Osteoarthritic Mice Exhibit Enhanced Prostaglandin E_2 and Unchanged Calcitonin Gene-Related Peptide Release in a Novel Isolated Knee Joint Model

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ABSTRACT. ***Objective.*** Osteoarthritis (OA) is a painful degenerative joint disease. To assess joint nociceptor activation indirectly, we used a novel *in vitro* knee joint preparation and determined the release of calcitonin gene-related peptide (CGRP) and prostaglandin E_2 (PGE_2) in osteoarthritic mice.

Methods. We studied STR/1N mice, which spontaneously develop OA, along with CD-1 mice as controls and C57/Bl6 mice with unilateral collagenase-induced OA and C57/Bl6 control mice. The release of CGRP and PGE_2 from tibial and femoral joint preparations was determined separately *in vitro* with enzyme immunoassays; we investigated both basal release and release induced by stimulation with capsaicin (CAP, 1 µM) or bradykinin (BK, 10 µM).

Results. Basal PGE_2 release from femoral and tibial preparations increased by 79% and 97%, respectively, in STR/1N mice between 6 and 18 weeks of age when they developed OA, while age-matched CD-1 mice exhibited only a weak increase (23%). BK-evoked PGE_2 release was significantly higher in 18-week-old STR/1N mice (931 ± 98 pg/ml and 759 ± 82 pg/ml from femoral and tibial preparations, respectively) than in age-matched CD-1 controls (236 ± 38 pg/ml and 246 ± 34 pg/ml). CAP stimulation induced a significant CGRP release, which, however, did not correlate with the temporal development of OA in STR/1N mice. Tibial but not femoral joint preparations from mice with collagenase-induced OA exhibited a significantly enhanced release upon BK stimulation compared to sham controls, while CAP-induced CGRP release did not reveal such difference.

Conclusion. Basal and evoked PGE_2 release from knee joint preparations rose while osteoarthritic alterations developed, whereas CGRP release remained unaltered. The increased PGE_2 release may contribute to enhanced nociceptor sensitivity underlying chronic OA pain. (J Rheumatol 2004; 31:2013–20)

Key Indexing Terms:
OSTEOARTHRITIS PAIN CALCITONIN GENE-RELATED PEPTIDE RELEASE
PROSTAGLANDIN E_2 RELEASE MOUSE KNEE JOINT MOUSE STRAINS

Osteoarthritis (OA) is a slowly progressive monoarticular disorder often leading to disability. It is the most common musculoskeletal disorder, and affects more than 80% of the population aged 55 years and older[1,2]. The knee joint is affected most frequently, followed by hand, hip, shoulder, and spine[3]. The disease is characterized by loss of cartilage and bone formation at joint margins[4]. One principal symptom of OA is chronic pain; however, little is known about its underlying pathophysiological processes probably affecting numerous joint tissues, including osteophyte growth with stretching of periosteum, elevated intraosseous pressure, microfractures, ligament damage, capsular tension, meniscal injury, and synovitis. Localized inflammation may be present in OA[5-7], causing pain either by direct activation of primary afferent nociceptive fibers of the OA joint or by sensitizing them to mechanical or chemical stimuli.

We developed an experimental model that allows measurement of the release of calcitonin gene-related peptide (CGRP) and prostaglandin E_2 (PGE_2) from the isolated knee joint of the mouse with enzyme immunoassays. CGRP is synthesized in primary afferent nociceptive neurons and is released from peripheral terminals upon noxious stimulation, e.g., by capsaicin (CAP)[8]. To stimulate CGRP release from the isolated knee joint, we used CAP because it is known to selectively activate nociceptive neurons[9,10]. Thus, nociceptor activation can be assessed indirectly by measuring the release of CGRP upon CAP stimulation. Once released, CGRP is able to induce vasodilatation and to promote prostaglandin production, thereby contributing to inflammation[11]. For stimulation of PGE_2 release we applied bradykinin (BK), an important endogenous inflammatory mediator that has been shown to contribute to synovitis in human OA[12]. PGE_2 is well known for its sensitizing effect on knee joint nociceptors, as shown when applied intraarticularly in cats[13].

