Neutrophil leukocyte-mediated inflammatory reactions in the periosteum and synovial membrane

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CONTENTS

List of abbreviations

CFA	complete Freund's adjuvant
CGRP	calcitonin gene-related peptide
C/K	carrageenan and kaolin
hCGRP	human calcitonin gene-related peptide
ICAM-1	intercellular adhesion molecule-1
IPC	ischemic preconditioning
IR	ischemia-reperfusion
IVM	intravital videomicroscopy
MPO	myeloperoxidase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	non-steroidal anti-inflammatory drug
PC	phosphatidylcholine
PMN	polymorphonuclear leukocyte
RBCV	red blood cell velocity
ROS	reactive oxygen species
RTX	resiniferatoxin
TNF-alpha	tumor necrosis factor-alpha
TRPV1	transient receptor potential vanilloid type-1
XOR	xanthine oxidoreductase

List of full papers relating to the subject of the thesis

Török L, **Hartmann P,** Szabó A, Varga R, Kaszaki J, Greksa F, Boros M. A csonthártya mikrokeringésének kísérletes vizsgálata intravitális fluoreszcens videó-mikroszkópiával. MAGYAR TRAUMATOLÓGIA, ORTOPÉDIA, KÉZSEBÉSZET, PLASZTIKAI SEBÉSZET 51(3): 239-246 (2008)

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1. INTRODUCTION

1.1. Microcirculatory changes are characteristic effectors of tissue inflammation

Inflammation is a complex biological response of cells, tissues and organs to harmful stimuli, such as pathogens, damaged cells or irritants. The cardinal symptoms (redness/*rubor*, swelling/*tumor*, increased heat/*calor*, pain/*dolor*, and loss of function/*functio laesa*) are all linked to changes in microcirculation. These microcirculatory responses are characterized by perfusion alterations, edema formation and the propagation of cell-to-cell interactions, mostly manifesting in the activation and tissue invasion of polymorphonuclear leukocytes (PMNs). Although the classical clinical signs are seen rather frequently at the bedside, the microcirculatory mechanisms underlying the clinical inflammatory events are mostly unmapped. Obviously, this is partly due to technical limitations; nevertheless, some of the diagnostic procedures are now available at the bedside. Our studies targeted the examination of inflammatory reactions taking place at the level of the microcirculation in the periosteum and the synovium. We planned to employ *in vivo* animal models of human diseases with high clinical incidence and impact, with special emphasis on orthopedic and trauma disorders and interventions. Our main goal was to understand the pathomechanism of microcirculatory alterations which could potentially lead to rational therapeutic approaches in clinical practice.

1.1.1. The major causes of inflammation affecting the integrity of the endothelium

It is currently considered that the activation and damage of endothelial cells and the enhancement of leukocyte-endothelial interactions in the microvessels can be ignited by direct or indirect mechanisms. Specifically, as a consequence of ischemia-reperfusion (IR) injuries, hypoxia/reoxygenation leads directly to microvascular endothelial injury through the formation of reactive oxygen species (ROS). After the ischemic period, most of the local and remote tissue destruction results from the oxido-reductive stress itself (Granger 1986; Parks 1986; Willy 2000; Ostman 2004). The main source of ROS is the xanthine/xanthine oxidase system, but infiltrating neutrophils also produce ROS and cytokines upon reperfusion (Granger 1988; Schoenberg 1991; Lojek 1997). Hypoxia-induced oxidative stress in the synovium is fundamental in the pathogenesis of arthritic disorders. It has been shown that oxidative damage is secondary to the increased pressure in the synovial cavity, reduced capillary density, vascular changes and an increased metabolic rate of synovial tissue in rheumatoid arthritis patients (Blake 1994; Mapp 1995; Tak 2000).

In other cases of arthritis, however, an endothelial dysfunction results from active regulatory elements of the inflammatory cascade, and the microvasculature mediates the spreading of inflammatory signals. Hence, the involvement of the microvessels in these reactions is indirect in nature.

It seems that both pathways will reach a common route, manifesting in microcirculatory disorders (decreased or increased tissue perfusion) and the activation of cell-to-cell reactions (e.g. leukocyte-endothelial interactions). These changes interact and promote each other, finally causing alterations at the macrocirculatory level too. We set out to investigate the background of direct and indirect forms of endothelial activation in specific experimental settings of the locomotor system.

1.1.2. Periosteal and synovial membrane damage in clinical practice

A tourniquet is often applied in both acute traumatological and elective orthopedic interventions when a bloodless operating field is required. This maneuver subjects all tissues of the extremity to IR-related changes. IR and reduced blood flow conditions during acute trauma surgery critically affect bone healing. The higher sensitivity of the periosteum (microcirculatory deterioration occurs after 60 min of ischemia (Gera 2007)) has been clarified previously by our research group. Impaired periosteal perfusion following fractures frequently results in delayed fracture repair and a non-union or manifest pseudarthrosis (Gustilo 1984,1990; Esterhai 1991; Kowalski 1996; Utvag 1998). Apart from traumatological interventions, elective orthopedic operations also frequently subject the limbs to iatrogenic IR injury. The application of a tourniquet most often occurs during arthroscopy, but limb hypoperfusion-reperfusion is a frequent consequence of the radical resection of tumors, reconstructions and autotransplantation via vessel anastomoses, too.

The term arthritis defines a group of conditions involving damage to the joints of the body. The most common forms are osteoarthritis, rheumatoid arthritis and gout, but the remaining forms (juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, systemic lupus erythematosus, scleroderma, fibromyalgia and chronic widespread pain) all display a high incidence (Oliver 2009). Although rheumatic diseases are diverse in etiology, all such conditions result in common symptoms, including different levels of pain, swelling and joint stiffness. In arthritic disorders, the synovial membrane is a major target in both the initiation and propagation of inflammation, where microcirculatory changes make an important contribution to the pathogenesis (Cremasco 2008; Smith 2008). Likewise attenuation of the inflammatory reactions taking place in the synovial membrane forms the

basis of current, up-to-date therapeutic approaches.

1.2. IR-induced microcirculatory disturbances in the periosteum and the synovium

1.2.1. Microcirculatory inflammatory reactions during IR injury

Leukocyte-endothelial cell interactions are decisive factors in the sequelae of IR injuries. The number of recruited PMNs and the extent of tissue damage during IR are inter-related phenomena (Harris 1996). At the beginning of this process, ROS-dependent direct endothelial damage occurs and the damaged/activated endothelial cells produce chemoattractants such as platelet-activating factor, leukotriene B4 and chemokines on their luminar surface thereby promoting the chemotaxic movement of PMNs (Kurose 1997; Wanner 1996). The process in which PMNs recognize and transiently interact with the vascular endothelium is termed “rolling”. The rolling step is mediated by selectins, carbohydrate-like structures present on the surface of endothelial cells and leukocytes. L-Selectin is constitutively present on PMNs, but its binding capacity is rapidly increased after leukocyte activation, possibly via receptor oligomerization (Li 1998). Unlike L-selectin, E- and P-selectins are expressed on inflamed endothelial cells. P-selectin is constitutively stored in the Weibel-Palade bodies of endothelial cells and expressed on the cell surface within minutes following stimulation by thrombin, histamine or ROS (Lorant 1991). E-selectin appears on the endothelial surface 1 or 2 h after cell stimulation (Fady 2000). The next step is the strengthening of adhesive forces, which results in the firm adherence of leukocytes to the endothelium. In this phase, PMNs also shed their L-selectin and increase their expression of ß2 integrins (CD11a,b,c/CD18) (Springer 1990). The leukocyte integrins engage ligands on the endothelial cells to develop a strong adhesive interaction. The major ligand for ß2 integrins is intercellular adhesion molecule (ICAM-1), which is constitutively expressed on the endothelium, and is up-regulated in response to cytokine stimulation (Groves 1992; Gaboury 1995). Finally, PMN transmigration occurs prominently at the borders of endothelial cells via tight junction discontinuities (Ginzberg 2001). Intravital videomicroscopy (IVM) is an excellent approach for the visualization and quantification of the interactions between PMNs and the endothelial lining.

1.2.2. The role of C-afferents in the inflammatory processes of the microcirculation

Peripheral nerve endings are specialized for the detection of painful stimuli; the major nociceptors are the myelinated A-delta and the unmyelinated C fibers of lower conduction velocity. Tissue damage resulting from ischemia produces numerous chemical

signals that activate or sensitize nociceptor terminals. Polymodular C-fibers are known to possess a double function: they are susceptible to various types of stimuli (mechanical, thermal or chemical) that act through the afferent neural system, also evoking local effects by the release of neuropeptides, e.g. neurokinin-A, substance-P and calcitonin gene-related peptide (CGRP) from the peripheral endings. The cell membrane of these C-fibers contains the transient receptor potential vanilloid type-1 (TRPV1) receptor, which is the pharmacological receptor of capsaicin. A special characteristic of the TRPV1 receptors, is that it can be activated by a warm sensation (>43 ^{o}C) and several chemical agents induced by tissue hypoxia (lactate, low pH and ion changes) (Caterina 1997), and also by resiniferatoxin (RTX), an ultrapotent analog of capsaicin (Szolcsányi 1990; Szállási 1999). Blood vessels in all vascular beds are surrounded by a dense perivascular capsaicin-sensitive innervation (Hill 1991; Dux 2009). The bone is endowed with a particularly rich innervation, including C-afferent fibers found in the bone and in the periosteum, and presumed to play important roles in the homeostasis of the bone (Bjurholm 1988; Gajda 2005).

Contradictory findings have emerged concerning the role of the C-afferent innervation in mediating the inflammatory pathways, because both pro- and anti-inflammatory features have been demonstrated in different models. CGRP induces chemotactic cytokine release *in vitro* (Ansel 1997), enhances the effect of inflammatory agents *in vivo* (Buckley 1991) and evokes neurogenic inflammation in the skin (Cappugi 1990). In contrast, exogenous CGRP improves the survival of skin grafts (Gherardini 1998; Jansen 1999) and in TRPV1 receptor knock-out mice, the thermal hyperalgesia resulting from the inflammatory reaction is missing (Caterina 2000; Bölcskei 2005). In sepsis, the anti-inflammatory effect of a high CGRP level has been proven to be anti-inflammatory by reducing the expression of PMN adhesion molecules (Monneret 2003).

Previous studies have demonstrated that C-afferent neurons may, in part, act as ischemic sensors, and that the IR-induced acidotic changes evoke a triggering effect on C-afferents (Ferdinandy 1997), with the resultant release of its mediators, including CGRP. In the present studies, we aimed to investigate the role of C-fibers in IR-related inflammatory reactions in the microcirculation.

1.2.3. Ischemic preconditioning (IPC) is a potential tool for modulation of IR-related injury

Benefits of brief periods of arterial occlusions on a subsequent, longer ischemic

period have already been demonstrated in several organs (Murry 1986; Gho 1996; Cheung 2006). Application of IPC to a limb has several advantages. Apart from its facile nature and low risk, the relatively large tissue mass provided by the limb potentially produces strong biochemical signals and a considerable extent of mediator release during the IPC process. It has been shown that IPC not only alleviates the local and systemic inflammatory consequences of limb ischemia (Szabó 2009), but also exerts considerable protection against remote organ injury (Gho 1996; Cheung 2006; Schmidt 2007). Limb IPC has even been employed in clinical practice (Kharbanda 2001; Hausenloy 2007; Loukogeorgakis 2007; Sullivan 2009).

The mechanism of protection provided by IPC includes the release of several endogenous vasodilator mediators, such as nitric oxide and carbon monoxide (for reviews, see Walsh 2007 and Tapuria 2008), and the activation of neurogenic processes with the release of neuropeptides, including CGRP (Li 1996; Brzozowski 2004). These mediators not only bring about biochemical effects and the induction of different signal transduction pathways (Yellon 2003), but also exert direct microcirculatory protective consequences in the postischemic vasculature (Koti 2002; Mallick 2005; Starr 2008).

1.2.4. The potential role of C-afferent innervation in the mechanism of IPC

Neurogenic mechanisms have been suggested to be involved in mediating both the local and the remote consequences of IPC. This suggestion is based on observations that the release of some neuropeptides can be detected at the site of preconditioning (Källner1998; Brzozowski 2004), and these pass into the blood stream and contribute to the transduction of the preconditioning stimulus to remote organs (Wolfrum 2005). CGRP, a major transmitter of capsaicin-sensitive sensory nerves, has already been addressed as a mediator of the preconditioning induced by brief ischemia (Ferdinandy 1997; Lu 1999). CGRP is responsible for the vasodilator effect of the primary afferent neurons (Tippins 1986), thereby attenuating the consequences of mesenteric ischemia (Pawlik 2000) and reperfusion injury in the heart (Li 1996; Peng 1996). In clinical studies, IPC proved to be associated with the local release of CGRP (Källner 1998; Brzozowski 2004). Furthermore, exogenous CGRP, or pretreatment with capsaicin to evoke the release of endogenous CGRP, produced preconditioning-like protection (Zhou 1999), and the protective effect of IPC was abolished by a CGRP antibody (Ouyang 2003).

Direct involvement/activation of a neuronal pathway is an alternative explanation for the protection provided by these mediators, which has been further characterized in

studies where the alleviating effects were inhibited by the ganglion blocker hexamethonium (Gho 1996; Xiao 2001; Liem 2002). Moreover, this pathway seems to be dependent on the type and locus of the ischemic stimulus. For instance, capsaicin-sensitive sensory neurons were identified as participating in the cardioprotection induced by intestinal preconditioning, which could be abolished in the presence of the hexamethonium-induced ganglion blockade (Xiao 2001). Local and remote effects of preconditioning by skeletal muscle ischemia, however, could not be affected by ganglion blockade (Liem 2002).

