbonyl] was performed to reveal what mediators might activate TRPV1. NDGA significantly inhibited edema, hyperalgesia, and arthritis score in TRPV1^{+/+}, but not in TRPV1^{-/-} mice. The effect of indomethacin was markedly smaller in knockouts. In TRPV1^{+/+} animals, HOE-140, but not desArgHOE-140, inhibited arthritis, whereas in TRPV1^{-/-} mice, HOE-140 produced limited effect. Thus, whereas bradykinin and lipoxygenase products seem to act exclusively via TRPV1 activation, prostanoids do not, or at least only partially, to enhance murine experimental arthritis and related hyperalgesia.

The transient receptor potential vanilloid (TRPV) subfamily of transient receptor potential cation channels consists of at least six members of membrane proteins in mammalian

cells. The first representative of this family, TRPV1, was discovered by a systemic search for the postulated capsaicin receptor (Szolcsányi, 1993). The TRPV1 cation channel, which was formerly called vanilloid receptor 1, is expressed in a major group of nociceptive primary afferent neurons (Caterina et al., 1997) and gated by noxious heat, protons (Caterina et al., 1997; Tominaga et al., 1998), endogenous lipids such as anandamide (Gauldie et al., 2001), oleoyldopamine (Chu et al., 2003), *N*-arachidonoyldopamine (Huang et al., 2002), arachidonyl-2-chloroethylamide (Baker and McDougall, 2004), and lipoxygenase products such as 12-(*S*-

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ABBREVIATIONS: TRPV1, transient receptor potential vanilloid 1; CGRP, calcitonin gene-related peptide; SP, substance P; CFA, complete Freund's adjuvant; NDGA, nordihydroguarectic acid, 1,4-bis(3,4-dihydroxyphenyl)-2,3-dimethylbutane; desArgHOE-140, D-arginyl-L-arginyl-L-prolyl-*trans*-4-hydroxy-L-prolylglycyl-3-(2-thyenylyl)-L-alanyl-L-seryl-D-1,2,2,4-tetrahydro-3-isoquinolinecarbonyl-L-(2*a*,3*b*,7*ab*)-octahydro-1*H*-indole-2-carbonyl; HOE-140, D-arginyl-L-arginyl-L-prolyl-*trans*-4-hydroxy-L-prolylglycyl-3-(2-thyenylyl)-L-alanyl-L-seryl-D-1,2,2,4-tetrahydro-3-isoquinolinecarbonyl-L-(2*a*,3*b*,7*ab*)-octahydro-1*H*-indole-2-carbonyl-L-arginine; ANOVA, analysis of variance; WT, wild-type; Th, T helper.

hydroxyperoxyicosatetraenoic acid (Hwang et al., 2000). Vanilloid compounds such as the plant-derived natural irritants capsaicin and resiniferatoxin activate only the TRPV1 channel members of the TRPV family. Consequently, the effects of capsaicin tested so far in vivo or in vitro were absent in TRPV1 gene-deleted mice (Caterina et al., 2000; Davis et al., 2000). On the other hand, more and more data indicate that further endogenous mediators of pain and inflammation also stimulate the nociceptive endings partially by gating the TRPV1 cation channels through intracellular pathways (Garcia-Martinez et al., 2002). TRPV1 is now considered as an integrator target molecule for a variety of noxious stimuli (Tominaga et al., 1998). This conclusion is in accordance with early observation on capsaicin-pretreated (desensitized) rats, since in these animals the characteristic feature is the selective analgesia to chemonociceptive stimuli (Szolcsanyi, 1993).

Several results indicate that sensory innervation of the joint by capsaicin-sensitive, TRPV1-expressing primary afferent neurons is not only involved in sensory input for stretch and pain but also these nerve fibers exert local and systemic effector functions (Maggi, 1995; Szolcsanyi, 1996). Neuropeptides released from these fibers into the surrounding tissues elicit neurogenic inflammation around the site of activation. Calcitonin gene-related peptide (CGRP) induces local vasodilatation and tachykinins, such as substance P (SP), evoke plasma protein extravasation in the innervated area (Maggi, 1995; Szolcsanyi, 1996). Furthermore, they have a trophic role, and they cooperate with the immune system (Ferrell and Lam, 1996). SP releases inflammatory mediators from mast cells and induces the secretion of prostaglandin E₂ and collagenase from synoviocytes and interleukin-1 from macrophages (Lam and Ferrell, 1991). These mechanisms are also implicated in chronic inflammatory reactions, such as rheumatoid arthritis (Jorgensen and Sany, 1994), which is a major problem in medicine. Arthritic joint characteristically displays hyperplasia of the synovial tissue contributing to pannus formation, mononuclear cell infiltration, and destruction of cartilage and subchondral bone. Spontaneous pain and allodynia elicited by activation and/or sensitization of nociceptors by inflammatory mediators such as bradykinin, lipoxygenase enzyme products, prostaglandins, and protons are also predominant symptoms of all forms of human arthritic diseases. Increased level of proinflammatory sensory neuropeptides has been demonstrated in the synovial fluid taken from patients with rheumatoid arthritis (Marabini et al., 1991) and also from arthritic experimental animals (Bileviciute et al., 1993). It is suggested that SP and CGRP constitute the most important group of neurogenic mediators of the inflammatory process and also participate in the nociceptive pathway.