We analyzed 2 murine models of knee joint OA: the

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STR/1N mouse, a spontaneous OA model, and a collagenase-induced instability model of OA in C57/Bl6 mice. The STR/1N mice are genetically prone to develop a bilateral instability of the knee joint and a varus deformity of the hindlegs leading to OA lesions, predominantly in the medial tibial plateau. Male animals show a higher degree of OA and less variability. Structural signs of OA are pronounced at the age of 18 weeks, but absent or mild at 6 weeks[14-19]. The collagenase-induced OA model is based on weakening ligaments in the knee joint due to intraarticular injection of highly purified bacterial collagenase, leading to increased joint laxity, which then results in OA lesions preferentially in the medial compartments of the collagenase-injected knee joint within 6 weeks in C57Bl6 mice[20,21]. The resulting changes, such as cartilage damage, fibrosis, osteophyte formation, and sclerosis of subchondral bone, mimic those in human OA. CD-1 mice were used as controls for STR/1N mice since they are known to gain weight to a similar extent as STR/1N mice.

Our aim was to assess nociceptor activation indirectly by measuring the release of CGRP and PGE_2 from isolated knee joints of control mice in comparison to mice that have developed knee OA.

MATERIALS AND METHODS

Preparation. We analyzed male STR/1N mice (strain bred and maintained at Aventis Pharma, Frankfurt, Germany) and male CD-1 mice (Charles River, Sulzfeld, Germany) at 2 different ages, 6 and 18 weeks (Table 1). In addition, we planned to analyze 6 male STR/1N mice at the age of 1 year; however, when 4 animals were lost between 40 and 49 weeks of age, we decided to include the data from the remaining 2 animals at the age of 49 weeks. In a subgroup of C57/Bl6 mice, 6 µl of a highly purified bacterial collagenase from *Clostridium histiolyticum* (Sigma type VII) dissolved in sterile saline (0.9%) was injected into the right knee joint (0.8 U/µl) at the age of 12 weeks in order to induce structural signs of OA. The left knee joints of these mice were sham-injected with sterile saline (0.9%). At age 18 weeks these animals were used in the release experiment. Another group of 18-week-old C57/Bl6 mice were used as untreated controls.

After the animals were killed by exposure to carbon dioxide, both hind limbs were disarticulated at the hips and truncated just proximal to the ankle joints. Leaving initially the knee joint capsule unattached, the femur was freed from skin, muscle, and soft tissue. The same was done with the tibia. Then the capsule was cautiously opened, and collateral and cruciate ligaments were cut in such a way that the tibia remained connected with the menisci and the femur connected with the patella.

Sampling and stimulation. Each femoral and tibial preparation was washed 30 min in carbogen-gassed synthetic interstitial fluid (SIF, pH 7.4, 37°C) containing: 108 mM NaCl, 3.48 mM KCl, 3.5 mM $MgSO_4$, 26 mM $NaHCO_3$, 11.7 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 9.6 mM sodium gluconate, 5.55 mM glucose, and 7.6 mM sucrose[22]. After the washout period each preparation was placed for 5 min in each of 6 consecutive glass tubes mounted in a shaking bath (37°C). All tubes were filled with 200 µl of SIF except the 3rd tube, which contained either BK 10 µM (Sigma) or CAP 1 µM (Sigma), and the 6th tube, which contained BK 10 µM in addition. The stimulation solution was prepared by diluting a stock solution that consisted of either BSA/water (0.1%) for BK or absolute EtOH for CAP 1:1000 in SIF. In the glass tubes, the preparations were positioned upright so that all joint preparations were covered with fluid, whereas the proximal part of the femur and the cut end of the tibial diaphysis stuck out of the fluid.

Enzyme immunoassays. Details of the analytical methods have been published[23]. For synchronous determination of CGRP and PGE_2 the samples were divided in 2 aliquots, and one was mixed with 5-fold concentrated commercial CGRP enzyme immunoassay buffer (200 µl sample + 50 µl buffer). The buffer consisted of potassium phosphate (0.1 M), NaCl (0.15 M), 0.1% bovine serum albumin (g/g), 0.01% sodium azide (g/g), and a combination of peptidase inhibitors to prevent neuropeptide degradation (SPIbio, Massy, France). The enzyme immunoassays (Cayman Chemical, Ann Arbor, MI, USA; SPIbio) were run on 96-well plates using a microplate reader as described by suppliers of immunoassay kits (Dynex, Germany). In our hands the minimum detection limit was 5 pg/ml for the CGRP enzyme immunoassay and 30 pg/ml for the PGE_2 enzyme immunoassay. The intra- and interassay coefficients of variation with repeated measurements were 10–15%.