The aim of the present work was to investigate the involvement of these neurogenic processes, with particular emphasis on the role of CGRP in mediating the microcirculatory effects of limb IPC.

1.3. Arthritis-induced microcirculatory disturbances in the synovium

1.3.1. Experimental approaches for the examination of the consequences of arthritis

Several animal models have been used to investigate the pathogenesis of arthritis and to test the effects of different anti-arthritic agents. Spontaneous osteoarthritis occurs in transgenic mice strains, guinea pigs and non-human primates (Bendele 1988; Carlson 1996). These models are appropriate for the assessment of long-term studies of the pathogenesis. Surgically induced instability models based on medial meniscectomy or/and ablation of the anterior crutial ligament mimic the lesions occurring in humans after traumatic injuries, but the development of signs takes a very long time. Hence, for investigation of the pathomechanism of the most common forms of arthritis (i.e. rheumatoid arthritis, osteoarthritis and gout), chemically induced models have been developed (Neugebauer 2007). Administration of carrageenan and kaolin (C/K), zymosan and chronic complete Freund's adjuvant (CFA) result in inflammatory monoarthritis. On the other hand, in animal models of polyarthritis induced by immunogenic (CFA and cartilage antigens) and non-immunogenic adjuvants, the knee joint is not the primary target and area of interest.

Carrageenan-induced arthritis belongs among the non-immunological rat models of arthritis, and represents an acute inflammatory phase well. C/K-induced arthritis is most probably initiated as a mechanical injury of the inner surface of the synovial membrane and the resultant inflammatory reaction leads indirectly to the activation/injury of the endothelial side of the synovial barrier. A macrophage-dependent inflammatory response takes place which is characterized by initial non-phagocytic edema and a late dual phagocytic inflammatory phase with rapid recruitment of PMNs at the site of the affected

area (Hansra 2000; Day 2004). Since this process is critically mediated by serotonin and arachidonic acid metabolites, these changes have been shown to be reversible by pretreatments with arachidonate cyclooxygenase inhibitors and anti-serotonin agents (Lodzki 2003). It has been shown that PMNs are mobilized within 1 h, and predominate up to 12 h after carrageenan injection, but are then replaced by monocytes/macrophages predominating up to their resolution at 48 h (Derek 1999). It has also been found that diminution of PMN infiltration in the synovial membrane influences the severity of inflammation directly (Cremasco 2008; Smith 2008).

Because of the crucial role of PMNs in the initiation and maintenance of arthritic disorders, in our studies we applied a PMN-driven inflammatory model of experimental arthritis.

1.3.2. Biological and anti-inflammatory effects of endogenous PC

PC is the most frequent membrane component in the body. It has been shown, however, that inflammation is associated with physical membrane defects which results in PC degradation and the exhaustion of endogenous PC sources (Jones 1989; Gross 1992; Bruhl 2004). This observation suggests that PC supplementation may be beneficial in various diseases. Several anti-inflammatory and anti-oxidant effects of PC and its metabolites have already been demonstrated *in vitro*. For instance, PC supplementation increases the bactericidal activity of PMNs by enhancing H_2O_2 production (Yan 2004), and effectively decreases lipid peroxidation (Pozharov 1990). Furthermore, a broad spectrum of experimental and clinical evidence suggests its beneficial effects. Exogenous PC administration exerts protection during cardiac ischemia (Duan 1990), possesses the ability to restore the damaged endothelial function in irradiated blood vessels (Soloviev 2002), ameliorates the multiple cellular dysfunction in alcoholic fatty liver (Lieber 2004), alleviates alcohol-induced hepatic oxidative stress (Aleynik 2003), and preserves the gastrointestinal mucosal barrier against lipopolysaccharide, non-steroidal drugs and biliary reflux-induced mucosal damage (Leyck 1985; Eros 2006; Dial 2008). PC has been proven to be protective in local ischemic insults (hindlimb and small intestinal ischemia (Gera 2007; Ghyczy 2008)) and in experimental sepsis (Mancilla-Ramírez 1995, Yan 2004; Demirbilek 2004).

It is important to note that phospholipid elements have particular aspects in joints. Specifically, PC is in important component of the synovial fluid (Hills 1984) and has a boundary lubrication function under severe loading conditions (Hills 2002; Gale 2007).

Moreover, the composition of the synovial fluid of an osteoarthritic joint is altered considerably, which leads to a reduced concentration of molecules responsible for lubrication (Swann 1984). It can also be hypothesized that PC supplementation may restore the normal PC content, which may contribute to the preservation of the epithelial part of the synovial barrier, causing a lesser degree of articular swelling.

Hence, in the present study we set out to examine the PMN functions in the synovial membrane in response to arthritis and also after PC therapy.

1.4. Tissue-specific characteristics of the synovial membrane in inflammatory states

Joints act as compartments of the body because they are protected from the local manifestation of systemic, antigen-dependent disorders (e.g. infections) (Granfors 1990). This may be explained by the special features of the synovial membrane. An important aspect of this special barrier function is the limited penetration of certain drugs into the joint cavity (Day 1999). However, systemic infections often lead to inflammatory reactions such as joint swelling and pain. During the reactive arthritis caused by Gram-negative bacteria, the causative agent is not present within the joint cavity; only the bacterial cell wall fragment lipopolysaccharide can be detected in the synovial fluid, and the etiological role of bacteria in the disease is only presumed (Sieper 2000). Importantly, in the majority of cases, an earlier trauma event precedes this state (Masson 1985). Interestingly, adhesion molecules are expressed not only on the endothelial cells, but also on interstitial cells (Edwards 1982; Burmester 1983; Henderson 1985). Hence, joints possess numerous special features, some of which provide increased protection against inflammatory reactions affecting the extremities.

All of the tissues of the limb undergo hypoxia/reoxygenation damage upon application of a tourniquet during surgery for orthopedic trauma. The relatively low ischemic sensitivity of the microvasculature of the muscle tissues, where the critical ischemia time is ~ 2-4 h (Belkin 1988; Wang 2008) and the enhanced sensitivity of the periosteum (microcirculatory deterioration occurs after 60 min of ischemia (Gera 2007)) have been clarified previously. In contrast, the consequences of short-term tourniquet ischemia on the microcirculation of the synovial membrane have not been investigated. Hence, our goal was to characterize the parallel microvascular inflammatory reactions of the tibial periosteum and the synovium of the knee joint in response to a standardized IR challenge. We also compared these changes with those seen in C/K-induced knee osteoarthritis, a classical model of PMN-driven inflammation.

2. MAIN GOALS OF THE STUDIES

- Our primary goal was to develop a standardized rodent model for examination of the PMN-driven inflammatory reactions in the synovial membrane in monoarthritis, using IVM. To this end, we employed a new method which allows direct IVM observations at the inner surface of the joint cavity. We also used IVM to characterize the *in vivo* microcirculatory alterations in the periosteum in a rat model of hindlimb IR. We investigated the nature and the time course of PMN accumulation in the periosteum and the expression of ICAM-1.
- A further aim was to clarify the anti-inflammatory properties of different therapeutic interventions in our models, and the contribution of neurogenic factors in the pathomechanism.
- In our monoarthritis model, we set out to ascertain the anti-inflammatory properties of PC by performing detailed microcirculatory and functional analyses relative to diclofenac sodium, a non-steroidal anti-inflammatory drug (NSAID) commonly used to treat knee joint inflammation.
- In the hindlimb IR, our aim was to reveal the possible involvement of neurogenic factors in the protective effects of limb IPC in the periosteum, with particular emphasis on the role of endogenous CGRP in the process. In line with this, we investigated whether a CGRP agonist treatment (hCGRP), a competitive antagonism of the CGRP receptor (by $CGRP_{8-37}$), or selective depletion of the chemosensitive C-fibers by the capsaicin analog RTX, influences the IPC-induced microcirculatory reactions in a rat model of experimental limb IR.
- Additionally, we set out to characterize the parallel microvascular inflammatory reactions of the tibial periosteum and the synovium of the knee joint in response to a standardized IR challenge. With this aim, we investigated cell-to-cell interactions, and hemorheological and biochemical consequences of IR in both compartments. We compared these changes with those seen in C/K-induced knee osteoarthritis, a classical model of local sterile inflammation known to be mediated by PMNs.

3. MATERIALS AND METHODS

The experiments were performed in accordance with the NIH Guidelines (Guide for the Care and Use of Laboratory Animals) and the study was approved by the Animal Welfare Committee of the University of Szeged.

3.1. Arthritis induction

The animals were anesthetized with intraperitoneal (ip) ketamine (50 mg kg^{-1}) and xylazine (12 mg kg^{-1}), the skin over the knee was disinfected with povidone iodide, and arthritis was then induced with a single intraarticular injection of 75 μl of a mixture of 2% λ-carrageenan (Sigma, St. Louis, MO, USA) – 4% kaolin (C/K) in saline (Day 2004). The contralateral knees were injected with saline.

3.2. Surgical procedures before intravital microscopy

Sprague-Dawley rats were used in Studies I and II, while Wistar rats were employed in Study II. The rats were anesthetized with sodium pentobarbital (45 mg kg^{-1} ip), the trachea was cannulated to facilitate respiration, and the right jugular vein and carotid artery were cannulated for fluid and drug administration and for the measurement of arterial pressure (a Statham P23Db transducer with a computerized data acquisition system; Experimetria Ltd., Budapest, Hungary), respectively. The animals were placed in a supine position on a heating pad to maintain the body temperature between 36 and 37 ^{o}C, and Ringer's lactate was infused at a rate of 10 ml kg^{-1} h^{-1} during the experiments, together with small supplementary doses of pentobarbital iv when necessary. The trachea was cannulated to facilitate respiration.

Preparation of the tibial periosteum in rats

The right femoral artery was dissected free, and the periosteum of the medial surface of the right tibia was exposed under a Zeiss 6x magnification operating microscope. By means of an atraumatic surgical technique (developed by our research group), the skin above the anterior tibia was dissected and the gracilis posterior muscle was cut through. This simple, novel, easily reproducible procedure provides a tissue window with good exposure of the proximal and medial microvascular architecture of the anterior tibial periosteum, without using local microcirculatory disturbances or inflammatory reactions.

Preparation of the synovial membrane of the knee joint in rats

We applied a novel model for investigation of the microcirculation in the synovial tissue of the rat knee joint. After the animals had been anesthetized with ip sodium pentobarbital (45

mg kg^{-1}) 6 h after the intra-articular C/K injection, the hindlimb was placed on a specially designed stage with the knee joint slightly flexed. Under an operating microscope at 3x magnification, the synovial membrane over the medial condyle of the proximal tibia was exposed with an atraumatic microsurgical technique. A longitudinal skin incision was made over the knee, and the tendon of the quadriceps femoris muscle was completely cut through transversally. Next, the articular capsule was opened beneath the tendon with an incision running on both sides close to the patella. The patella was turned gently upwards, the inner surface of the joint cavity was visualized and the synovia rising along the medial condyle of the tibia was exposed.

3.3. Intravital videomicroscopy (IVM)

The objective of the intravital microscope was directed toward the synovial membrane, which was superfused with 37 ^{o}C saline. The microcirculation of the synovia was visualized by fluorescent IVM (Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury lamp, Acroplan 20x water immersion objective), fluorescein isothiocyanate (Sigma Chemicals, USA) being used to label the erythrocytes (0.2 ml), and rhodamine-6G (Sigma, St. Louis, MO, 0.2%, 0.1 ml iv) to stain the leukocytes. The IVM images were recorded with a charge-coupled device video camera (AVT HORN-BC 12) attached to an S-VHS video recorder (Panasonic AG-MD 830) and a personal computer.

3.4. Video analysis

Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images, using image analysis software (IVM, Pictron Ltd., Budapest, Hungary). The red blood cell velocity (RBCV, μm s^{-1}) was measured in 5 separate fields in 5 capillaries at each time point of each experiment. Leukocyte-endothelial cell interactions were analyzed within 5 postcapillary venules (diameter 11-15 μm) per animal. Adherent leukocytes (stickers) were defined in each vessel segment as cells that did not move or detach from the endothelial lining within an observation period of 30 s, and are given as the number of cells per mm^2 of endothelial surface.

3.5. Nociceptive tests

Mechanical hyperalgesia was quantified by the von Frey test (LaBuda 2000) in duplicates exactly 24 h after arthritis induction. Briefly, the animals were placed into transparent

plastic chambers on a metal mesh surface. Pulling-back reactions of the hind limbs were provoked with monofilaments of ascending thickness (Senselab Aesthesiometer 600, Somedic Sales AB, Hörby, Sweden) applied to the plantar surface. The test was classified as positive if at least 3 withdrawals could be evoked out of 10 trials. The pressure relating to a monofilament was calculated via the following polynomial function: $y = 0.7558x^2 - 9.4077x + 29.43$ (where x was the force of the applied microfilament, which lay in the range 0.026-110 g).

For thermal hyperalgesia detection, we used the paw withdrawal test (Hargreaves 1988), applying a Hargreaves apparatus. The animals were placed individually into plastic boxes, and were allowed to acclimatize to their surroundings for 15 min. Radiant heat from a halogen bulb was then delivered to the plantar surface of the hindpaws through the base of the box. The time that elapsed until the animals withdrew their hindpaws was measured (paw withdrawal latency). In order to prevent tissue damage, a 20-s cut-off time was employed. Both nociceptive tests were performed in duplicates.