Participation of the TRPV1 receptor in acute inflammatory and nociceptive models has been investigated. In TRPV1-null mutant mice, neurogenic inflammation induced by mustard oil (Banvolgyi et al., 2004), edema evoked by carrageenin, or mechanical hyperalgesia 1 day after the injection of complete Freund's adjuvant into the hindpaw were similar to the TRPV1^{+/+} controls (Caterina et al., 2000). On the other hand, hyperalgesia to noxious heat in these acute inflammatory conditions was markedly inhibited or abolished in the TRPV1 receptor knockout mice (Davis et al., 2000). However,

there are no data on the role of this receptor under long-term inflammatory conditions.

Therefore, the aim of the present study was to adapt adjuvant-induced arthritis originally developed in Lewis rats to mice and to analyze the development of chronic inflammation and related mechanical hyperalgesia in animals lacking the TRPV1 receptor. Further experiments were designed to reveal the role of cyclooxygenase/lipoxygenase enzyme products and bradykinin in the activation or sensitization of the TRPV1 receptor in the present model using enzyme inhibitors and receptor antagonists. It was intended to shed light on the participation of TRPV1 in the effects of inflammatory mediators released during long-term systemic arthritis.

Materials and Methods

Animals. Experiments were performed on male TRPV1 receptor gene knockout mice (TRPV1^{-/-}) and their wild-type counterparts (TRPV1^{+/+}) weighing 20 to 25 g. Two breeding pairs of TRPV1^{+/-} heterozygote mice were generous gifts from Dr. J. B. Davis (Glaxo-SmithKline, Harlow, UK). The genotype of the animals in the first generation was determined by Southern blot analysis and polymerase chain reaction. The TRPV1^{+/+} and TRPV1^{-/-} were successfully bred as wild-type and knockout mice in the Laboratory Animal Centre of the University of Pécs, from where they were obtained for the experiments, under standard pathogen-free conditions at 24–25°C. Mice were provided with standard chow and water ad libitum.

Generation of TRPV1 Receptor Knockout Mice. The generation of TRPV1 receptor knockout mice was by homologous recombination in embryonic stem cells (129 ES) to generate a mouse lacking transmembrane domains 2 to 4 of the mTRPV1 gene. Germ-line chimeras were crossed onto C57BL/6 females to generate heterozygotes, which were intercrossed giving rise to healthy homozygous mutant offspring in the expected Mendelian ratio, as described by Davis et al. (2000). After genotyping by PCR, they were bred from homozygous knockout breeding pairs, so all offspring were also homozygous knockouts (TRPV1^{-/-}).

Induction of Arthritis. Arthritis of the left tibiotarsal joint of the mice (20–25 g; *n* = 8–12) was evoked by s.c. injection of complete Freund's adjuvant (CFA; killed mycobacteria suspended in paraffin oil; 0.05 ml, 1 mg/ml) into the plantar surface of the left hindpaw and the root of the tail. To enhance systemic effects, an additional injection was given into the tail on the following day (Helyes et al., 2004).

Measurement of Paw Edema and Mechanonociceptive Threshold. The volume of the paws was measured by plethysmometry (plethysmometer 7140; Ugo Basile, Comerio, Italy) and the mechanical touch sensitivity of the paws by aesthesiometry (Dynamic plantar aesthesiometer 37400; Ugo Basile) before the experiment and 2, 5, 8, 12, 15, 18, and 21 days after CFA administration.

The plethysmometer consists of two vertical interconnected water-filled Perspex cells, the larger of which is used to measure volume displacement induced by immersion of the mouse paw. The water level in an interconnected smaller tube, which contains a force transducer, generates a proportional volume measurement of the mouse paw, which is expressed in cubic centimeters.

The aesthesiometer is used to assess touch sensitivity on the plantar surface of the paw. The mice move about freely in one of the two compartments of the enclosure positioned on the metal mesh surface. After acclimation and cessation of exploratory behavior, the operator placed the touch stimulator unit under the animal's paw, using the adjustable angled mirror to position the filament below the target area of the paw. After pressing the start key, an electrodynamic actuator of proprietary design lifts a straight metal filament, which touches the plantar surface and begins to exert an increasing upward force at a preset rate of application until a stop signal (when the animal removes the paw) is attained. The paw withdrawal

threshold is numerically shown in grams on the digital screen. Edema and hyperalgesia were expressed as percentage of initial control values.

Histological Processing. The left tibiotarsal joints were excised after killing the animals by pentobarbital sodium (Nembutal) overdose (100 mg/kg i.p.) on the 18th day after CFA administration. The specimens were fixed in 4% formaldehyde for 8 h, decalcified in a demineralizing solution containing 7% (w/v) AlCl₃, 5% (v/v) formic acid, and 8.5% (v/v) HCl for 8 h at 4°C (Helyes et al., 2004). Noninflamed joints decalcified in parallel at the same time were used as control samples to make sure that the decalcination procedure was appropriate. Once the joints had become sufficiently soft, they were washed in Sörensen phosphate buffer. They were dehydrated at 4°C for 8 h in 5% (w/v) in saccharose followed by immersion into 10 and 15% (w/v) saccharose for subsequent two periods of 8 h. Then, the samples were embedded in paraffin, sectioned with microtome (5 mm), and stained with hematoxylin and eosin.

Assessment of Joint Inflammation. Arthritic changes were scored by an observer blinded to the treatment the animals received using a grading scale of 0 to 3 according to the proportion of areolar tissue that was densely infiltrated with mononuclear cells. Synovial lining cell hyperplasia and the number of leukocytes observed in the synovial tissue were graded similarly on the same scale. Cartilage destruction was scored on a scale of 0 to 3, ranging from no damage to fully destroyed cartilage layers. Bone erosion scores were obtained for the following features: 0, normal; 1, mild loss of cortical bone at a few sites; 2, moderate loss of cortical trabecular bone; and 3, marked loss of bone at many sites. The score values given for these four different histopathological features were added to generate a composite arthritis score ranging between 0 and 12 (Helyes et al., 2004). From every joint (six to eight mice in each group), four or five sections were taken from different depths to give a representative appreciation of the whole joint. Mean scores were determined from the different sections of the individual animals, and composite score values of the different experimental groups were calculated from these mean scores.