Statistical analysis. The amounts of CGRP and PGE_2 release were calculated as pg/ml of sample volume. Due to the small size of the preparations it was impossible to determine the surface of the joint tissue around the femoral or tibal condyles, so that the release values could not be referred to the surface of the incubated tissue. For each trial 6 successive data points (samples s1 to s6) were obtained for CGRP and PGE_2. CAP-evoked CGRP release was calculated as peak plus poststimulus values minus baseline values; BK-induced PGE_2 release was calculated as peak minus baseline value. Results from groups of identical experiments are given as means ± SEM; n refers to the number of animals or joint preparations, respectively. Statistical comparison within one series of experiments was made using the Wilcoxon matched-pairs test. Comparison between different groups of experiments was performed using the Mann-Whitney U-test. P values < 0.05 were considered significant and are indicated in the figures.

Table 1. Basal PGE_2 release from femoral and tibial joint preparations.

	STR/1N			CD-1		
	Basal PGE_2 Release, pg/ml		Body Weight, g	Basal PGE_2 Release, pg/ml		Body Weight, g
	Femur	Tibia		Femur	Tibia	
6 weeks (n)	367 ± 55 (12)	299 ± 58 (12)	23.7 ± 2.2 (6)	591 ± 51 (18)	358 ± 10 (18)	28.1 ± 1.3 (9)
18 weeks (n)	658 ± 64 (34)	589 ± 70 (34)	36.7 ± 1.0 (17)	727 ± 46 (14)	442 ± 33 (14)	42.4 ± 1.1 (7)
p	0.003	< 0.001	< 0.001	NS (0.062)	0.017	< 0.001
Percentage increase	79	97	55	23	23	51

Values are means ± SEM. N refers to the number of femoral or tibial joint preparations, or to the number of animals used, respectively. There were 2 femoral and tibial joint preparations per animal. Intrastrain comparisons between 6 and 18-week-old animals by U-test.

RESULTS

Basal release of PGE_2 in knee joint preparations of STR/1N mice. Basal PGE_2 release from both femoral and tibial preparations almost doubled between 6 and 18 weeks of age, when OA is developing in STR/1N mice[14-19] (Table 1). The release increased even further in STR/1N mice at age 49 weeks (776 ± 110 and 685 ± 60 pg/ml in femoral and tibial preparations, respectively; n = 4 each). On the other hand, control CD-1 mice exhibited only a small change of their basal PGE_2 release over time, which was significant only for tibial joint preparations (Table 1). Similarly, between 6 and 18 weeks, both strains gained weight by about 50% (Table 1); therefore weight gain alone can be excluded as the sole reason for the strong increase in basal PGE_2 release observed in the STR/1N mice.

Basal PGE_2 release from femoral preparations was significantly lower in young STR/1N mice compared to young CD-1 mice ($p < 0.01$, U-test; Table 1), whereas release from tibial preparations was similar in both groups of young mice ($p > 0.05$, U-test; Table 1). The 18-week-old STR/1N and CD-1 mice exhibited no significant differences in basal PGE_2 release from tibial and femoral preparations (Table 1).

Basal release of CGRP in knee joint preparations of STR/1N mice. In all mouse strains tested, basal CGRP release was around 10 pg/ml and thus was close to the detection limit of the CGRP enzyme immunoassay.

Evoked CGRP release in knee joint preparations of STR/1N mice. In 18-week-old STR/1N mice, the stimulation with CAP (1 µM) resulted in a pronounced CGRP release from femoral and tibial joint preparations ($p < 0.001$, Wilcoxon test; Figure 1A). The enhanced release declined slowly, reaching baseline values in sample 6. The subsequent stimulation with BK (10 µM) in sample 6 had no effect on the CGRP release. However, when stimulating with BK without a previous CAP stimulation in another group of 18-week-old STR/1N mice, a small but significant increase in CGRP release could be detected from both femoral and tibial joint preparations (8.9 ± 0.3 to 14.2 ± 2.4 and 9.5 ± 0.3 to 15.5 ± 2.1 pg/ml, respectively; $p < 0.04$, Wilcoxon test, n = 6). The 6-week-old mice showed a similar time course of stimulated CGRP release from joint tissue compared to 18-week-old animals (data not shown).