3.6. Knee joint swelling

Joint inflammation was characterized by the increase in the diameter of the joints 48 h after C/K injection. The anteroposterior and mediolateral diameters (2 *a* and 2 *b*, respectively) were measured with a caliper square and the cross-sectional area was calculated via the formula $a \times b \times \pi$.

3.7. Histological analysis of PMN infiltration and immunohistochemical analysis of ICAM-1 expression

Tissue samples taken from the synovium and the periosteum were embedded in paraffin, 4-µm sections were placed on silanized slides and, after conventional methods of dewaxing and rehydration (initiated in xylene, followed by decreasing concentrations of ethanol and methanol), the samples were either stained with hematoxylin-eosin (to characterize inflammation by the number of infiltrating neutrophils) or subjected to immunohistochemical analysis for tissue ICAM-1 expression. In the latter samples, the endogenous peroxidase activity was blocked with a mixture of methanol and 1% H_2O_2 for 5 min, and nonspecific tissue antigens with conventional 2.8% cow milk. Mouse monoclonal anti-rat ICAM-1 antibody (BD Pharmingen, BD Biosciences, San Jose, CA, USA) was used as primary antibody (1:200; 30 min), this being followed by a biotinylated goat anti-mouse

antibody conjugated to HRP polymer (Envision® System; Dako, Denmark) for 30 min, which employs 3,3'-diaminobenzidine as chromogen. The sections were counterstained with hematoxylin (for 1 min) and examined by two independent histologists by means of light microscopy at 200x magnification. During the semiquantitative analysis, vessels in the synovial membrane were evaluated separately and the extent of ICAM-1 immunopositivity and perivascular PMN infiltration were determined by means of the following score systems:

Score	% of ICAM-1-positive vessels	Staining
1	< 5%	local
2		diffuse
3	5-25%	local
4		diffuse
5	25-50%	local
6		diffuse
7	> 50%	local
8		diffuse

Score	% of vessels displaying perivascular infiltration	Localization
1	< 5%	focal
2	5-25%	
3	25-50%	
4	> 50%	
5	< 5%	diffuse
6	5-25%	
7	25-50%	
8	> 50%	

3.8. Immunofluorescence detection of the periosteal localization of TRPV1 and CGRP

TRPV1 receptor and CGRP peptide immunoreactivity was demonstrated according to a modified method of Baiou *et al.* (Baiou 2007). To detect TRPV1 immunoreactivity, rabbit anti-TRPV1 (Alomone, Jerusalem, Israel; 1:1000 dilution) and Cy3-conjugated donkey anti-rabbit (Jackson, West Grove, USA; 1:500 dilution) antibodies, and to detect CGRP immunoreactivity, mouse anti-CGRP (Abcam, Cambridge, UK; 1:2500 dilution) and Daylight 488-conjugated anti-mouse (Jackson, West Grove, USA; 1:500 dilution) antibodies were used.

3.9. Xanthine oxidoreductase (XOR) activity

Tissue biopsies were homogenized in phosphate buffer (pH 7.4) containing 50 mM Tris.HCl, 0.1 mM EDTA, 0.5 mM dithiotreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg ml^{-1} soybean trypsin inhibitor and 10 μg ml^{-1} leupeptin. The homogenate was centrifuged at 4 °C for 20 min at 24,000g and the supernatant was loaded into centrifugal concentrator tubes. The activity of XOR was determined in the ultrafiltered supernatant by fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total XOR) or absence (XO activity) of the electron acceptor methylene blue.

3.10. Myeloperoxidase (MPO) activity

Tissue MPO activity was measured in synovium and periosteum biopsies by the method of Kuebler *et al.* (Kuebler 1996). Briefly, the tissue was homogenized with Tris.HCl buffer (0.1 M, pH 7.4) containing 0.1 M polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4 °C for 20 min at 24,000g. The MPO activities of the samples were measured at 450 nm (UV-1601 spectrophotometer; Shimadzu, Japan), and the data were referred to the protein content.

3.11. Experimental protocols

Study I.

In this study, the possible involvement of neurogenic factors in the protective effects of limb IPC in the periosteum were investigated, with particular emphasis on the role of endogenous CGRP in the process. The experiments were performed in two major series, where the animals were allotted into one or other of the following experimental groups. In the first group, the periosteal microcirculatory responses to 60-min total limb ischemia followed by a 180-min reperfusion period were examined (IR, n=6). After recording of the baseline microcirculatory variables (t = -60 min) with IVM, complete hindlimb ischemia was induced by placing a tourniquet around the proximal femur, with simultaneous occlusion of the femoral artery with a miniclip (Figure 1). The occlusions were then released (t = 0 min), and the periosteal microcirculation was observed via IVM at 60, 120 and 180 min during the reperfusion phase. In further experiments, the CGRP agonist hCGRP (0.3 μg kg^{-1} iv over 5 min (n=6); Shen 2003) or IPC (2x10-min IR) was applied prior to the IR insult. IPC was performed in three subgroups, in which the animals recieved the CGRP antagonist $CGRP_{8-37}$ (30 μg kg^{-1} h^{-1} iv during a 3-h reperfusion; Shen 2003), the capsaicin analog RTX, which selectively depletes the chemosensitive afferents (15 μg kg^{-1}

sc diluted with saline from a stock solution dissolved in ethanol; injections were repeated three times, every second day, ending 14 days before the experiments), or vehicle (saline) (n = 6-8). The effect of the systemic RTX treatment on the sensory functions of the chemosensitive primary sensory neurons was confirmed by the abolition of the eye-wipe reaction induced by the intraconjunctival instillation of 50 µl of 0.1% capsaicin solution.

In the second series of experiments, we investigated the consequences of $CGRP_{8-37}$ and RTX treatment on the periosteal inflammatory reactions (n=6-7) after limb IR without IPC to determine the direct effects of the compounds on the postischemic microcirculation.

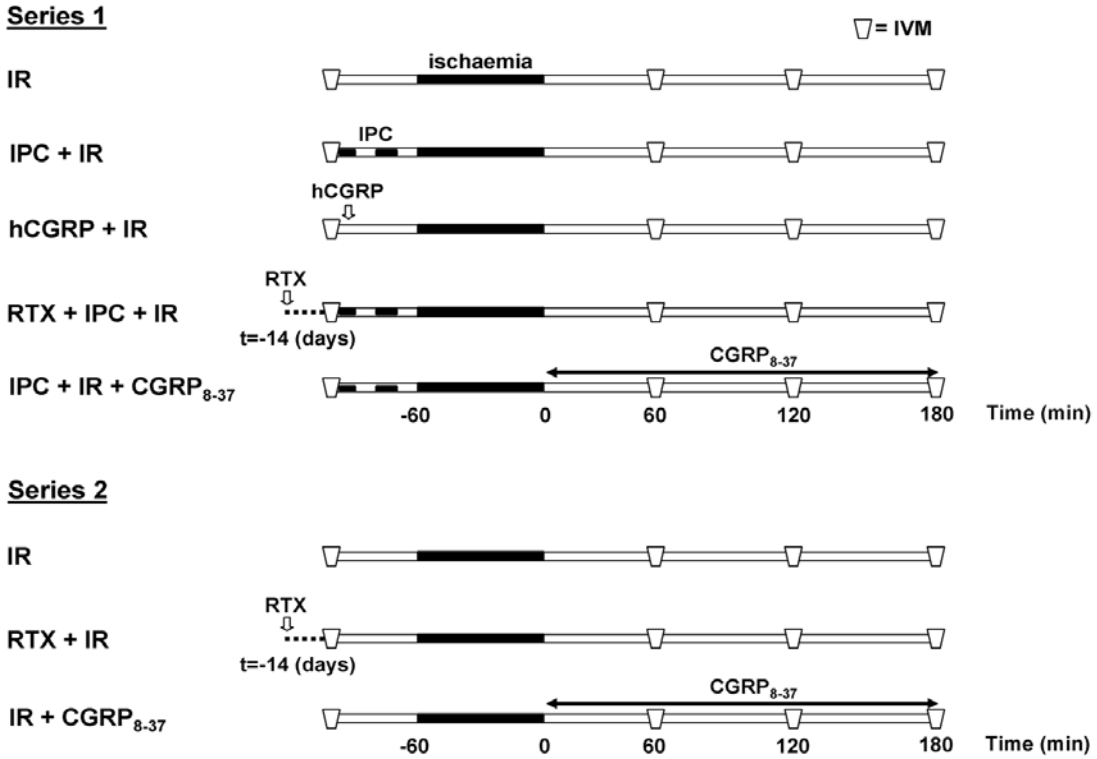

Figure 1. The time sequence of treatments and measurements in Study I. 60-min total limb ischemia was followed by 180-min reperfusion. In the first series, ischemic preconditioning was elicited in 2 cycles with 10-min ischemia followed by 10-min reperfusion (IPC). In another group of experiments, the CGRP receptor agonist hCGRP (0.3 µg kg^{-1} iv over 5 min) was applied prior to the IR insult. The capsaicin analog chemosensitive afferent depletor RTX was given in a dose of 15 µg kg^{-1} sc, repeated three times, every second day, ending 14 days before the experiments (RTX). The CGRP receptor antagonist $CGRP_{8-37}$ (30 µg kg^{-1} h^{-1} iv) was infused during the entire 3-h reperfusion period. In the second series of experiments, the consequences of $CGRP_{8-37}$ and RTX treatments were investigated after limb IR without IPC. IVM examinations of the periosteal microcirculation were performed at the indicated time points before ischemia and during reperfusion. At the end of the experiments, a histology specimen was taken from the periosteum for immunofluorescence localization of CGRP and TRPV1 and immunohistochemical determination of ICAM-1, respectively.

Study II

These experiments were performed to develop a standardized rodent model of PMN-driven monoarthritis, and to assess the anti-inflammatory properties of PC by performing detailed

nociceptive and morphological analyses of the knee joint and IVM investigation of the synovium, in two experimental series (Figure 2). In the first series, the animals participated in functional nociceptive tests reflecting the degree of inflammation, while in the second series IVM examinations of the knee joint were performed. The animals in the first group (n=8) were pretreated with PC solution (1,2-diacylglycero-3-phosphocholine in 5% glucose, Phospholipon 90G, Phospholipid GmbH, Cologne, Germany) in a dose of 150 mg kg^{-1} (1 ml kg^{-1}) through a plastic gastric tube 4 h before arthritis induction (t = -4 h), and the gavage was repeated 8 h later (t = 4 h). The typical fatty acid composition for PC was 20% palmitic acid, 5% stearic acid, 10% oleic acid, 60% linoleic acid and 5% linolenic acid). A further two groups were gavaged with identical volumes (1 ml kg^{-1}) of diclofenac sodium (0.5 mg kg^{-1}, Novartis Hungaria Kft., Budapest, Hungary) or saline at t = -4 and 4 h (n=6 and n=8, respectively). On days 2 and 3, the same oral treatments were repeated twice daily. In the second series, identical treatment protocols were used, except that IVM examinations of the knee joints were started 6 h after arthritis induction (2 h after the second oral treatment with PC (n=8), diclofenac sodium (n=6) or saline (n=8). At the end of the experiments, tissue samples from the synovium were taken for histological assessment.

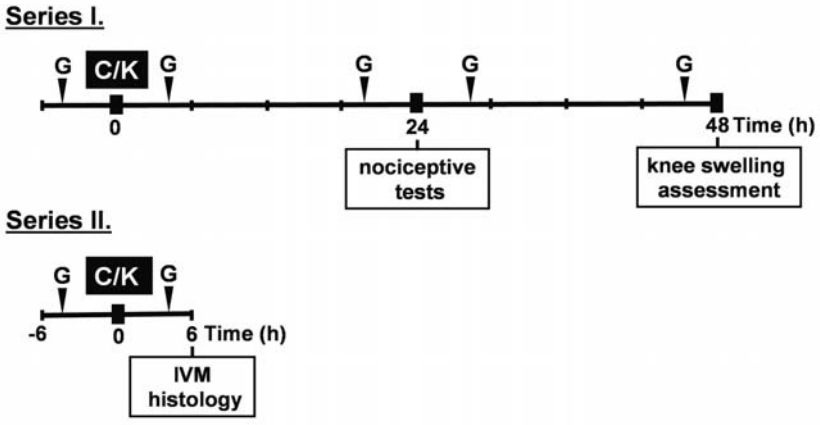

Figure 2. Time sequence of interventions in Study II. In the first experimental series, tests for secondary mechanical touch sensitivity (von Frey) and heat-provoked paw withdrawal were performed 24 h after arthritis induction with C/K and for knee joint swelling measurements 48 h after the challenge. Animals were gavaged (G) with diclofenac, PC or the saline vehicle. In the second series, IVM of the synovial membrane was performed 6 h after the challenge**.**

Study III

These experiments were conducted to compare the microvascular inflammatory reactions of the tibial periosteum and the synovium of the knee joint in response to a standardized IR challenge. The experiments were performed in two major series, with the animals allotted to one or other of the following experimental groups. In the first group, the periosteal and

synovial microcirculatory responses to 60-min total limb ischemia followed by a 180-min reperfusion period were examined (IR, n=6). After recording of the baseline microcirculatory variables (t = -60 min) with fluorescence IVM, complete hindlimb ischemia was induced by placing a tourniquet around the proximal femur, with simultaneous occlusion of the femoral artery with a miniclip. The occlusions were then released (t = 0 min), and the periosteal and synovial microcirculation were observed via IVM at 180 min in the reperfusion phase. In the second series, arthritis was induced in anesthetized animals with a single intra-articular injection of 75 µl of the C/K mixture in saline. IVM examinations of the knee joints were started 6 h after arthritis induction. Tissue specimens for immunohistochemical analysis and biochemical determinations were taken at the end of the experiments. Tissue biopsies were fixed in buffered formalin, and biochemical samples were stored at -20 ^{o}C.