Drug Treatments. For examining the role of lipoxygenase products in the activation of TRPV1 receptors under long-term inflammatory conditions, one group of animals was treated every day throughout the whole 18-day period with nordihydroguaric acid (NDGA; 25 mg/kg i.p.; Chacur et al., 2001), a nonselective lipoxygenase inhibitor. In the second group, the bradykinin B1 receptor antagonist desArgHOE-140 (250 µg/kg i.p.; Ferreira et al., 2001), in the third one the B2 receptor antagonist HOE-140 (250 µg/kg i.p.; Wirth et al., 1991) was administered daily. Mice in the fourth group received daily treatment of indomethacin (1 mg/kg i.p.; Gauldie et al., 2004), the nonselective cyclooxygenase enzyme inhibitor. There were 6 to 12 animals in each experimental group.

Ethics. All experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988) and complied with the recommendations of the International Association for the Study of Pain and the Helsinki Declaration. The studies were approved by the Ethics Committee on Animal Research of Pécs University according to the Ethical Codex of Animal Experiments and license was given (license no. BA 02/200-6-2001).

Statistical Analysis. For determining statistically significant differences between the results of paw edema and hyperalgesia measurements of different groups, two-way analysis of variance (ANOVA) followed by Bonferroni's modified *t* test was used. Analysis of the composite arthritis score was performed by nonparametric Mann-Whitney *U* test. In both cases, *p* < 0.05 was considered significant.

Drugs and Chemicals. Complete Freund's adjuvant, desArgHOE-140, HOE-140, NDGA, and indomethacin [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid] were purchased from Sigma-Aldrich (St. Louis, MO). Nembutal was from Serva (Heidelberg,

Germany). Indomethacin was dissolved in 5% NaHCO₃ and diluted with sterile saline. Stock solutions of desArgHOE-140, HOE-140, and NDGA were made with 96% ethanol. The final solution contained 10% of this stock solution, 5% Tween 80, and 85% isotonic saline.

Results

The weights of the animals remained unchanged over the period of the study with no significant differences between the TRPV1^{+/+} and TRPV1^{-/-} groups.

CFA-Induced Paw Edema, Mechanical Hyperalgesia, and Arthritic Histological Changes in TRPV1^{+/+} and TRPV1^{-/-} Mice. One day after CFA injection, neither edema (TRPV1^{+/+}, 46.38 ± 4.22; TRPV1^{-/-}, 36.69 ± 3.91%; *p* = 0.06), nor mechanical hyperalgesia (TRPV1^{+/+}, 31.24 ± 4.52%; TRPV1^{-/-}, 26.86 ± 3.68; *p* = 0.18) differed significantly in the two groups. In TRPV1^{+/+} mice, the volume of the CFA-injected paw gradually increased, reaching a maximal swelling of 130% 16 days after the induction of inflammation, whereas 30% edema developed on the contralateral paw. In TRPV1^{-/-} mice, edema of both the CFA-treated and the contralateral paws was significantly smaller, with a maximum of 80 and 15%, respectively, throughout the 18-day experimental period (*p* < 0.05; two-way ANOVA; Fig. 1). In TRPV1^{+/+} animals, mechanical touch sensitivity threshold of the CFA-treated paws decreased by 45 to 50% and on the contralateral paw 10 to 15% hyperalgesia developed. In TRPV1^{-/-} mice mechanonociceptive threshold drop of the CFA-treated paw was only 30 to 35% and no hyperalgesia developed on the contralateral side (*p* < 0.05; two-way ANOVA; Fig. 2).

Compared with the normal joint structure (Fig. 3a), the left tibiotarsal joints of CFA-treated TRPV1^{+/+} mice were damaged by expanding synovial pannus. Widening of the synovial cavity, mononuclear cell infiltration, thickening of the synovial membrane, disruption of the cartilaginous tissue, and massive bone damage were apparent (Fig. 3b). Joint samples of TRPV1^{-/-} mice showed moderate inflammatory changes, there was small cartilage erosion, bone damage was not seen, enlargement and mononuclear cell infiltration of the synovial tissue was less pronounced than in TRPV1^{+/+} animals (Fig. 3, c and d). There was no prominent difference between the additive scores given for synovial swelling/cellular infiltration; it was 4.57 ± 0.67 in the TRPV1^{+/+} and 3.17 ± 0.88 in the TRPV1^{-/-} group. On the other hand, cartilage/bone destruction was minimal in mice lacking the TRPV1 receptor, the scores for these two parameters were 3.16 ± 0.78 and 1.41 ± 0.61 in TRPV1^{+/+} and TRPV1^{-/-} animals, respectively. Composite arthritis score calculated by adding the values for all these four parameters was significantly smaller in TRPV1^{-/-} mice (4.6 ± 1.2; *n* = 8) than in TRPV1^{+/+} animals (7.8 ± 1.1; *n* = 8) (Fig. 6).