The extent of CGRP release evoked by CAP stimulation did not change in STR/1N mice between 6 and 18 weeks, when OA is developing, in either the femoral or the tibial joint preparations (Figure 2A). Control CD-1 mice also did not exhibit significant changes of CAP-evoked release with age (Figure 2A). Young as well as 18-week-old STR/1N mice delivered a higher mean peak CGRP release from femoral and tibial joint preparations than age-matched CD-1 mice; these differences were significant for young animals ($p < 0.04$, U-test; Figure 2A).

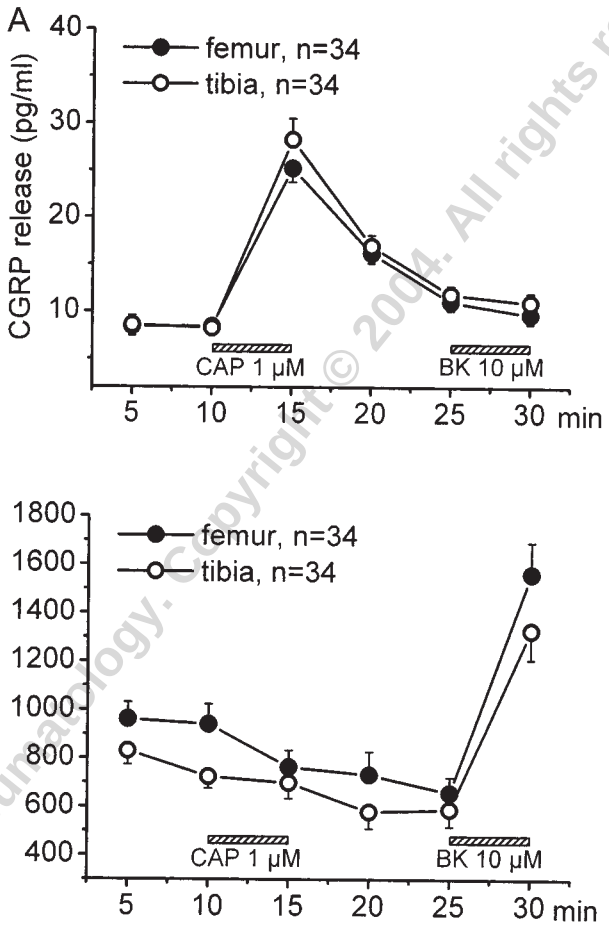

Figure 1. Release of CGRP (A) and PGE_2 (B) determined synchronously from femoral and tibial joint preparations of 18-week-old STR/1N mice. Data show mean release ± SEM of 34 femoral and 34 tibial joint preparations. Release was measured over 30 min and every 5 min one sample was collected. Joint preparations were stimulated first with capsaicin (CAP, 1 µM), resulting in an almost 3-fold increase in CGRP release in the 3rd sample (A) without inducing any detectable PGE_2 release (B). Subsequently, joint preparations were stimulated with bradykinin (BK, 10 µM), leading to a 2-fold increase in PGE_2 release in the 6th sample (B), whereas no CGRP release was induced (A).

Evoked PGE_2 release in knee joint preparations of STR/1N mice. Stimulation of joint preparations from STR/1N and CD-1 mice with BK (10 µM) evoked a significant increase in PGE_2 release over baseline ($p < 0.001$, Wilcoxon test). Between 6 and 18 weeks, the BK-evoked PGE_2 release increased significantly in tibial (+ 77%) and femoral (+ 89%) joint preparations from STR/1N mice; in contrast, in preparations from control CD-1 mice no such change was detected (–16% and +27%; nonsignificant, U-test; Figure 2B). The BK-evoked PGE_2 release increased even further with age in STR/1N mice (979 ± 96 pg/ml for femoral and 1153 ± 246 pg/ml for tibial preparations at the age of 49 weeks; n = 4 each). The BK-induced release from joint preparations was significantly more pronounced in STR/1N mice than in age-matched control CD-1 mice ($p < 0.02$, U-test; Figure 2B). Stimulation of joint preparations with CAP