3.12. Statistical analyses

In all studies, the statistical software package SigmaStat version 2.03 (Jandel Corporation, San Rafael, CA, USA) was used. In Study I, changes in variables within and between groups were analyzed by the two-way repeated measures ANOVA test followed by the Holm-Sidak test. In Studies II and III, changes in variables within and between groups were analyzed by two-way ANOVA followed by the Bonferroni test. p values < 0.05 were considered statistically significant. Data in all Figures are expressed as means±standard error of the mean (SEM).

4. RESULTS

4.1. The effects of IPC on the postischemic inflammatory reactions of the periosteum. The involvement of sensory nerve activation in the effects of IPC

4.1.1. Macro- and microcirculatory changes

Reperfusion after 60-min limb ischemia was not associated with significant changes in femoral artery blood flow (Figure 3). When IR was preceded by IPC, however, the postischemic femoral blood flow was significantly higher than the preischemic values throughout the examination period. Both CGRP receptor antagonism and sensory denervation with RTX reversed the elevation in femoral blood flow during reperfusion after IPC+IR. Exogenous CGRP treatment followed by IR evoked effects similar to those seen after IPC+IR.

The periosteal capillary RBCV in the IR group decreased during reperfusion in comparison with the preischemic value and no recovery was observed during the examination period (Figure 4). In this respect, IPC+IR caused a temporal restoration to the control value (at 60 min of reperfusion). The RBCV during reperfusion after $CGRP_{8-37}$ and RTX pretreatment combined with IPC was similar to that observed in IR animals. No decrease in postischemic RBCV was seen, however, when hCGRP was administered before ischemia.

When $CGRP_{8-37}$ and RTX pretreatment was combined with IR without IPC (Series 2), both the femoral blood flow and the periosteal capillary RBCV were similar to those observed in vehicle-treated IR animals (Table 1).

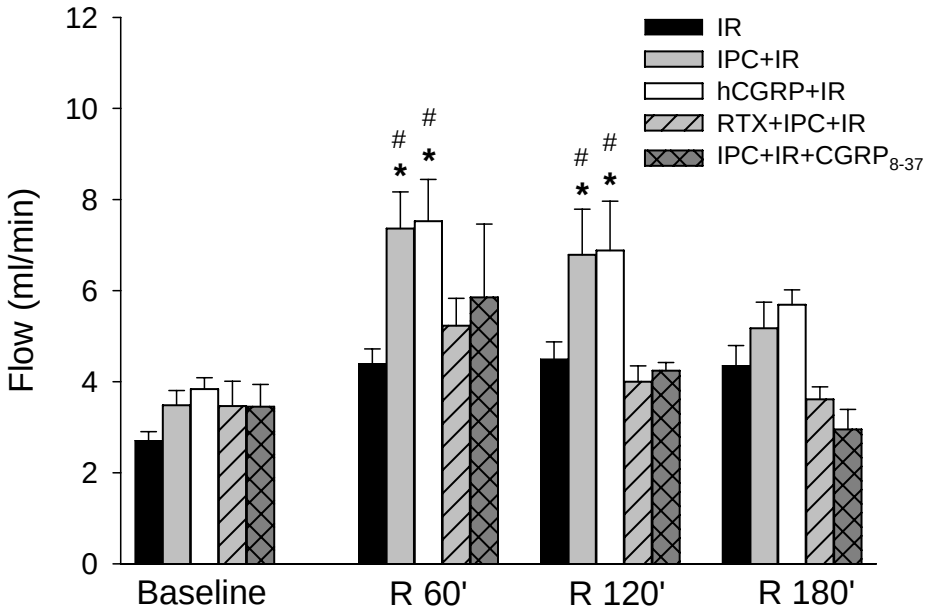

Figure 3. Changes in femoral artery blood flow in rats subjected to 60-min ischemia followed by 180-min reperfusion (IR), to IPC + IR, to hCGRP administration, to RTX pretreatment or to $CGRP_{8-37}$ treatment (see above). Values are presented as means ± SEM. *p<0.05 vs baseline, #p<0.05 vs IR group.

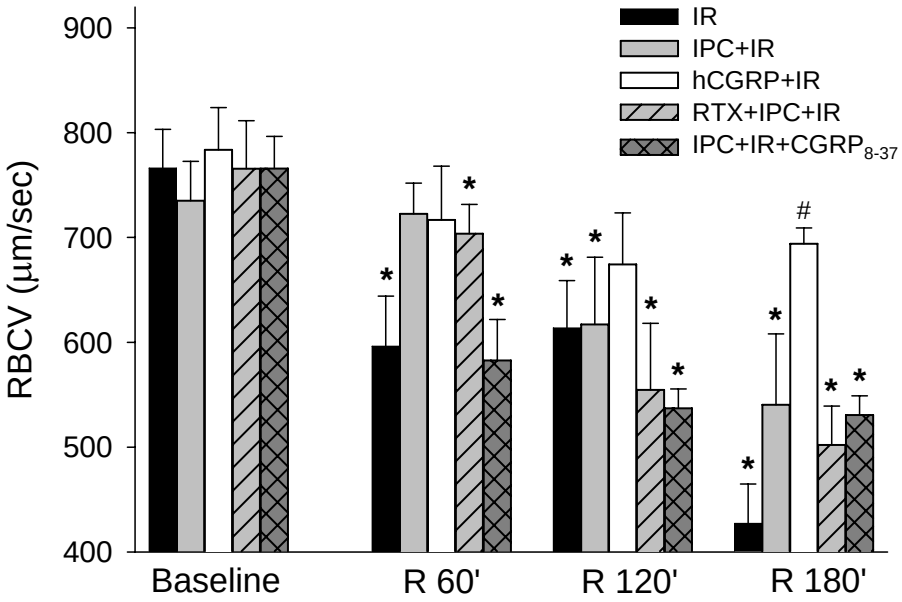

Figure 4. Changes in RBCV in rats subjected to 60-min ischemia followed by 180-min reperfusion (IR), to IPC + IR, to hCGRP administration, to RTX pretreatment or to $CGRP_{8-37}$ treatment (see above). Values are presented as means ± SEM. *p<0.05 vs baseline, #p<0.05 vs IR group.

Group	Parameter	Baseline	R 60 min	R 120 min	R 180 min
IR	Flow (ml/sec)	2.705 ± 0.197	5.745 ± 0.566*	5.125 ± 0.830*	4.390 ± 0.328*
	RBCV (µm/sec)	*766.1 ± 37.1*	*596.2 ± 47.8**	*613.8 ± 45.1**	*427.1 ± 37.6**
IR + $CGRP_{8-37}$	Flow (ml/sec)	2.829 ± 0.493	5.286 ± 1.229*	6.290 ± .291*	5.852 ± 1.607*
	RBCV (µm/sec)	*765.8 ± 30.7*	*582.6 ± 39.1**	*536.9 ± 18.5**	*530.6 ± 18.2*#*
IR + RTX	Flow (ml/sec)	2.834 ± 0.546	4.674 ± 0.292	4.158 ± 0.435*	5.228 ± 0.605*
	RBCV (µm/sec)	*765.6 ± 45.7*	*703.5 ± 28.0*	*554.5 ± 63.5**	*501.0 ± 37.2**

Table 1. Changes in femoral artery blood flow and red RBCV in Series 2, in which $CGRP_{8-37}$ or RTX pretreatment was combined with IR without IPC. *p<0.05 vs baseline #p<0.05 vs IR.

4.1.2. Changes in primary and secondary intravascular leukocyte activation and in periosteal ICAM-1 expression

The extent of leukocyte rolling in the periosteal postcapillary venules was approximately doubled in the IR group during the reperfusion phase. When limb IR was preceded by IPC, no significant increase in postischemic rolling was observed as compared with the baseline value (Figure 5). A rise in rolling leukocytes similar to that seen with IR was evident in the presence of CGRP receptor antagonism with $CGRP_{8-37}$, except for the moderately reduced values at the end of the examination period. Two weeks after RTX treatment, a significantly higher rolling PMN fraction was observed in the examined structures, with a subsequent postischemic rise similar to that seen after IR alone. In contrast, administration of the CGRP analog at the corresponding time point as in the IPC protocol (i.e. prior to the ischemia) resulted in significantly reduced interactions in comparison with that of IR.

IR produced a significant, approximately 3-fold increase in the number of leukocytes showing firm adhesion (sticking) to the walls of postcapillary venules. The number of sticking leukocytes was significantly reduced if IR was preceded by IPC (Figure 6). This effect of IPC was reversed by both CGRP antagonism and depletion of the chemosensitive afferent nerves with RTX. The hCGRP pretreatment exerted a protective effect similar to that seen with IPC.

The data from the second series of the experiments showed that $CGRP_{8-37}$ or RTX alone did not influence the IR-induced leukocyte rolling or adhesion (Table 2). RTX caused a significant rise in the baseline values of rolling PMNs.

In the IPC+IR and hCGRP group, a significantly lower ICAM-1 expression in the periosteal venules was observed than in the IR group (Figure 7). In the $CGRP_{8-37}$- and RTX-treated IPC+IR animals, the ICAM-1 values observed were similar to those seen in the IR group (Figure 7), and similar changes were evident when $CGRP_{8-37}$ and RTX treatment was applied in the presence of IR (data not shown).

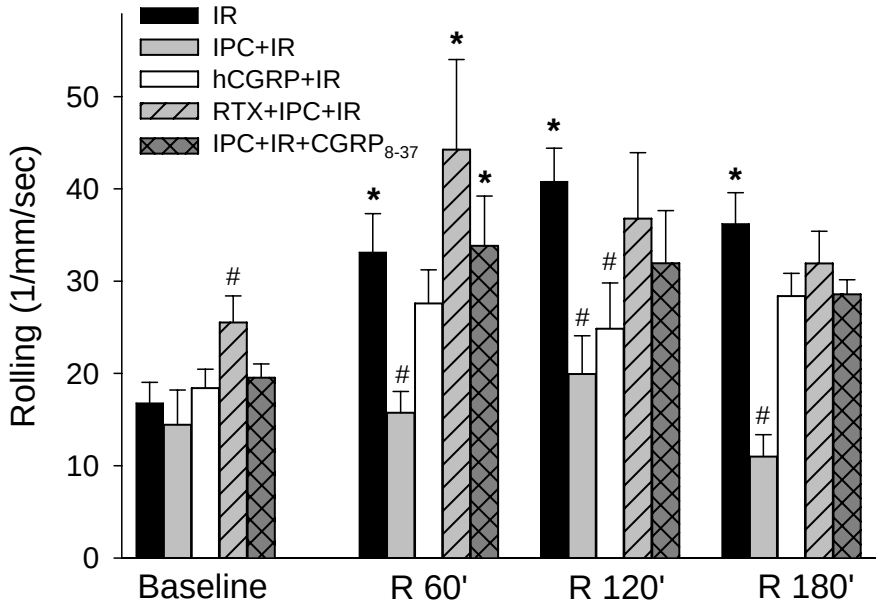

Figure 5. Changes in primary leukocyte-endothelial cell interactions (rolling) in postcapillary venules of the tibial periosteum in rats subjected to 60-min ischemia followed by 180-min reperfusion (IR), to IPC + IR, to hCGRP administration, to RTX pretreatment or to $CGRP_{8-37}$ treatment (see above). Values are presented as means ± SEM. *p<0.05 vs baseline, #p<0.05 vs IR group.

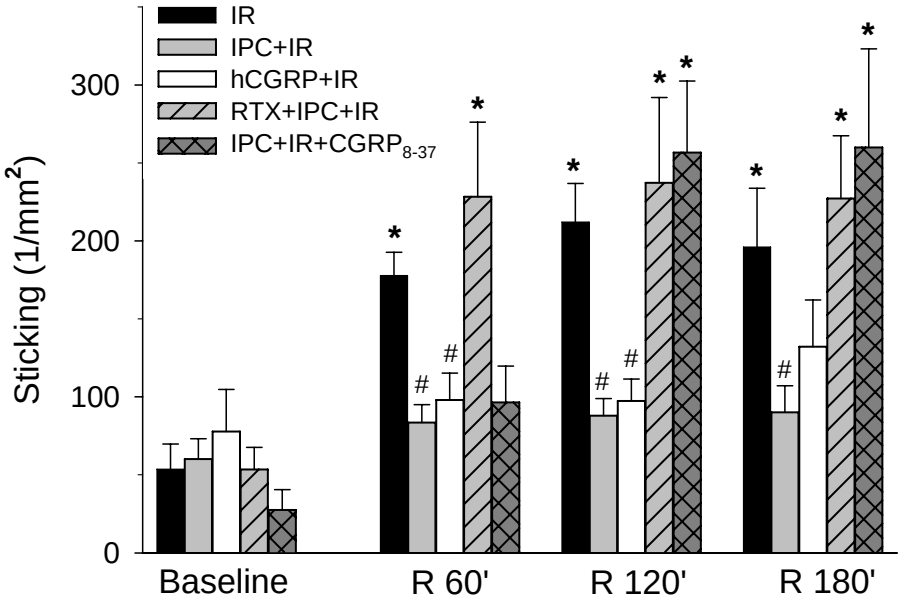

Figure 6. Changes in secondary leukocyte-endothelial cell interactions (sticking) in postcapillary venules of the tibial periosteum in rats subjected to 60-min ischemia followed by 180-min reperfusion (IR), to IPC + IR, to hCGRP administration, to RTX pretreatment or to $CGRP_{8-37}$ treatment (see above). Values are presented as means ± SEM. *p<0.05 vs baseline, #p<0.05 vs IR group.