Effect of desArgHOE-140, HOE-140, Indomethacin, and NDGA on Paw Edema, Mechanical Hyperalgesia, and Arthritic Histological Changes in TRPV1^{+/+} Mice. In TRPV1^{+/+} mice, daily i.p. administration of the bradykinin B1 receptor antagonist desArgHOE-140 (250 µg/kg) did not influence significantly either CFA-induced paw swelling or mechanical hyperalgesia, and it had no action on the composite arthritis score. On the other hand, the B2 receptor antagonist HOE-140 (250 µg/kg) induced 30 to 40% inhibi-

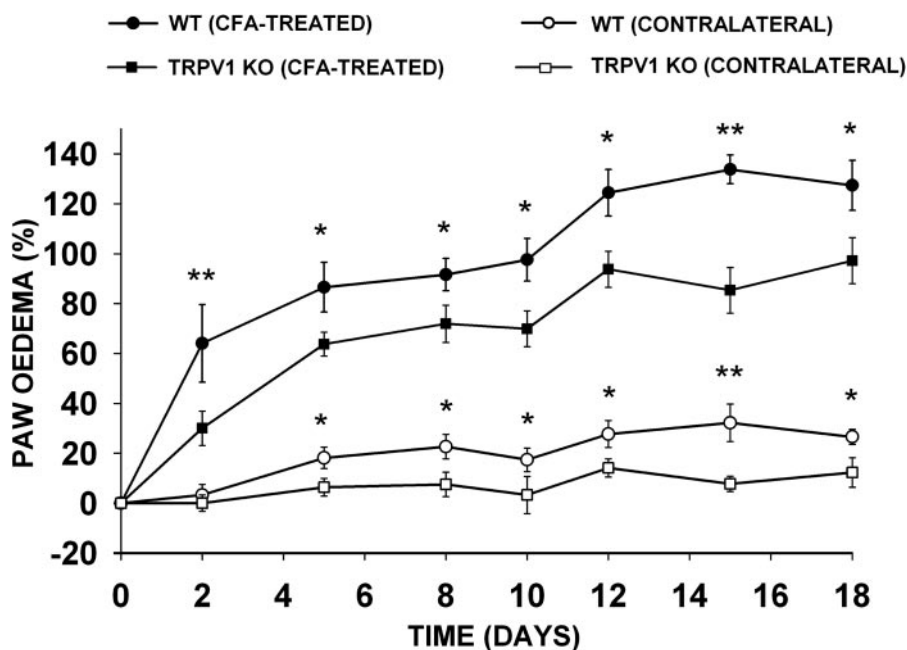


Fig. 1. Paw edema of TRPV1^{+/+} (WT) and TRPV1^{-/-} (knockout; KO) mice induced by CFA injection throughout the 18-day experimental period. Results are expressed as percentage of swelling compared with the initial paw volumes and shown as means \pm S.E.M. of $n = 12$ to 15 mice/group. ANOVA followed by Bonferroni's modified t test was used to determine statistically significant differences, *, $p < 0.05$; **, $p < 0.01$ WT versus KO.

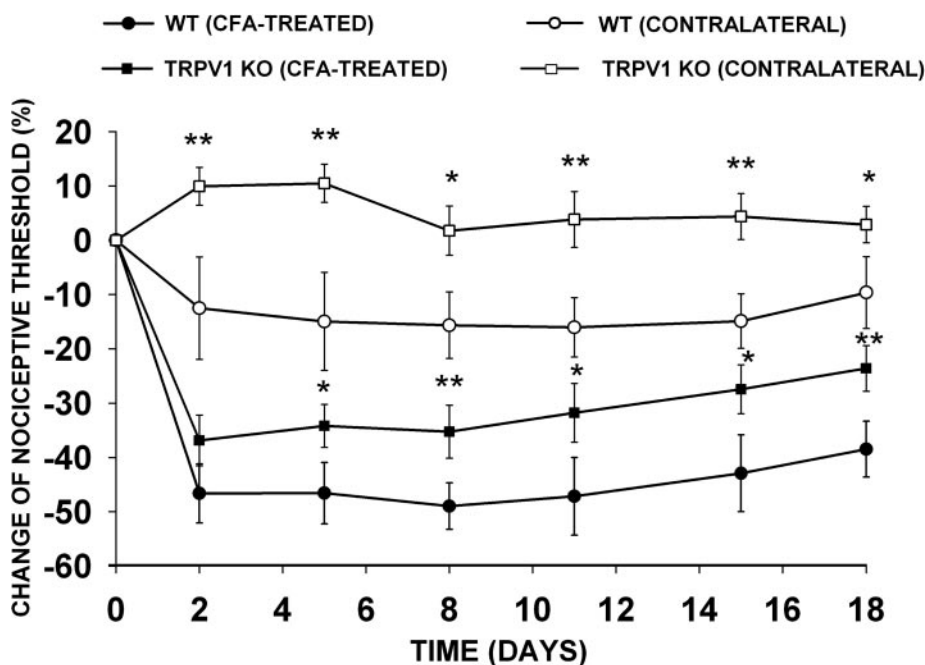


Fig. 2. Mechanical hyperalgesia of the paw of TRPV1^{+/+} (WT) and TRPV1^{-/-} (knockout; KO) mice induced by CFA injection throughout the 18-day experimental period. Results are expressed as percentage of change of touch sensitivity compared with the initial paw values and shown as means \pm S.E.M. of $n = 12$ to 15 mice/group. ANOVA followed by Bonferroni's modified t test was used to determine statistically significant differences, *, $p < 0.05$; **, $p < 0.01$ WT versus KO.

tion of edema and hyperalgesia throughout the whole experimental period of 18 days and decreased histological score by 55%. The nonselective lipoxygenase enzyme inhibitor NDGA (25 mg/kg) caused 40 to 50% inhibition of inflammatory hyperalgesia, and the cyclooxygenase enzyme inhibitor indomethacin (1 mg/kg) elicited even greater, 60 to 80% inhibitory action. These agents decreased edema by 25 to 30% and 40 to 55% and arthritis score by 40 and 65%, respectively ($p < 0.05$; two-way ANOVA; Fig. 4, a and b; Fig. 6).