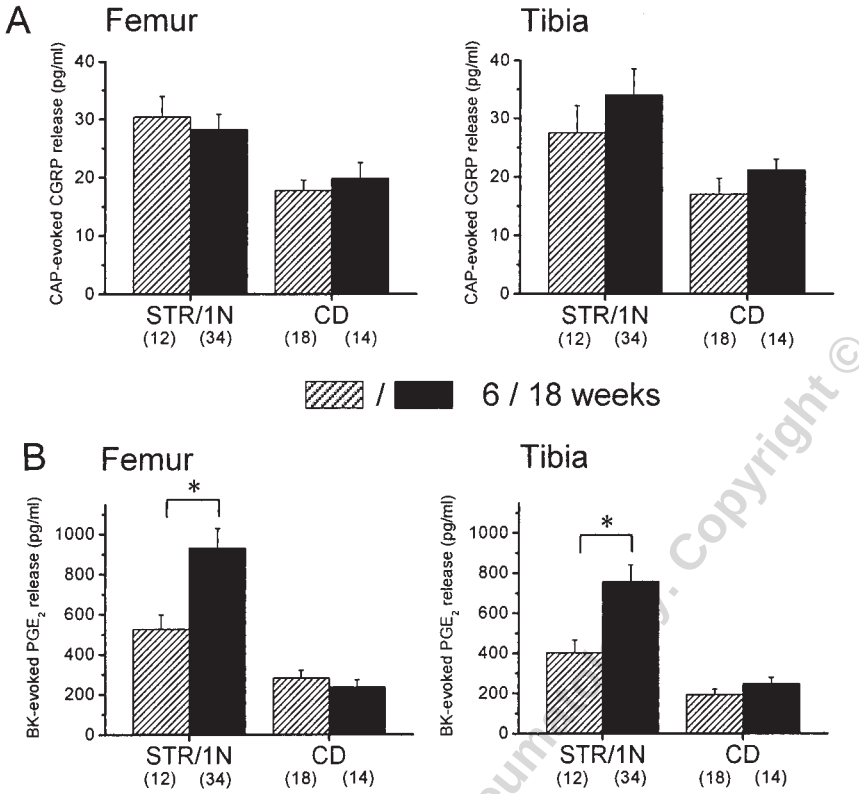

Figure 2. Capsaicin-evoked CGRP (A) and BK-evoked PGE_2 (B) release from joint preparations of 6- and 18-week-old STR/1N and CD-1 mice. CAP-evoked CGRP release was calculated as peak plus poststimulus values minus baseline values. BK-induced PGE_2 release was calculated as peak minus baseline value. Data are presented as mean ± SEM. Numbers in parentheses are numbers of femoral or tibial preparations. BK-evoked PGE_2 release rose significantly between 6 and 18 weeks in the OA STR/1N mouse strain ($p < 0.01$, U-test) whereas control CD-1 mice showed no age-related change in release.

(1 µM) did not induce a significant PGE_2 release over baseline.

Evoked CGRP and PGE_2 release in the collagenase-induced OA model. Additional knee joint preparations were studied from 18-week-old C57/Bl6 mice 6 weeks after a unilateral intraarticular collagenase injection. This period is sufficiently long to allow for development of structural signs of OA in C57Bl6 mice[20,21].

CAP induced a CGRP release from the femoral and tibial tissue preparations of collagenase-treated joints. There was, however, no significant difference in CAP-evoked CGRP release compared to comparable preparations obtained from sham-treated joints and from control joints (Figure 3A).

The BK-evoked PGE_2 release was significantly higher from tibial joint preparations of collagenase-treated joints compared to sham-treated and control joints ($p < 0.05$, U-test; Figure 3B). The BK-evoked PGE_2 release from femoral preparations did not exhibit such significant differences (Figure 3B).

DISCUSSION

Knee joint model to determine the release of CGRP and PGE_2. Pain sensations arising from joints are mediated in the first instance by primary afferent nerve fibers linking joint tissues with second-order neurons in the spinal cord. The vast majority (70–80%) of all knee joint afferents are unmyelinated, roughly half of them containing neuropeptides like CGRP and substance P[24-31]. The neuropeptides are released from the peripheral endings of the C-fiber nociceptors upon noxious stimulation[8]. Thus, nociceptor activity can be assessed indirectly by measuring the neuropeptide release from the peripheral nerve endings. We introduced a novel *in vitro* knee joint model that allows measurement of the release of CGRP from the isolated mouse knee joint. In addition to CGRP release, we assessed the release of PGE_2, an endogenous algogenic substance that very likely plays an important role in OA pain, as inferred from the known efficacy of cyclooxygenase inhibitors to reduce OA pain[32]. In our release experiment, joint preparations were exposed to the