Group	Parameter	Baseline	R 60 min	R 120 min	R 180 min
IR	Rolling	16.8 ± 2.2	33.1 ± 4.1*	40.8 ± 3.6*	36.9 ± 3.1*
	Sticking	*53.7 ± 16.1*	*177.9 ± 14.8**	*212.1 ± 24.8**	*196.0 ± 37.8**
IR + $CGRP_{8-37}$	Rolling	19.3 ± 3.2	42.1 ± 5.4*	44.7 ± 6.8*	36 ± 2.8*
	Sticking	*59.5 ± 10.1*	*167.7 ± 40.7**	*202.5 ± 53.5**	*172.0 ± 56.5**
IR + RTX	Rolling	32.0 ± 2.5 #	36.3 ± 7.3	35.2 ± 3.5	25.5 ± 6.4
	Sticking	*43.9 ±14.4*	*224.5 ± 41.1**	*171.8 ± 27.2**	*159.4 ± 25.8**

Table 2. Effects of $CGRP_{8-37}$ and RTX treatment on total limb IR-induced primary and secondary leukocyte-endothelial cell interactions (rolling, mm^{-1} s^{-1}; sticking, mm^{-2}), without IPC. *p<0.05 vs baseline #p<0.05 vs IR.

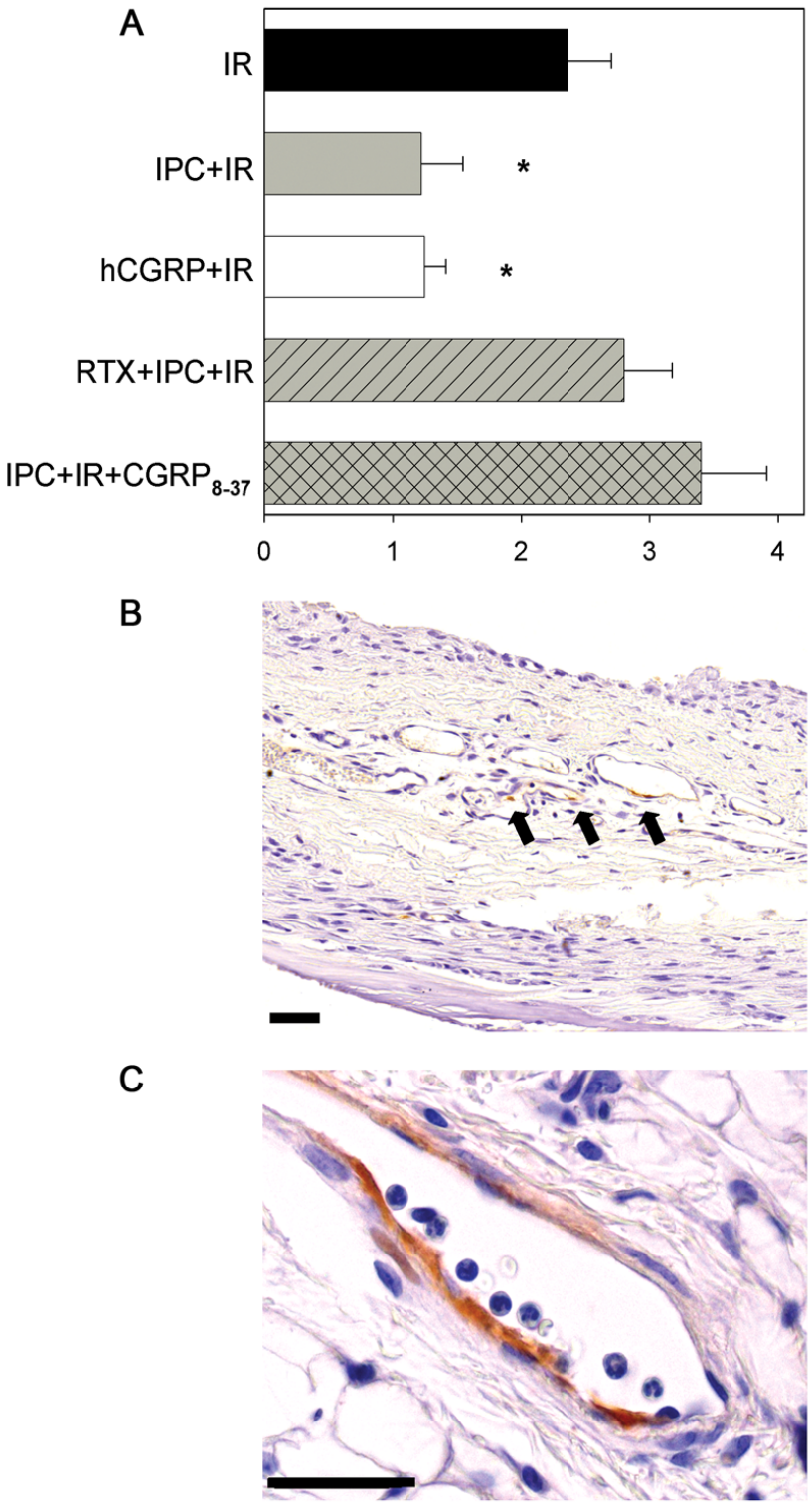

Figure 7. Effects of hCGRP, RTX and $CGRP_{8-37}$ treatment on IR or IPC+IR-induced periosteal ICAM-1 expressions (**A**). Data are presented as means ± SEM. *p<0.05 vs the IR group. Representative micrographs demonstrate moderate focal (see arrows, **B**) and diffuse staining for ICAM-1 in the periosteal postcapillary venules (**C**). Bar corresponds to 50 µm.

4.1.3. The effects of sensory nerve depletion of periosteal TRPV1 and CGRP expression

In periosteal tissue samples obtained from control animals, numerous nerve fibers and fiber bundles exhibited TRPV1 and CGRP immunoreactivity. The perivascular nerve fibers were frequently associated with small blood vessels which could be identified as arteries and arterioles. These fibers exhibited an almost complete overlap of the TRPV1 and CGRP immunoreactivities, suggesting their colocalization in periosteal sensory nerves (Figure 8).

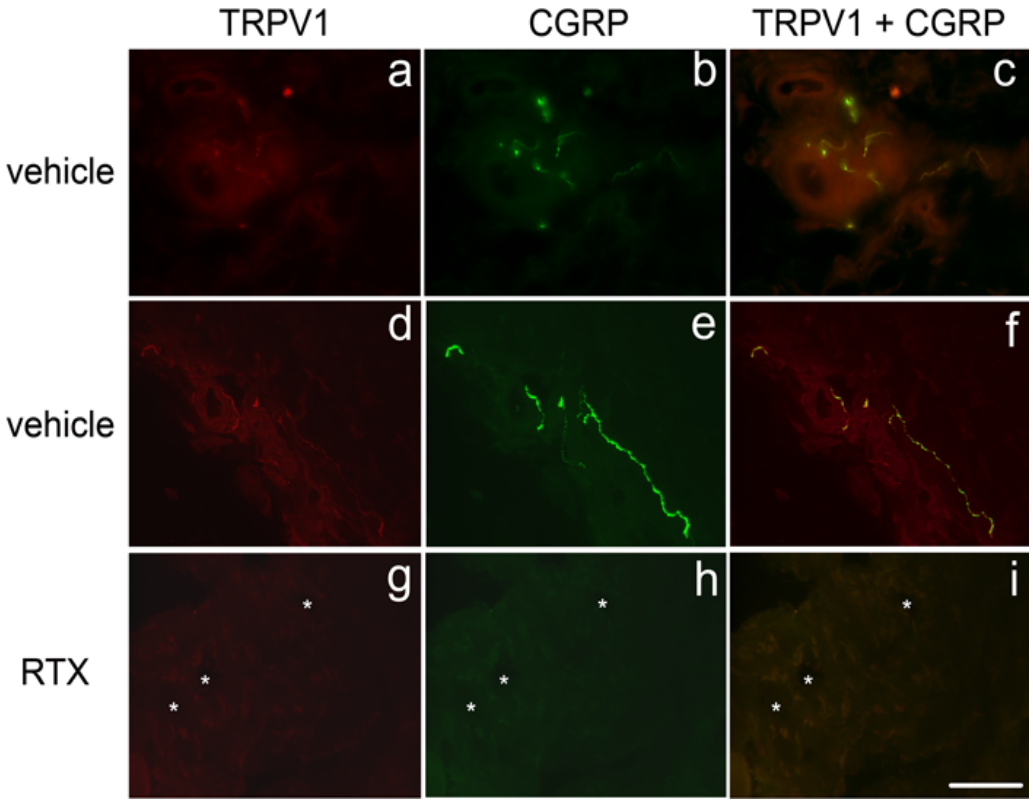

Figure 8. Photomicrographs illustrating the localization of TRPV1 and CGRP in perivascular nerve fibers of the periosteum in vehicle- (**a-f**) and RTX-treated animals (**g-i**), using wide-field (**a-c**) and confocal (**d-i**) fluorescence microscopy. Nerve fibers were stained for CGRP (red channel; **a,d,g**) and TRPV1 (green channel; **b,e,h**); color composite images (**c,f,i**) illustrate the co-localization of the labeling. Note the depletion of TRPV1/CGRP double-positive nerve fibers following systemic RTX treatment **(g-i**). Asterisks mark transections of blood vessels in RTX-treated animals. Bar represents 50 μm.

4.2. Consequences of C/K-induced knee joint inflammation. The effects of diclophenac and PC on the consequences of arthritis induced by C/K

4.2.1. Changes in secondary hyperalgesic reactions of the hindlimb and knee joint swelling

In the first series, the extent of inflammation was estimated by means of functional tests 24 h after arthritis induction. The mechanical touch sensitivity was considerably increased in response to arthritis, as the C/K-injected limbs responded to a lower level of trigger (24.2±4.1 g mm^{-2}) than the saline-injected control limbs (77.7±5.1 g mm^{-2}) in animals receiving the saline vehicle (Figure 9A). This parameter was significantly diminished in response to diclofenac and PC treatments, albeit complete restoration was not achieved. The thermal nociceptive latency (Figure 9B) was also significantly decreased in the saline-treated group (from the control level of 13.1±0.5 s to 7.2±0.7 s in the injured leg), and diclofenac and PC treatments exerted similar protective effects to that seen with the von Frey test.

The changes in knee cross-section (Figure 10) furnish a direct and objective measure of joint inflammation. The cross-sectional area in the C/K-injected knees was ~ 35% larger than that in the contralateral knees 48 h after the challenge, but was significantly reduced, by ~ 20%, by diclofenac and PC treatments; in the case of diclofenac, complete restoration to the level for the saline-injected knees was achieved.

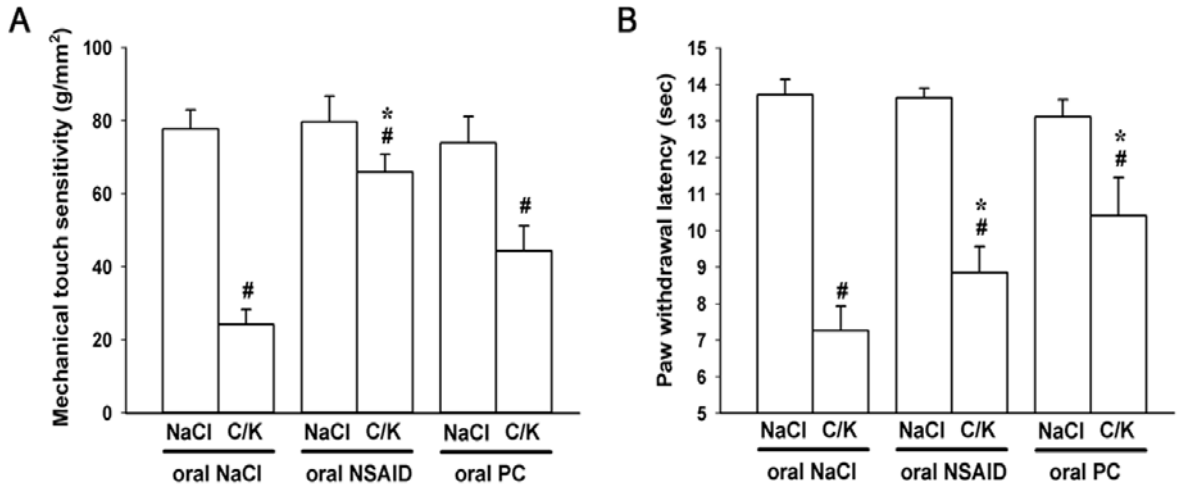

Figure 9. The effects of oral treatments with diclofenac (NSAID), PC or the saline vehicle on the C/K-induced changes in mechanical touch sensitivity (von Frey test) (**A**) and heat-provoked paw withdrawal (**B**) in limbs where the knees were injected with C/K or the saline vehicle (contralaterally). Data are presented as means ± SEM. # $p < 0.05$ vs control limb; * $p < 0.05$ vs C/K + oral saline.