Effect of HOE-140, Indomethacin and NDGA on Paw Edema, Mechanical Hyperalgesia, and Arthritic Histological Changes in TRPV1^{-/-} Mice. In TRPV1^{-/-} mice, NDGA had no effect on any of the CFA-induced inflammatory symptoms. Inhibition of paw edema induced by HOE-140 was only significant on the day 18 (19.76%), whereas it

exerted no significant action on mechanical hyperalgesia. These drugs did not notably change the arthritis score either. Indomethacin decreased hyperalgesia by 50 to 60%, edema by 20 to 30%, and arthritis score by 40%, respectively ($p < 0.05$; two-way ANOVA; Fig. 5, a and b; Fig. 6). It is worthy to mention, that in contrast to TRPV1^{+/+} animals indomethacin in TRPV1^{-/-} mice did not decrease edema formation in the early stage of CFA-induced arthritis. The first significant inhibition, 30.07%, was detected on the day 11, and on the second and third weeks the inhibitory effect of indomethacin on paw swelling in TRPV1^{-/-} mice was about one-half of the action observed in TRPV1^{+/+} animals. On the contrary, in both groups of mice mechanical hyperalgesia was similarly diminished by indomethacin throughout the whole experimental period,

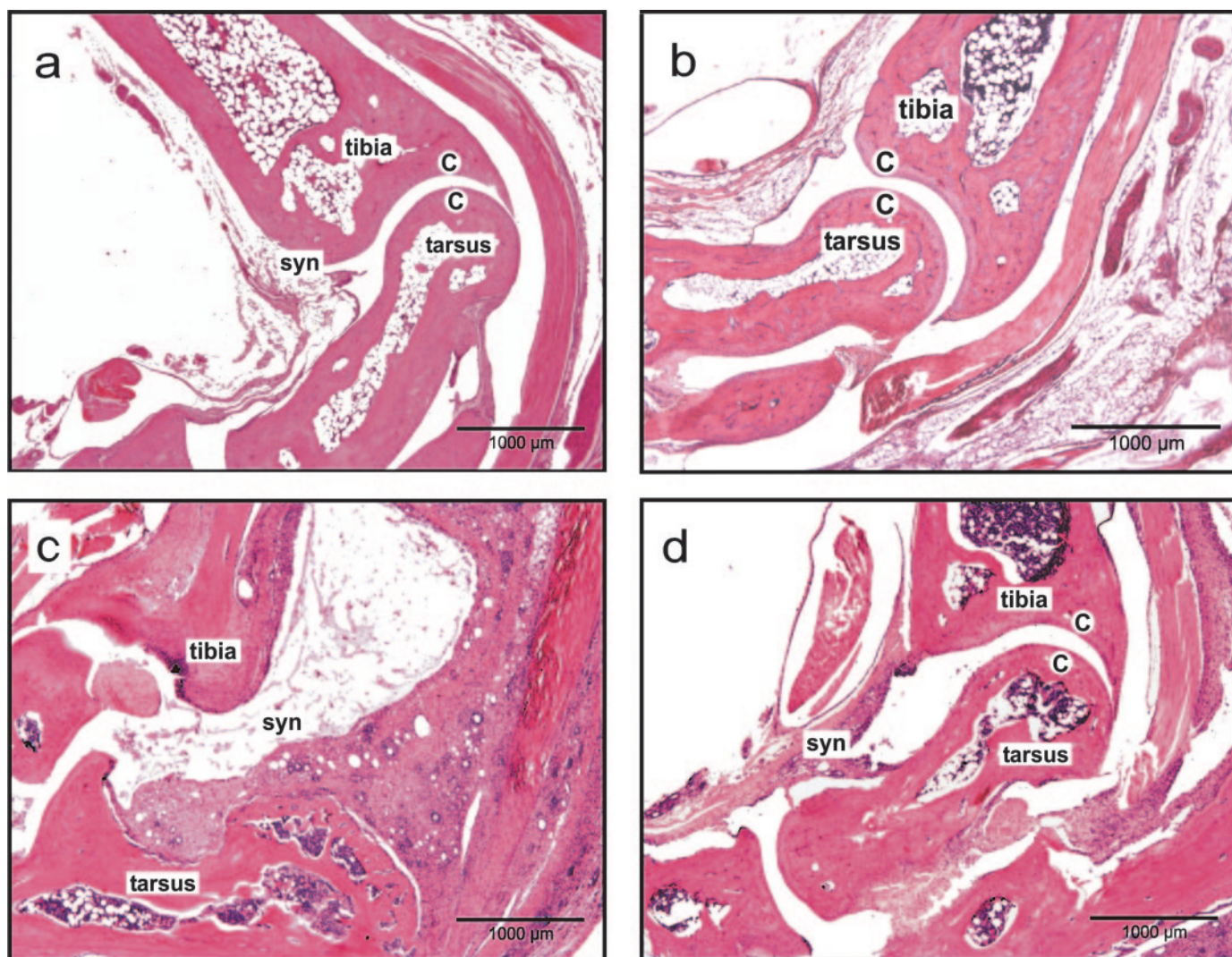


Fig. 3. Histological samples of the left tibiotarsal joints stained with hematoxylin and eosin taken from an untreated TRPV1^{+/+} (WT) (a), an untreated TRPV1^{-/-} (knockout; KO) mouse (b), a CFA-treated WT mouse (c), and a CFA-injected TRPV1^{-/-} mouse (d). Magnification, 40× in all micrographs. Syn, synovial tissue; C, cartilage; the tibia and the tarsus are indicated accordingly. Note that in the joint of the TRPV1^{+/+} mouse the articular cavity is markedly larger, the extensively expanding synovial pannus is more densely infiltrated with mononuclear cells, cartilage erosion is more pronounced, and there is a massive bone destruction by the invading synovium.

although the percentage inhibition values were always higher in TRPV1^{+/+} mice.