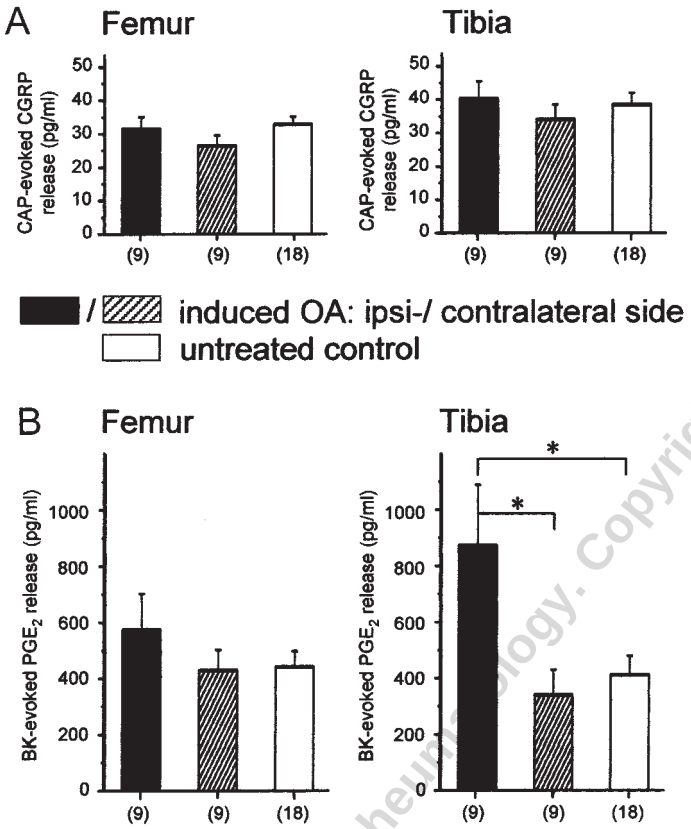

Figure 3. Capsaicin-evoked CGRP (A) and BK-evoked PGE_2 (B) release from joint preparations of C57/Bl6 mice. In 9 mice, OA had been induced in the right knee joint by intraarticular injection of collagenase 6 weeks prior to the release experiment. Data obtained from sham-injected contralateral joints and from joints of age-matched untreated C57/Bl6 mice ("control") are illustrated. Data calculation as described in legend of Figure 2. Numbers in parentheses are numbers of femoral or tibial joint preparations. BK-evoked PGE_2 release was significantly enhanced in OA tibial preparations ($p < 0.05$, U-test); CAP-evoked CGRP release remained unchanged.

elution buffer after the capsule was opened and after transection of collateral and cruciate ligaments, thereby obtaining 2 preparations, the tibia connected with the menisci and the femur connected with the patella, which were incubated separately. In terms of the measurability of neuropeptide release, the release model described here is comparable to the *in vivo* rat knee joint perfusion model[33,34], which is not practical in mice due to the small size of these animals.

To stimulate the knee joint preparations, we used CAP and BK. CAP, the hot ingredient of chili peppers, is known to activate the vanilloid receptor TRPV1[35], which is expressed selectively by a subgroup of nociceptive fibers, leading to release of neuropeptides such as CGRP and substance P[9,10]. CGRP was found to be significantly released upon stimulation of (femoral and tibial) joint preparations with CAP. This is in accord with findings obtained in other isolated tissue preparations such as peripheral nerves, skin, bladder, and muscle tissue[36-39]. Besides CAP, the endogenous inflammatory mediator BK induced a significant CGRP release from joint preparations. However, the release was much smaller than that evoked by CAP (60% vs 300% increase in STR/1N mice). Studies using tissue preparations of the heart, trachea, and skin have also shown that BK is a relatively weak stimulus for neuropeptide release[23,40,41]. The finding that BK was unable to increase CGRP release after a previous CAP stimulation might be due to a CAP-induced nociceptor desensitization or a release-induced depletion of CGRP. We also tried to determine the substance P release evoked by CAP stimulation: however, this was found to be at the detection limit of the enzyme immunoassay (data not shown). Although many substance P-containing fibers have been found in knee joint preparations, their number is lower than the number of CGRP-containing fibers[26,27].

In this study, BK induced a pronounced PGE_2 release from joint preparations, as reported for preparations of several different tissues[42-44]. In the joint, the major source for prostaglandins upon BK stimulation is supposed to be the synovial tissue[45]. Neurons play a minor role as a source

for evoked prostaglandin release, since CAP, which selectively excites neurons, did not cause any detectable PGE_2 release either in joint preparations (in our study) or in an isolated skin preparation[38]. Moreover, prior denervation did not reduce BK-evoked PGE_2 release from isolated skin[46].