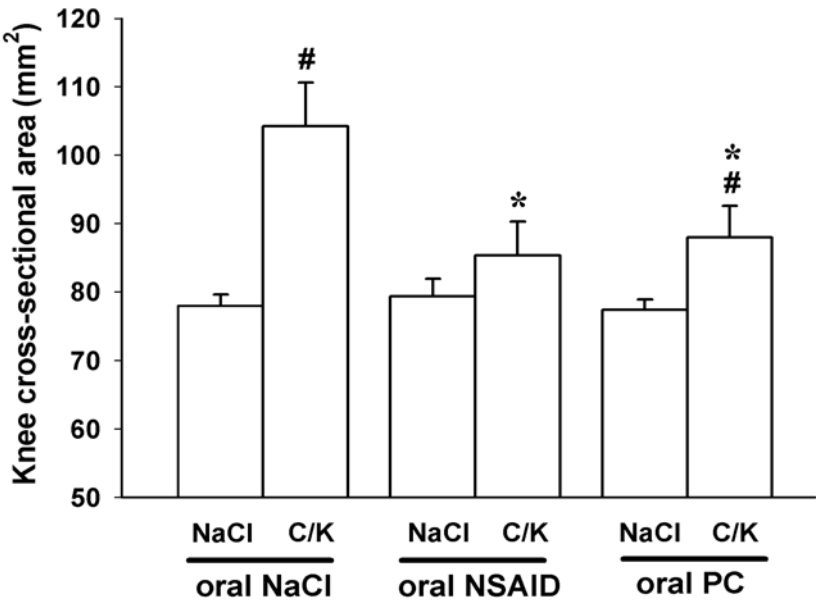

Figure 10. The effects of oral treatments with diclofenac (NSAID), PC or the saline vehicle on the C/K-induced changes in knee joint swelling (expressed as knee cross-section). The contralateral knees were injected with saline. Data are presented as means ± SEM. #p<0.05 vs control limb; *p<0.05 vs C/K + oral saline.

4.2.2. Microcirculatory changes

In the second experimental series, the microcirculatory consequences of the joint inflammation were quantified via IVM, and the leukocyte-endothelial interactions (rolling and sticking) in the postcapillary venules of the synovial membrane were determined. The data relating to the rolling fraction of the PMNs in the postcapillary synovial venules exhibited a large degree of dispersion and no baseline differences could be observed between the C/K- and saline-injected knees or the groups which participated in the treatment protocols (data not shown). However, the injection of C/K was accompanied by a statistically significant, ~ 6-fold increase in PMN leukocyte adherence (sticking) to the endothelial layer as compared with the control side (Figure 11). This reaction was considerably reduced by PC (by ~ 40%, p < 0.05), but was only moderately ameliorated by diclofenac treatment (by ~ 22%).

The RBCV in the capillaries of the synovial membrane was very high in both the control and the C/K-injected legs (1374±75 and 1111±89 μm s^{-1}, respectively) of the saline-treated animals. Neither diclofenac nor PC treatment influenced this parameter (1238±120 and 1150±129 μm s^{-1}, respectively).

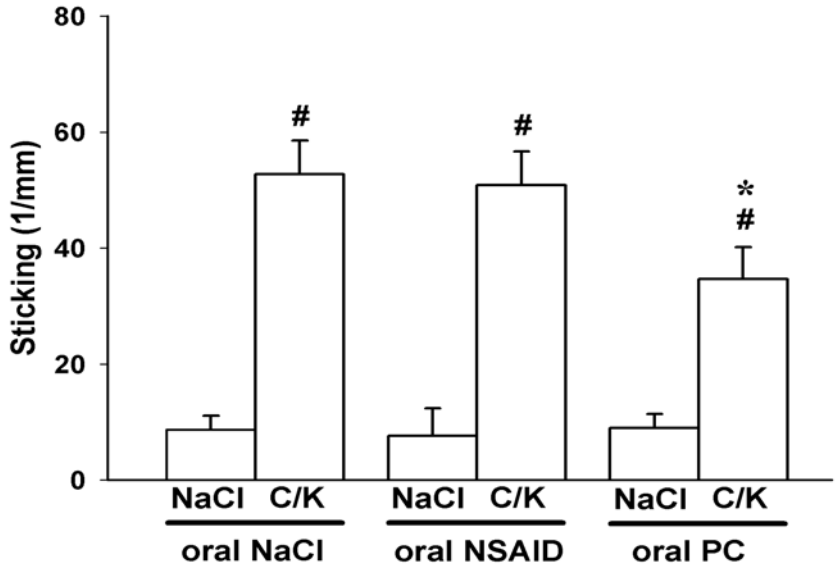

Figure 11. The effects of oral treatments with diclofenac (NSAID), PC or the saline vehicle on the number of sticking leukocytes in the postcapillary venules of the synovial membrane. Contralateral knees were injected with saline. Data are presented as means ± SEM. #p<0.05 vs control limb; *p<0.05 vs C/K + oral saline.

4.2.3. Histomorphometric changes

Histomorphometric analysis revealed a definite increase in the tissue accumulation of PMNs, as evidenced by the increase in the number of infiltrating granulocytes in the perivascular regions at the end of the experimental protocol in each group (Figure 12A,C). Likewise, immunohistochemical analysis indicated a considerable increase in the ICAM-1 immunoreactivity positivity in the venules, but not in the arterioles of the synovial membrane (Figure 12B,D). Diclofenac administration did not influence these changes, whereas oral PC treatment significantly reduced the increases in both parameters (Figure 12).

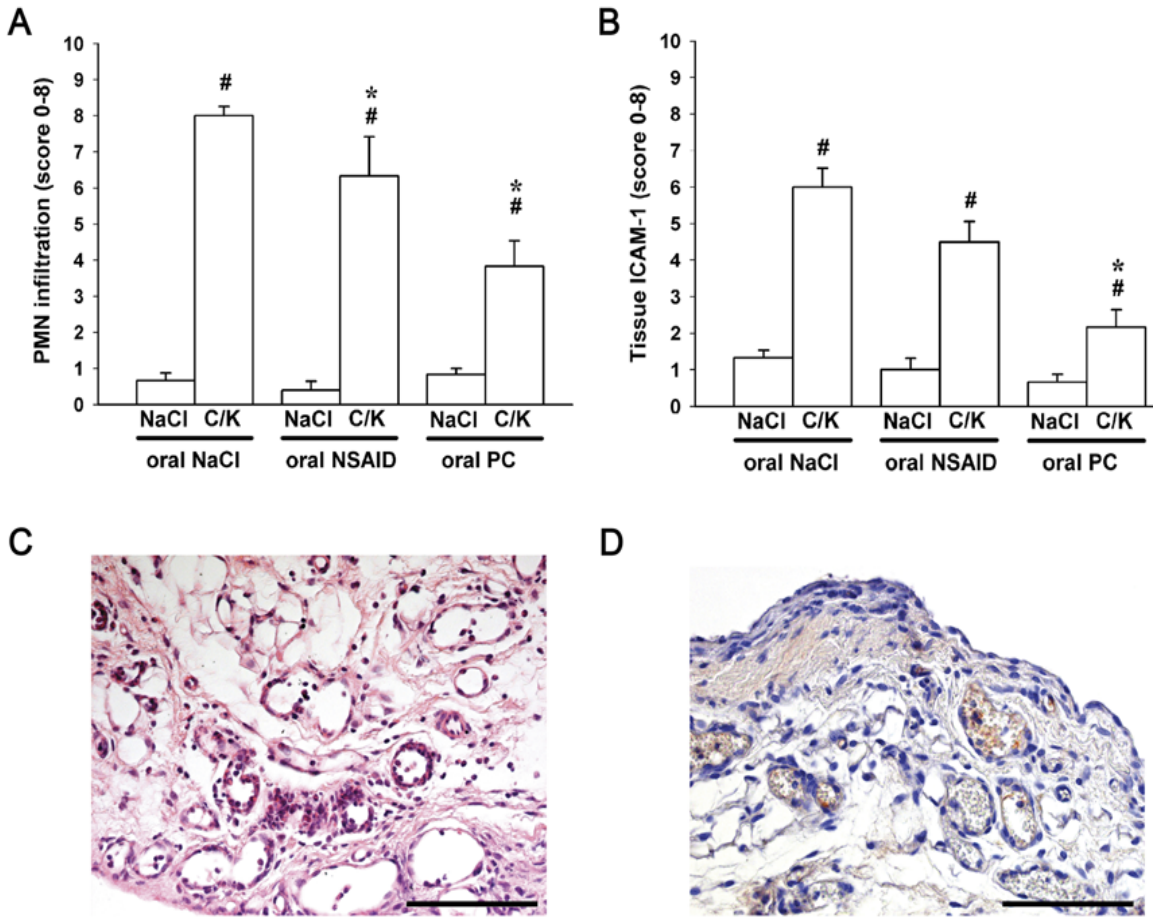

Figure 12. The effects of oral treatments with diclofenac (NSAID), PC or the saline vehicle on the synovial leukocyte infiltration (**A**, H&E staining) and ICAM-1 expression (**B**, immunohistochemistry). The contralateral knees were injected with saline. PMN infiltration (**C**) and intravascular ICAM-1 positivity (**D**) are also shown in representative micrographs. The bar represents 100 µm. Data are presented as means ± SEM. #$p<0.05$ vs control limb; *$p<0.05$ vs C/K + oral saline.

4.3. Differences between periosteal and synovial microvascular inflammatory reactions in response to limb IR

4.3.1. Microhemodynamic changes

The baseline RBCVs in the periosteum were significantly lower than those in the synovial membrane (Figure 13). In response to IR, the RBCV decreased only in the periosteum by 180 min of reperfusion. The RBCVs in the capillaries of the synovial membrane did not change substantially in response to the IR insult, but were reduced in the limbs injected with C/K.

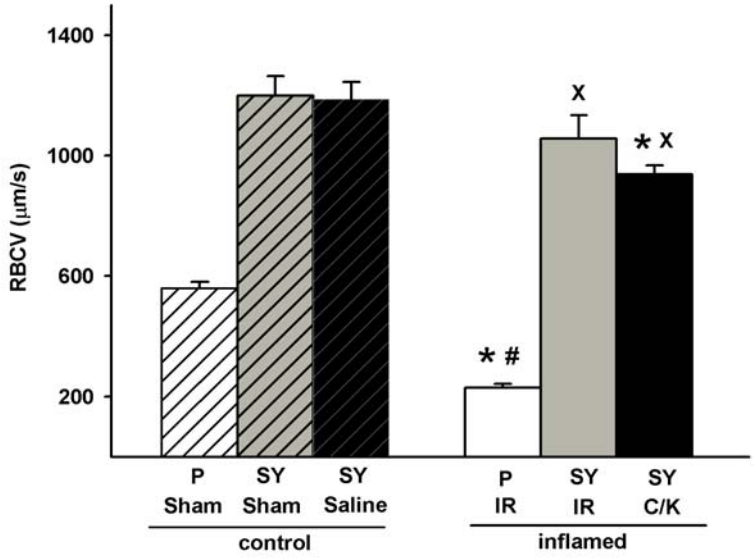

Figure 13. RBCVs measured in the capillaries of the periosteum (P) and the synovial membrane (SY) in sham-operated (Sham) limbs and in limbs subjected to total limb IR injury. C/K-injected limbs served as positive control, while the contralateral limbs were injected with saline (Saline). Data are presented as means ± SEM. *p<0.05 vs control limb; ^{x}p<0.05 vs P+IR; #p<0.05 vs SY+IR.

4.3.2. Microcirculatory leukocyte activation

No baseline differences were observed between the periosteum and the synovium in PMN rolling and adherence (sticking) to the endothelial layer of the postcapillary venules. Reperfusion after 60-min total limb ischemia was accompanied by a statistically significant (~ 160%) increase in PMN rolling and a more than 2-fold increase in PMN sticking in the periosteal venules (Figure 14A,B). Significant increases were not observed in these parameters in the synovium at 180 min of reperfusion. The rolling and adherence attained significantly higher values in response to C/K injection.

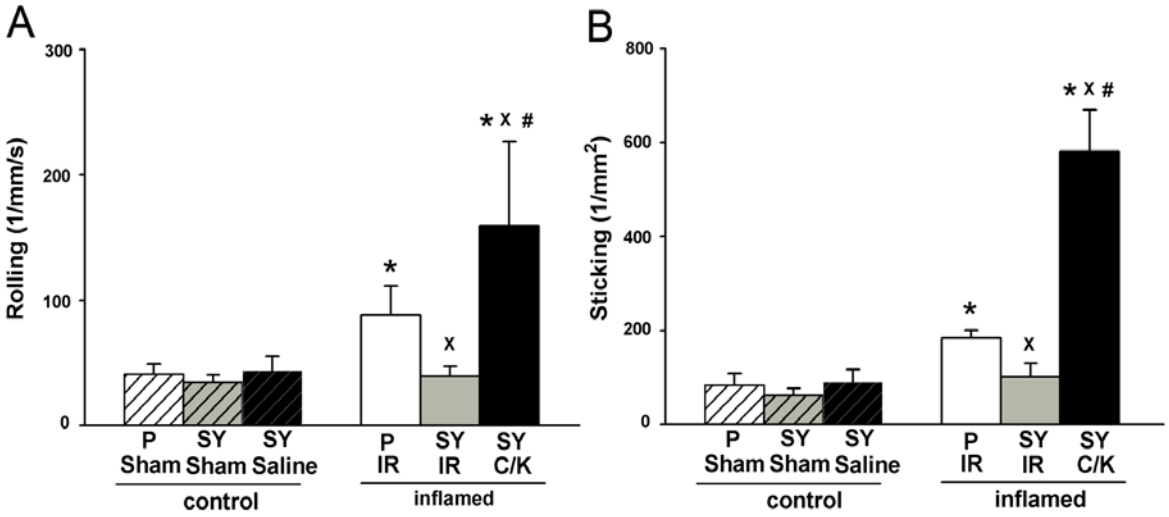

Figure 14. Rolling and sticking leukocytes in the postcapillary venules of the periosteum (P) and the synovial membrane (SY) in sham-operated (Sham) limbs and in limbs subjected to total limb IR injury. C/K-injected limbs served as positive control, while the contralateral limbs were injected with saline (Saline). Data are presented as means ± SEM. *p<0.05 vs control limb; ^{x}p<0.05 vs P+IR; #p<0.05 vs SY+IR.