Discussion

The present data revealed that adjuvant-induced arthritis originally developed in Lewis rats (Helyes et al., 2004) can be adapted to mice. The other commonly used experimental model, collagen-induced arthritis, which can be used in CD1 or BALB/c mice, does not work in the C57BL/6 strain due to different responsiveness of these animals for the antigen challenge (Lariviere et al., 2001). Since transgenic mice are usually constructed from the C57BL/6 strain, this is a suitable experimental model for the examination of long-term joint inflammation in genetically manipulated animals. The significance of this technique in studying chronic inflammation in gene-deficient mice can be emphasized by the fact that the presently available data on TRPV1 knockout animals were obtained exclusively in acute models (Caterina et al., 2000; Davis et al., 2000; Banvolgyi et al., 2004). Mustard

oil-induced acute neurogenic inflammation (Banvolgyi et al., 2004) and carrageenin-evoked (Davis et al., 2000) and mechanical hyperalgesia 1 day after the CFA injection into the hindpaw did not differ in TRPV1^{-/-} and TRPV1^{+/+} mice (Caterina et al., 2000). These results are supported by our findings, since we also found no significant difference between these groups 24 h after the induction of inflammation. On the other hand, as arthritis proceeded, in TRPV1 knockout mice CFA-induced edema was significantly smaller from the second day and mechanical hyperalgesia from the fifth day compared with wild-type (WT) animals. Therefore, it seems that TRPV1 activation plays an important role in the development of the chronic but not the acute phase of adjuvant arthritis.

Capsaicin-sensitive peptidergic afferent fibers are sensitized in arthritis, and these activated nerve endings release both proinflammatory (tachykinins and CGRP) and anti-inflammatory neuropeptides (somatostatin) (Szolcsanyi et al., 1998a,b). Systemic resiniferatoxin pretreatment has been shown to increase CFA-induced paw edema, histological

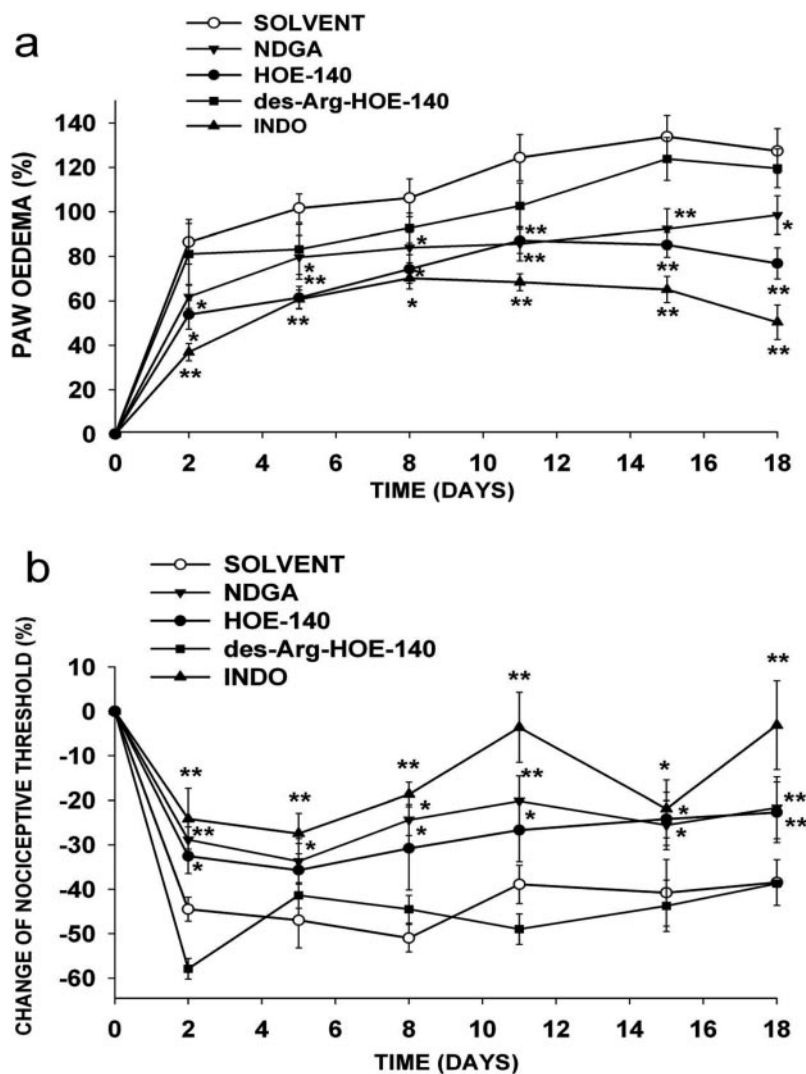


Fig. 4. Effect of daily administration of the bradykinin B1 receptor antagonist desArgHOE-140 (250 $\mu\text{g}/\text{kg}$), the B2 antagonist HOE-140 (250 $\mu\text{g}/\text{kg}$), the cyclooxygenase enzyme inhibitor NDGA (25 mg/kg) on CFA-induced paw edema (a) and mechanical hyperalgesia (b) in TRPV1^{+/+} (WT) mice. Results are means \pm S.E.M. of $n = 6$ to 8 mice/group. ANOVA followed by Bonferroni's modified t test was used to determine statistically significant differences, *, $p < 0.05$; **, $p < 0.01$ versus solvent-treated control group.