CGRP and PGE_2 release in OA mice. We used 2 different animal models of OA, the STR/1N mouse as a spontaneous OA model and the collagenase-induced OA in C57/Bl6 mice as an instability-based OA model. In both models the animals develop severe structural changes in the affected knee joints, such as cartilage damage, fibrosis, osteophyte formation, and sclerosis of subchondral bone[14-21]. To correlate OA changes of the knee joints and the release of CGRP and PGE_2 we compared young, 6-week-old animals, which do not exhibit such structural signs of OA (STR/1N and CD-1 mice) and 18-week-old animals showing either no (CD1C57Bl6) or severe (STR/1N and collagenase-injected C57/Bl6 mice) structural signs of OA[14-21].

CGRP release evoked by CAP stimulation did not correlate with the temporal development of OA in STR/1N mice, i.e., we did not find a significant difference in CGRP release between 6-week-old and 18-week-old animals. In the collagenase-induced OA model there was also no difference between the collagenase-treated joints and the sham-treated or control joints. This means that structural signs of OA were not reflected by a change of the CAP-evoked CGRP release. There are different possible explanations for this observation. First, the innervation of joint tissues by peptidergic nociceptive nerve fibers and the sensitivity of these nociceptors remains unaltered in OA mice. Second, some immunohistochemical analyses of OA tissues of mice showed less immunostaining of neuropeptide-containing nerve fibers and signs of degenerated axonal profiles in areas with severe tissue damage[26,27]. However, the distribution of neuropeptide-containing fibers in human or animal OA tissue is controversial. In addition to the finding of less immunostaining of neuropeptide-containing nerve fibers in OA tissue, some authors describe no change or increased neuropeptide staining compared to control tissue[28,29]. It remains to be determined whether there is a reduction of the number of joint-innervating peptidergic nociceptors in OA mice (18-week-old STR/1N mice and C57/Bl6 mice after intraarticular collagenase injection). A reduction of peptidergic nociceptive fibers in OA mice, together with an unchanged neuropeptide release from OA joints compared to non-OA joints, would imply that the CGRP release per remaining joint nerve fiber has increased in OA mice.

We found that both basal and BK-evoked PGE_2 release from joint preparations increased substantially in STR/1N mice between 6 and 18 weeks, while comparable changes in PGE_2 release were not detected in CD-1 mice. Increases in PGE_2 release have been observed while structural changes are known to occur in joints of STR/1N mice[16-19]. The enhanced PGE_2 release is not a consequence of increase in body weight, since the release did not lead to comparable alterations in CD-1 mice that gain weight similarly to STR/1N mice between 6 and 18 weeks of age. These results match descriptions of signs of localized inflammation in human OA synovium[6,7]. In the collagenase-induced instability OA model we found an enhanced BK-evoked PGE_2 release from tibial preparations of collagenase-treated joints compared to release from sham-treated and control joints. It is unclear why a similar increase was not observed in the release from femoral preparations. In other studies using C57Bl6 mice a distinction of the histopathological score was made for medial and lateral compartments[20,21]. In those studies the medial compartments of the collagenase-treated joints showed a higher score, with the medial tibial plateau revealing the worst cartilage lesions[20,21]. This is in agreement with our finding in C57Bl6 mice that preparations of collagenase-treated tibial joints showed a significantly higher PGE_2 release than tibial preparations of sham-treated and control joints. Histopathological alterations of synovial tissues that may contribute even more to the altered PGE_2 release have not yet been investigated in the collagenase-induced OA model.

The enhanced PGE_2 release we detected in 2 murine OA models mirrors the elevated prostaglandin concentrations found in synovial fluid and in cartilage and synovial tissue of human patients and horses with OA[47-51]. The enhanced PGE_2 release may lead to increased nociceptor sensitivity[52], thereby contributing to chronic OA pain.

The mouse knee joint release model allows determination of basal and evoked release of CGRP and PGE_2 from joint preparations. It has been shown that PGE_2 release increased in the same period when structural changes of osteoarthritic joints are known to occur. This is similar in human patients with OA, and thus demonstrates that both murine OA models used in our study reproduce different aspects of the human pathophysiology of OA.

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