4.3.3. Changes in XOR activity and MPO activity

Significant increases in XOR activity were observed in response to all of the challenges in all structures (Figure 15A). The highest degree of increase was observed in response to C/K injection. IR caused similar extents of increase in this parameter in the synovium and the periosteum.

Significant increases in MPO levels were not detected in response to IR in the periosteum or the synovium (Figure 15B). In the C/K-treated limbs, however, the synovial MPO activity was ~ 10-fold higher than in the contralateral limbs.

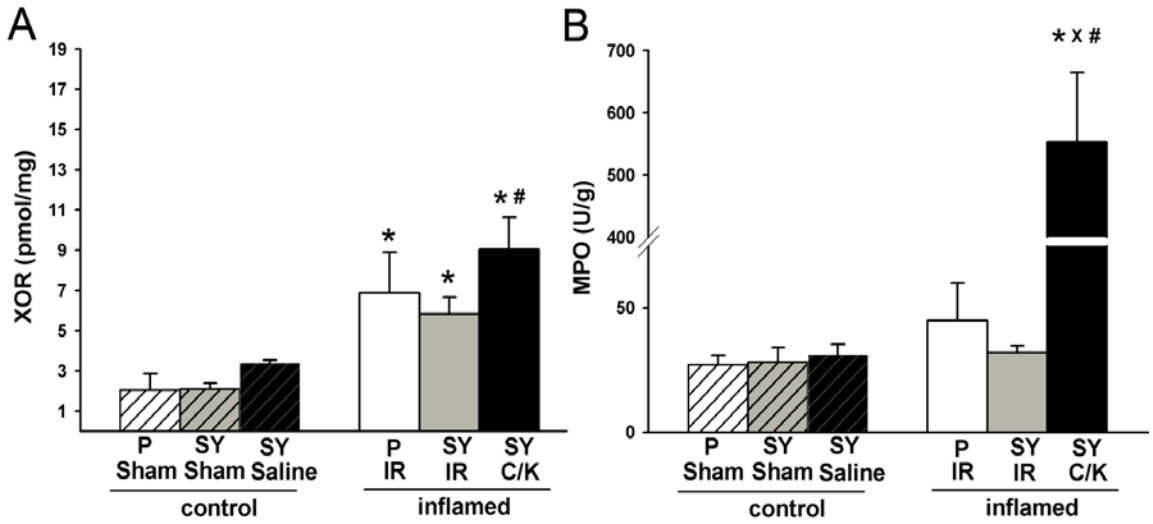

Figure 15. XOR activity and MPO activity measured in the periosteum (P) and in the synovial membrane (SY) in sham-operated (Sham) limbs and in limbs subjected to total limb IR injury. C/K-injected limbs served as positive control, while the contralateral limbs were injected with saline (Saline). *p<0.05 vs control limb; ^{x}p<0.05 vs P+IR; #p<0.05 vs SY+IR.

4.3.4. Tissue ICAM-1 expression

In response to IR, considerable increases in ICAM-1 immunoreactivity were observed in the venules of the periosteum, but not in those of the synovium (Figure 16). Injection of the joint cavity with C/K resulted in an increase in ICAM-1 expression similar to that in the postischemic periosteum.

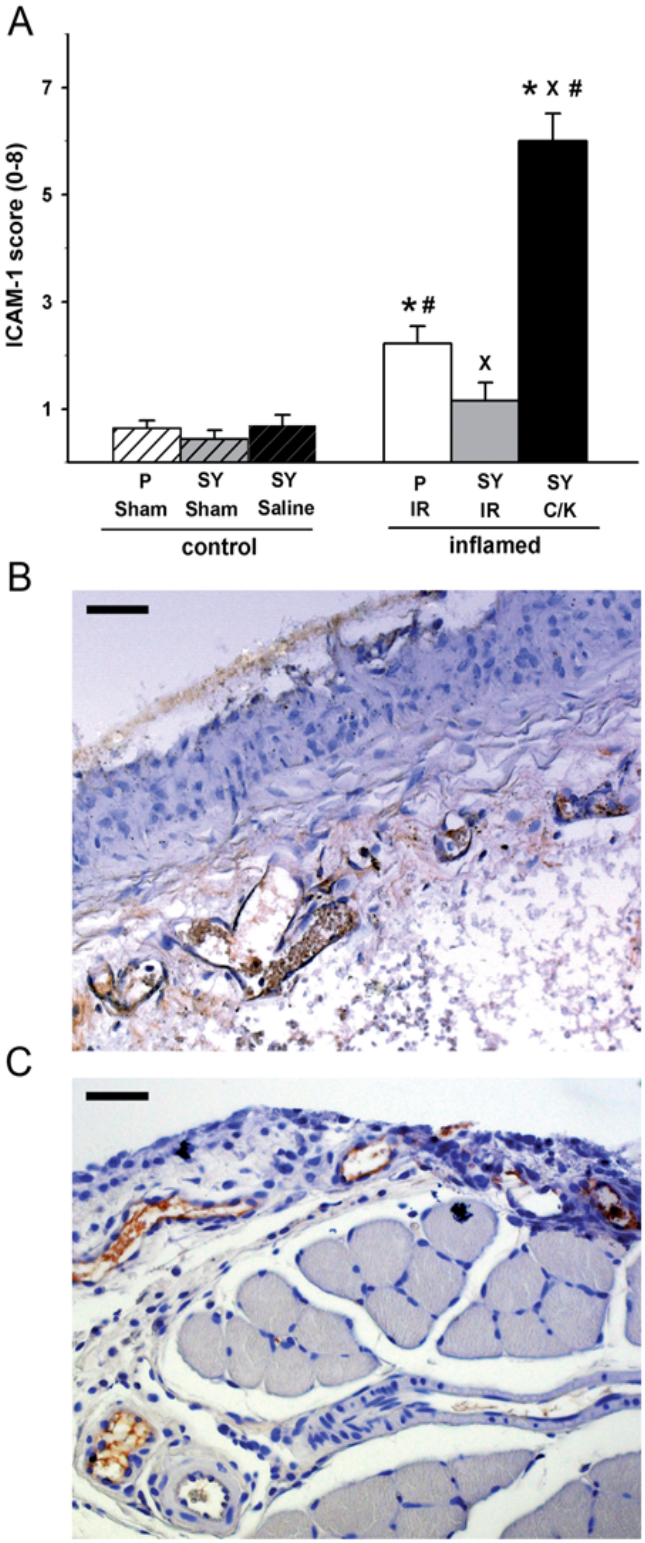

Figure 16. ICAM-1 expression in the periosteum (P) and the synovial membrane (SY) in sham-operated (Sham) limbs and in limbs subjected to total limb IR injury (**A**). C/K-injected limbs served as positive control, while the contralateral limbs were injected with saline (Saline). Representative micrographs show positive reactions for ICAM-1 in the IR-challenged periosteum **(B)** and in the synovium in response to C/K injection **(C).** The bar denotes 50 μm. Data are presented as means ± SEM. *p<0.05 vs control limb; ^{x}p<0.05 vs P+IR; $^{\#}p$<0.05 vs SY+IR.

5. DISCUSSION

5.1. Effects of different therapeutic interventions on the PMN-endothelial interactions

In our models of limb IR and knee C/K arthritis, the local injury was manifested in significant increases in the primary and secondary forms of PMN-endothelial interactions (rolling and firm adherence) in the postcapillary venules of the periosteum and the synovium. These reactions can be quantitatively assessed by IVM, and the efficacy of various therapeutic interventions can also be judged objectively.

Study I revealed that the local inflammatory consequences of limb IR, as evidenced by the enhanced leukocyte-endothelial interactions in the periosteum, could be ameliorated effectively by IPC. The major finding of this study is that the protective effects of IPC against IR-induced inflammatory injury are mediated by sensory nerves which express the TRPV1 receptor and contain CGRP. The contributions of chemosensitive C-fiber afferents and the sensory neuropeptide CGRP to this phenomenon are supported by the observations that the protective effects of IPC were eliminated by administration of the CGRP antagonist $CGRP_{8-37}$ or by the RTX-induced depletion of C-fiber afferents. Moreover, systemic application of a CGRP analog produced decreases in leukocyte-endothelial cell interactions similar to those seen after IPC. These findings collectively suggest that activation of the chemosenstive C-fiber afferents and the release of CGRP from the activated nerve terminals during the induction of IPC may reduce the inflammatory reactions produced by IR.

The possible involvement of sensory nerves in the development of both the local and the remote effects of IPC have already been implicated, since the activation of TRPV1 receptors expressed by nociceptive C-fiber primary afferent neurons has been demonstrated under ischemic conditions, due to their responsiveness to elevated proton concentrations, a frequent consequence of tissue ischemia and inflammation (Tominaga 1998). These sensory neurons are also activated by lipid peroxidation products via TRPA1 receptors, which also sensitize TRPV1 receptors through a Ca^{2+}-dependent mechanism (for a review, see Stucky 2009). Upon activation of C-fibers during ischemia, orthodromic impulses mediate the perception of pain, whereas antidromic impulses elicit CGRP release from the peripheral nerve endings (Jancsó 2009). The significance of the release of sensory neuropeptides from the stimulated sensory nerve terminals has been confirmed by the detection of these peptides at the site of preconditioning (Källner 1998; Brzozowski 2004) and in the bloodstream (Chai 2006), which is presumed to contribute to the transduction of preconditioning stimulus in remote organs (Wolfrum 2005). Furthermore, the depletion of

capsaicin-sensitive neurons not only terminates the protection provided by IPC, but also prevents the release of CGRP (Tang 1999).

Selective destruction of the chemosensitive afferents was elicited by RTX, which is known to bring about the metabolic damage of cells as a result of Ca^{2+} accumulation (Szallasi 1989; Pecze 2009), causing a long-lasting desensitization to pain. Our present results demonstrate that RTX pretreatment abolished the beneficial effects of IPC in alleviating the microcirculatory changes induced by IR. Similarly to RTX pretreatment, administration of the CGRP antagonist $CGRP_{8-37}$ during the reperfusion phase prevented the development of IPC-induced protection. Similarly to the effect of IPC, systemic application of the CGRP analog hCGRP, administered at a matching time with the preconditioning trigger, resulted in an alleviation of the IR-induced changes in the microcirculatory reactions. These observations provide further proof of the effector role of CGRP in the mechanisms of limb IPC. These findings suggest, therefore, that the marked protective effects of IPC against the IR-induced (micro)circulatory injuries are mediated by these chemosensitive afferent nerves which express TRPV1 receptor. It can be assumed that transient occlusion of the blood circulation activates chemosensitive afferent nerves and/or nerve endings, which in turn release the (neurogenic) mediator(s) producing the protective anti-inflammatory effects of IPC.

The C/K-induced monoarthritis model (Study II) is based on the inability of macrophages to process the high molecular weight carrageenan, resulting in its accumulation within lysosomes and an inflammatory response (Hansra 2000). Carrageenan is a sulfated polysaccharide extracted from red algae. Upon injection into the joint, carrageenan elicits a time-dependent local, acute inflammatory reaction, which is a suitable method for evaluating anti-inflammatory agents. The basis of this immediate response is direct physical damage and the consequent rapid recruitment of PMNs at the site of the affected area, resulting in the production of serotonin and arachidonic acid metabolites; most of these reactions can be inhibited effectively by arachidonate cyclooxygenase inhibitors and antiserotonin agents (Lodzki 2003). Shifting of the content of participating inflammatory cells during these carrageenan-induced events is a typical reaction. PMNs are mobilized within 1 h, but then are replaced by monocytes and macrophages up to their resolution at 48 h (Derek 1999).

Because of the crucial roles of PMN reactions in the early phase of inflammation (Cremasco 2008; Smith 2008), we set out to examine the effects of PC on leukocyte activation in the synovial membrane itself. IVM is a powerful tool for the on-line

visualization of microcirculatory events which may be coupled to structural, histological analyses in the synovial tissue. In previous studies, however, the synovium was observed without opening of the joint capsule (Coelho 2008; Veihelmann 1998) and this technique permitted visualization only of Hoffa's fatty body, intra-articular fatty tissue containing synovial cells on the interior surface of the joint. The methodology presented by our study allowed direct visualization of the synovial membrane. The medial condyle of the rat is large and relatively flat, the site is easily accessible for microscopy, and a considerable mass of tissue can be harvested for histological examinations. The synovial tissue along the condyle contains well-defined microcirculatory structures, which can be visualized in several fields under the objective of the IVM.