changes, and related inflammatory hyperalgesia in rats (Helyes et al., 2004). This pretreatment, however, does not induce a selective loss of TRPV1 cation channels; they act by damaging the whole TRPV1-expressing capsaicin-sensitive nerve ending. Thus, the present results provide the first definite evidence for the regulatory role of TRPV1 receptors in the long-term inflammatory and hyperalgesic symptoms of the adjuvant arthritis model. Several mediators, such as bradykinin, prostaglandins, protons, histamine, serotonin, nerve growth factor (Ferrell and Lam, 1996; Szolcsanyi et al., 1998a), and endocannabinoids (Gauldie et al., 2001; Baker and McDougall, 2004) released during arthritis have been shown to activate or sensitize the capsaicin-sensitive nerve endings. TRPV1 activation induces the release of inflammatory neuropeptides such as substance P and CGRP, which evoke local plasma protein extravasation, arteriolar vasodilatation (Lam and Ferrell, 1991, 1993), and stimulate inflammatory cells in the joint. These peptides have also been shown to evoke cytokine secretion from Th0, Th1, and Th2 antigen-specific T cells (Levite and Chowder, 2001), enhance tumor necrosis factor- α production of monocytes, induce generation of interleukin-1 in macrophages (Brunelleschi et al., 1998), and potentiate the proliferation, enzyme secretion, and adhesion molecule expression of fibroblast-like synovio-

cytes (Lambert et al., 1998). Therefore, it is suggested that leukocytes and fibroblasts/synovial cells are at the interface between the immune and the nervous system during long-term inflammation (Lambert et al., 1998). On the other hand, other neuropeptides, such as somatostatin (Helyes et al., 2004) or endomorphin-1 (McDougall et al., 2004) have been found to be anti-inflammatory in the joint.

The lipoxygenase inhibitor NDGA markedly diminished paw edema, hyperalgesia, and histological damage in TRPV1^{+/+} mice, but it had no effect in TRPV1^{-/-} animals. It suggests that lipoxygenase products, e.g., 12-(*S*)-hydroxyperoxyeicosatetraenoic acid, synthesized in a great amount in the inflamed tissues activate TRPV1. They bind to the same site as capsaicin located in the cytosolic domain of the receptor, and on this ground, it has been suggested that this lipid might be the endogenous ligand for TRPV1 (Hwang et al., 2000).

Bradykinin, a nonapeptide released in the inflamed tissues, causes pain and hyperalgesia. It is known to activate as well as sensitize sensory neurons to other stimuli (Shin et al., 2002). We found the bradykinin B1 receptor antagonist des-ArgHOE-140 was ineffective in TRPV1^{+/+} mice in the CFA-induced arthritis model. On the other hand, the B2 receptor antagonist HOE-140 induced significant inhibitory action in

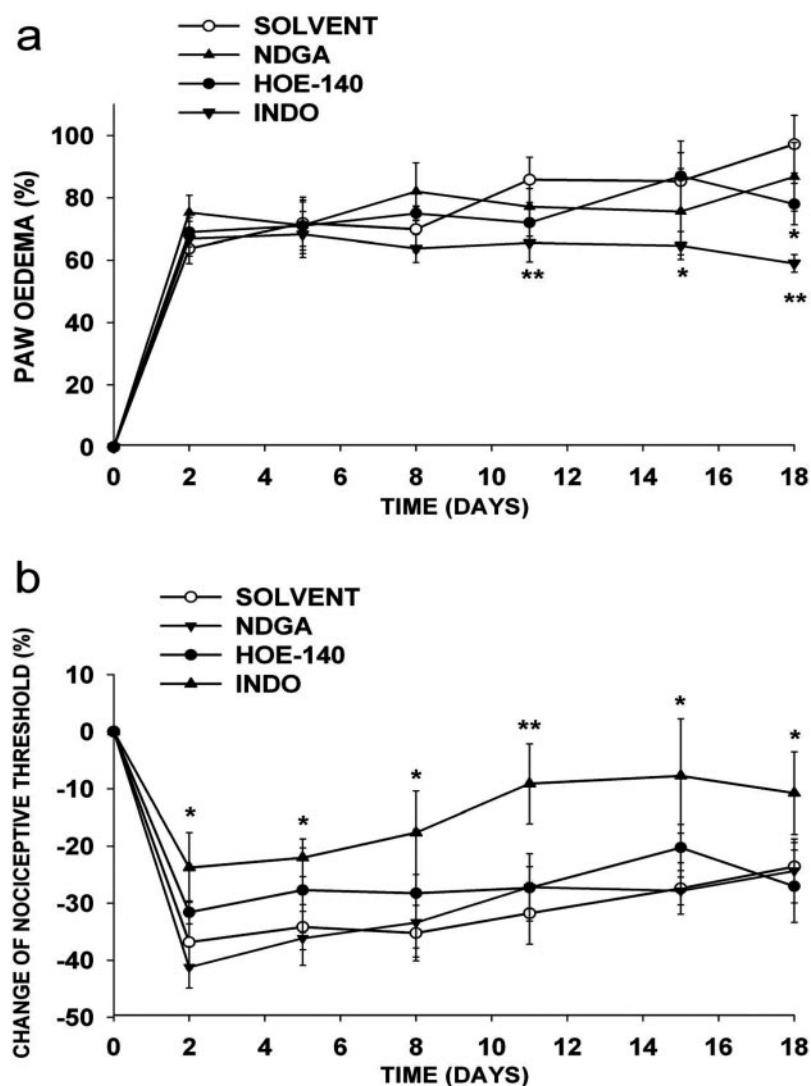


Fig. 5. Effect of daily administration of the bradykinin B1 receptor antagonist desArgHOE-140, the B2 antagonist HOE-140, the cyclooxygenase enzyme inhibitor INDO, and the lipoxygenase enzyme inhibitor NDGA on CFA-induced paw edema (a) and mechanical hyperalgesia (b) in TRPV1 gene-deficient (KO) mice. Results are means \pm S.E.M. of $n = 6$ to 8 mice/group. ANOVA followed by Bonferroni's modified t test was used to determine statistically significant differences, *, $p < 0.05$; **, $p < 0.01$ versus solvent-treated control group.