Injection of carrageenan resulted in an inflammatory response which was characterized by increases in primary and secondary leukocyte-endothelial interactions. Considerable differences between groups occurred only in the number of sticking leukocytes, depending on the treatment applied. Specifically, although diclofenac obviously reduced the symptoms of arthritis (swelling and nociceptive reactions), only PC supplementation ameliorated the microcirculatory inflammatory reactions significantly. In a previous study, the beneficial effects of exogenous PC administration had already been proven in relation to the leukocyte-endothelial interactions in the periosteum (Gera 2007). Independent studies confirmed our observations that PC supplementation acts as an immune-modulator for the inflammatory responses of leukocytes (Tonks 2005; Miranda 2008). However, conflicting reports have been published on the effects of NSAID administration on leukocyte-endothelial interactions. It has been shown that non-selective cyclooxygenase inhibitors can even function as pro-inflammatory agents in the microcirculation (Arndt 1995); moreover, diclofenac can augment the upregulation of the expression of adhesion molecules *in vitro* (Diaz-Gonzales 1995). The majority of studies, however, report that diclofenac diminishes the number of PMN-endothelial interactions (Scheja 1985; Martinez 2004). In our study, administration of diclofenac did not influence the PMN-endothelial interactions. It is interesting that PC with another fatty acid constitution (i.e. the lysophosphatidylcholine form) has been shown to induce endothelial damage (atherosclerosis and advanced adhesion molecule expression (for a review, see Dart 1999) and induces leukocyte-endothelial cell interactions (Scalia 1997). This observation could indicate that the altered fatty acid constitution of the PC molecule may contribute to the mechanism of action. In our study, histomorphometric analysis of leukocyte recruitment in the synovial tissue supported the IVM results: C/K injection induced a marked increase

in the number of PMNs in the perivascular region of the synovium, which was moderated by PC supplementation. However, administration of diclofenac did not modify the perivascular infiltration of leukocytes significantly, suggesting that the anti-inflammatory effect of the NSAID compound could not be mediated by factors other than influencing the recruitment of PMNs in this case.

As for the simultaneous comparison of the synovial and the periosteal microcirculatory inflammatory reactions induced by IR (Study III), the increases in both the primary and secondary forms of PMN-endothelial interactions (rolling and firm adherence) were confined to the periosteal postcapillary venules, but these IR-induced reactions were virtually missing in the synovial membrane. The causes of tissue-specific ischemic tolerance have not yet been clarified, and many factors should be considered in this phenomenon. These include differences in oxidant-induced injury, ICAM-1 expression in the postcapillary endothelial cells and functional microhemorheology. The biochemical consequences of hypoxia-reoxygenation after tourniquet ischemia have not been compared previously in the synovium and periosteum. As regards the synovial membrane, both inflammatory joint diseases and arthroscopic interventions lead to oxidative stress (Willy 2000; Ostman 2004). The generation of ROS has been demonstrated in the synovial fluid and membrane under clinical conditions and postulated as a causative factor in joint disorders (Beckman 1989; Kennett 2009). Specifically, oxido-reductive stress has been shown to play a fundamental role in the pathogenesis of rheumatoid arthritis as result of an increased pressure in the synovial cavity, a reduced capillary density and vascular changes, and the increased metabolic rate of the synovial tissue (Blake 1994; Mapp 1995; Tak 2000). The activity of XOR, a source of ROS in vascular endothelial cells (Smith 1989), has been shown to be increased in the skeletal muscle after tourniquet ischemia (Saricaoglu 2005). Moreover, joint inflammation is associated with enhanced XOR activity in the synovium (Klocke 2005) and the synovial fluid (Hanachi 2009). Our present findings indicate that increases in XOR activity also occur in response to tourniquet ischemia and arthritis, the latter being more accented. Furthermore, the similar extents of increase in XOR activity in the periosteum and the synovium in response to IR suggest that the differences in the microcirculatory responses are most probably not due to differences in XOR-derived ROS production. Moreover, infiltrating PMNs are also important sources of ROS through NADPH oxidase 2 activity (Bedard 2007). In our study, C/K-induced osteoarthritis was used as a positive control because it is associated with severe tissue destruction known to be mediated by infiltrating PMNs (Day 2004; Hartmann 2009). The MPO activity data relating

to the final step of PMN-endothelial interactions showed that only C/K-induced arthritis was sufficient to bring about significant increases in tissue infiltration by PMNs within the examined time frame. Taken together, our above data clearly suggest that PMN-mediated inflammatory reactions are less severe in the IR-challenged synovium than those in the periosteum or in response to arthritis.

5.2. Effects of different therapeutic interventions on the ICAM-1 expression

The increased tissue expression of ICAM-1 parallels the changes in PMN-endothelial interactions in all of the models examined. Among the endothelium-derived compounds, ICAM-1 is constitutively expressed on the endothelial surface and up-regulated in response to a variety of proinflammatory cytokines.

In Study I, the decreased tissue ICAM-1 expression in response to IPC and to exogenous CGRP clearly suggests a protective effect of physiological levels of CGRP on the leukocyte-endothelial cell interactions. Previous findings regarding the role of CGRP in ICAM-1-dependent PMN adhesion is rather controversial. An increased adhesion of PMNs to endothelial cells can be demonstrated by CGRP *in vitro* (Zimmerman 1992) and by capsaicin *in vivo* in human volunteers (Quinlan 1998), suggesting a pro-inflammatory effect of TRPV1 stimulation on the endothelial cells. On the other hand, an ameliorating effect of CGRP on the activation of endothelial cells (Harada 2003), adhesion molecule CD11b expression (by a cAMP-dependent mechanism (Monneret 2003)) and the superoxide production of PMNs (Tanabe 1996) have been demonstrated *in vitro*. It is conceivable that, during the course of the capsaicin-induced neurogenic inflammatory response, the acute and massive release of neuropeptides produces high local tissue neuropeptide concentrations which most probably exceed the amount of mediators released by pathophysiological stimuli (e.g. IR injury) *in vivo*. Hence, a careful consideration of TRPV1-positive stimulation is warranted in the different experimental settings using capsaicin-evoked neurogenic inflammation.

In Study II, arthritis was associated with a significant increase in the expression of ICAM-1 on the endothelial cells of the postcapillary venules. The relationship between ICAM-1 and carrageenan-induced inflammation has been clearly proven in other models (such as pleurisy and air pouch inflammation (Salvemini 2001)). Inflammatory cytokines increase ICAM-1 expression primarily through the activation of gene transcription by NF-κB binding to the ICAM-1 promoter (Roebuck 1999). The beneficial effect of PC on vascular ICAM-1 formation can be explained by its influence on TNF-α-induced NF-κB

transcription (Treede 2007). In a supplementary experimental series, we set out to validate the effect of PC on TNF-α-induced NF-κB transcription, and we therefore measured the TNF-α level in the synovial lavage fluid with ELISA (data not shown). C/K injection resulted in an increase, but, owing to the high data dispersion, the increase was not found to be statistically significant. Interestingly, the lowest TNF-α values were found in the PC-treated animals. On the basis of these novel *in vivo* data, a TNF-α-related effect of PC cannot be excluded, but this issue requires further clarification.

In Study III, IR injury was associated with a significant increase in the amount of ICAM-1 expressed on the surface of the periosteal postcapillary endothelial cells, but not in the synovium. The presence of ICAM-1 on postcapillary endothelial cells in the inflammatory synovial microenvironment has been shown previously (Hale 1989; Hartmann 2009) and has been proven to be an appropriate marker for the estimation of inflammation. Furthermore, based on previous observations of the inflamed synovium, adhesion molecules are expressed not only on the endothelial, but also on the interstitial cells (Edwards 1982; Burmester 1983; Henderson 1985). Accordingly, the C/K arthritis model provided a broad spectrum of microcirculatory inflammatory reactions, including enhanced ICAM-1 expression, demonstrating the lower microcirculatory vulnerability of the synovial membrane to IR.

5.3. Possible role of microhemorheological/perfusion changes in PMN-endothelial interactions

Microvascular injury is often accompanied by an increased heterogeneity of tissue perfusion, a reduction in functional capillary density and deteriorations in microvascular velocity (Wolfard 2002) and red blood cell deformability (Mikó 2006). It has also been recognized that expression levels of adhesion molecules and the consequent extent of leukocyte-endothelial interactions are strongly influenced by microhemodynamic parameters. As such, perfusion changes and secondary shear stress *per se* influence PMN activation and adhesive interactions by changing the dynamics and half-lives of molecular bonds (Marshall 2003). A higher velocity of PMNs results in higher degree of shear stress along the vessel walls, which reduces the possibility of firm adhesion to the endothelial cells (Kubes 1997). Owing to methodological limitations, we could determine velocities at the capillary level, but not at the venular part, where these cellular interactions take place. It is presumable, however, that potential relatively high (or preserved) microvascular velocities also have anti-inflammatory features in our settings. Alterations in microvascular

perfusion are typical manifestations of the postischemic injury in the periosteum (Wolfard 2002; Szabó 2009); hence, reduced microvascular velocities may influence the adhesion molecule expressions.

Study I targeted the role of CGRP in the beneficial effects of limb IPC. CGRP is known to be one of the most potent vasodilator in the body (Brain 1985) and the main source of the circulating CGRP is the spillover release of CGRP from the perivascular nerve terminals. Blood vessels in all vascular beds are surrounded by a rich perivascular capsaicin-sensitive innervation (Hill 1991; Dux 2009), and receptors for CGRP have been identified in the media and intima of resistance vessels (Sigrist et al. 1986). A local vasodilatory effect of exogenous CGRP has also been shown (De Hoon 2003), but the application of its specific antagonist $CGRP_{8-37}$ was without any effect. Hence, the role of endogenous CGRP in regulating the regional hemodynamics under resting conditions (Shen 2001) was reported to be rather unlikely. Furthermore, the postischemic macro- and microhemodynamic changes, and the anti-inflammatory effects of CGRP are probably independent of its presence in the (micro)circulation in view of the rather short half-life (~10 min) of the peptide. Nonetheless, in pilot studies we investigated the possible effects of different treatments on the hemodynamics in sham-operated animals. The mean arterial pressure and the cardiac output were measured by means of a flow probe situated on the mesenteric superior artery. RTX, applied as pretreatment 3 weeks before the experiments, or $CGRP_{8-37}$ given in a 3-h infusion, had no effects on these parameters of the macrohemodynamics as compared with control animals not treated with antagonists. The hCGRP bolus that was given prior to the ischemia resulted in a 5-min decrease in blood pressure, although no further changes occurred during the reperfusion period. Furthermore, the postischemic deterioration in microvascular velocity values was not basically influenced by either CGRP receptor antagonism or TRPV1 neuronal depletion. These data suggest that IPC and CGRP bring about their alleviating effect on cell-to-cell interaction not by affecting microhemodynamic changes, but rather by influencing adhesion molecule expressions directly.

C/K-induced local inflammation led to pronounced alterations in PMN-endothelial interactions, but not in RBCV values. PC did not influence microvascular velocities either. Hence, again, PC may potentially directly modulate adhesion molecule expressions and its action on PMN-endothelial interaction does not seem to be based on microhemodynamic differences. Clarification of the exact mechanism of its action requires further investigations.

Study III, however, provides clear evidence that the periosteum and the synovium are characterized by different microvascular velocities. Specifically, the velocities in the synovial membrane were considerably higher than those measured in the periosteum, even under resting circumstances. Furthermore, the IR affected microcirculatory velocities in the periosteum, but not in the synovium. The impact of these findings cannot be accurately judged because, owing to methodological limitations, these studies did not provide data on velocities in the venular side. It is likely, however, that higher velocities in the capillaries are most probably accompanied by higher perfusions in the venules where PMN-endothelial interactions take place. The relatively high microvascular velocities most probably have potential anti-inflammatory consequences. Consequently, we propose that a higher synovial microvascular velocity and a lesser degree of deterioration in this parameter may be responsible, at least in part, for lower PMN-endothelial interactions in the postischemic microvasculature of the synovial membrane. This assumption is supported by the notion that C/K induces not only a deterioration in RBCV, but also significant increases in both primary and secondary PMN-endothelial interactions in the synovial membrane. Further studies are required to assess the potential link between microhemorheological and adhesion molecule expression changes in the synovium and other tissue types.

6. SUMMARY OF NEW FINDINGS

- Osteoarthritis is accompanied by PMN-driven inflammatory reactions in the synovial membrane, which can be quantified appropriately by our new method using IVM.
- Limb IPC reverses the periosteal microcirculatory inflammatory reactions in which CGRP, released from the chemosensitive afferent nerves, plays a decisive role. This protection is manifested in an amelioration of leukocyte-endothelial interactions and endothelium-derived adhesion molecule expression. Controlled activation of chemosensitive afferent nerves by IPC may provide potential therapeutic benefits against IR-induced PMN reactions in the periosteum.
- Limb IR induces different microcirculatory consequences in the periosteum and the synovium. A postischemic deterioration of the microvascular perfusion, activation of PMN-endothelial interactions and an increased adhesion molecule expression occur in the periosteum, but these reactions are less intense in the synovium. The higher baseline microcirculatory velocities and a lesser degree of postischemic perfusion deficit in the synovium may explain the lower susceptibility of the synovial microcirculation to IR

injury.

- Similarly to diclofenac, exogenous PC, ameliorates knee joint inflammation, adhesion and the tissue accumulation of PMNs; the potential therapeutic benefit provided by PC may result from the reduction of PMN-mediated inflammatory pathways.

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9. ANNEX