TRPV1^{+/+} animals, but not in the knockouts. Up-regulation of B2 receptors in joint diseases has been suggested by immunolocalization studies (Cassim et al., 1997). There are data demonstrating that this receptor subtype plays role in the sensitization of nociceptors through the protein kinase C pathway, which regulates the sensitivity of TRPV1 (Sugiura et al., 2002). Furthermore, bradykinin via B2 receptors also activates TRPV1 by generating lipoxygenase products (Hwang et al., 2000). Several results suggest that lipoxygenase products are involved in the bradykinin-induced biological effects (Hwang et al., 2000).

The cyclooxygenase enzyme inhibitor indomethacin significantly diminished arthritis and related hyperalgesia in both TRPV1^{+/+} and TRPV1^{-/-} mice, but the percentage of inhibition compared with the respective solvent-treated control groups was smaller in the TRPV1 receptor gene-deficient animals. A large body of literature describes the role of prostaglandins in inflammation (Sabata et al., 1986) and increased production of prostaglandins and thromboxane A₂ was found in the synovial fluid of arthritic experimental animals (Sabata et al., 1986) and patients (Basu et al., 2001), which was inhibited by indomethacin. Nonsteroidal anti-inflammatory agents exert direct inhibitory actions on vasodilatation, plasma protein extravasation, leukocyte functions

and also Th1 and to a lesser extent Th2 immune responses (Yamaki et al., 2003). On the other hand, prostaglandin E₂ and I₂ have been reported to act directly on capsaicin-sensitive nociceptors with cAMP as a second messenger to sensitize them to noxious stimulation (Hingtgen and Vasko, 1994). Furthermore, high concentrations of prostanoids directly evoke substance P and CGRP release from these neurons (Hingtgen and Vasko, 1994). These data can explain the greater inhibitory action of indomethacin in TRPV1^{+/+} animals. The present results support the conclusion that TRPV1 on capsaicin-sensitive nerve endings play an important role in the mediation of prostanoids in CFA-induced arthritis, particularly in its early stage, whereas the prostanoid-induced hyperalgesia is only slightly affected by sensitization of the TRPV1 receptors.

There is strong evidence that not only the sensitivity but also the density of the expression of TRPV1 is enhanced during inflammatory conditions. The proportion of TRPV1-labeled unmyelinated axons in the digital nerve significantly increases after CFA-induced chronic inflammation in the rat (Carlton and Coggeshall, 2001). These results raise the possibility that up-regulation of TRPV1 at the peripheral nerve terminals is involved in the increased sensitivity of this receptor to several stimuli.

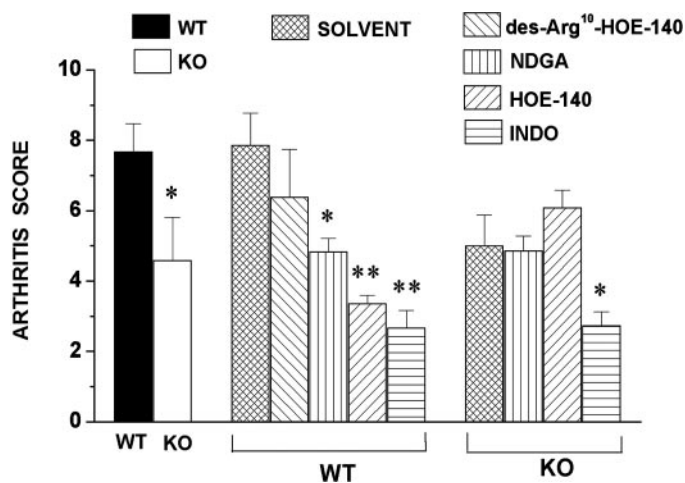


Fig. 6. Histological scores. Arthritic changes such as inflammatory cell infiltration, synovial proliferation/enlargement, articular cartilage breakdown, and bone destruction were graded from 0 to 3 and added. Results are means \pm S.E.M. of $n = 6$ to 8 samples/group. Nonparametric Mann-Whitney U test was used to determine statistically significant differences, *, $p < 0.05$; **, $p < 0.01$.

It has previously been suggested that C-polymodal nociceptors and the TRPV1 membrane protein localized on these nerve endings are integrators of noxious chemical stimuli to signal pain under inflammatory conditions (Szolcsányi, 1993; Tominaga et al., 1998). In the present study, we provided evidence that activation/sensitization of the TRPV1 receptor by lipoxygenase products, bradykinin, and prostaglandins (“inflammatory mixture”) enhances adjuvant-induced arthritis and related hyperalgesia in the mouse. Furthermore, although bradykinin and lipoxygenase products seem to act exclusively via TRPV1 activation, prostanoids do not, or at least partially. Based on these findings, TRPV1 channel blocking agents, endogenous ligand antagonists, or their release inhibitors as lipoxygenase blocking agents all could effectively inhibit inflammatory processes and hyperalgesia. Therefore, they might provide promising novel perspectives on drug development for the treatment of arthritis and other inflammatory diseases.

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