Reperfusion injury of the periosteal microcirculation

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CONTENTS

List of abbreviations		**3**
List of papers related to the subject of the thesis		**4**
1.	**INTRODUCTION**	**5**
1.1.	The microcirculatory aspects of bone surgery. Importance of the periosteal microcirculation in bone autotransplantation	**5**
1.2.	Microvascular architecture of the bones and periosteum	**5**
1.3.	Methods of examination of the periosteal microcirculation	**7**
1.4.	Clinical causes of periosteal damage	**8**
1.5.	The ischemia-reperfusion (I-R) injury	**8**
1.5.1.	The role of leukocytes	**9**
1.6.	Endothelial cell-derived vasoactive mediators in microvascular homeostasis and pathologies	**10**
1.6.1.	Nitric oxide	**10**
1.6.2.	The endothelins. The role of endothelin-1 (ET) in I-R injury	**11**
1.7.	Endogenous protective mechanisms against oxido-reductive stress The potential role of endogenous phosphatidylcholine (PC)	**12**
2.	**GOALS**	**14**
3.	**MATERIALS AND METHODS**	**15**
3.1.	Surgical procedures	**15**
3.2.	Experimental protocol	**16**
3.3.	Intravital videomicroscopy	**17**
3.4.	Video analysis	**17**
3.5.	Histological analyses	**18**
3.6.	Biochemical analysis	**18**
3.7.	Statistical analysis	**18**
4.	**RESULTS**	**19**
4.1.	Effect of ET on the postischemic periosteal microcirculatory changes	**19**
4.2.	Effects of PC on the microcirculatory, biochemical and histological alterations caused by limb I-R	**22**
5.	**DISCUSSION**	**27**
6.	**SUMMARY OF NEW FINDINGS**	**31**
7.	**ACKNOWLEDGMENTS**	**32**
8.	**REFERENCES**	**33**
9.	**ANNEX**	**41**

List of abbreviations

I-R	ischemia-reperfusion
NO	nitric oxide
PMN	polymorphonuclear leukocyte
ET	endothelin
ET-A	endothelin A receptor
PC	phosphatidylcholine
MC	mast cell
FCD	functional capillary density
RBCV	red blood cell velocity
MPO	myeloperoxidase
MAP	mean arterial pressure

List of full papers relating to the subject of the thesis

Wolfárd A, Császár J, **Gera L,** Petri A, Simonka JA, Balogh A, Boros M. Endothelin-a receptor antagonist treatment improves the periosteal microcirculation after hindlimb ischemia and reperfusion in the rat. *Microcirculation* 9(6):471-6. 2002. IF (2002) = 2.125

Gera L, Varga R, Török L, Kaszaki J, Szabó A, Nagy K, Boros M. Beneficial effects of phosphatidylcholine during hindlimb reperfusion. *Journal of Surgical Research* 2006. (accepted for publication). IF (2005) = 1.956

Varga R, **Gera L,** Török L, Kaszaki J, Szabó A, Nagy K, Boros M. Effects of phosphatidylcholine therapy after hindlimb ischemia and reperfusion. *Magyar Sebészet* 2006. (accepted for publication). IF = 0

Abstract relating to the subject of the thesis

Varga R, **Gera L,** Kaszaki J, Szabó A, Ghyczy M, Boros M. Phosphatidylcholine treatment improves the periosteal microcirculation following hindlimb ischemia and reperfusion. European Surgical Research 36 (S1):14. 2004.

1. INTRODUCTION

1.1. The microcirculatory aspects of bone surgery. Importance of the periosteal microcirculation in bone autotransplantation

Vascularized bone autografts are frequently used in reconstructive surgery for the replacement of large bone defects. Despite the mastering of surgical techniques, reestablishment of the vascular supply in cases of vascular malperfusion still poses a clinical problem which jeopardizes the survival of transplanted grafts. A cascade of biochemical and microcirculatory mechanisms is implicated in this phenomenon during reperfusion (Schoenberg *et al.* 1985). Mainly because of methodological limitations, the exact components involved in the pathogenesis of bone ischemia-reperfusion (I-R) remain unknown.

Protection and feeding of the bone cortex are the major functions of the periosteum. It is a double-layered membrane covering the outer surface of the bones, except for the parts enclosed in the joint capsules, and is composed of an outer fibrous layer and an inner osteogenic cellular layer. The functions of the periosteum include the isolation of the bone from the surrounding tissues, providing a route for the circulatory and nerve supply. Moreover, the overall status of the bone is critically affected by the activity of the periosteum since it also influences the osteogenesis. Its elastic and contractile features preserve the shape of the bone, and it additionally plays a role in the maintenance of the metabolic and electrochemical gradient between the two sides of the membrane. A proprioceptive role too has also been attributed to the periosteum. As concerns the blood supply, the bone cortex exhibits predominance as compared with that of the centromedullary part, since about 70-80% of the arterial supply is directed toward the cortex (Chanavaz *et al.* 1995). The importance of the periosteal microcirculation is hallmarked by the observation that restoration of the periosteal microcirculation *per se* guarantees the survival of the bone graft even in an environment involving a moderate blood supply (Berggren *et al.* 1982). For this reason, the periosteal microcirculation is a good indicator of the perfusion changes of the whole bone, particularly during the early reperfusion phase after bone autotransplantation.

1.2. Microvascular architecture of the bones and periosteum

The blood supply of the bones is of fundamental importance in the mechanisms of growth, development and mineral exchange in bones. Despite many investigations there is

still much to learn about the blood circulation in human bone. This is due to many factors, such as the anatomical complexity of the vessels, the difficulty of demonstrating them in hard tissue and species differences between experimental animals and man.

The long bones receive their vascular supply from the nutritive arteries and the epiphyseal and metaphyseal vessels (for a review, see Hooper *et al.* 1987).

Normally, long bones have one major nutrient artery, which enters the medullary cavity through a canal in the shaft, accompanied by a vein (or veins) and nerve fibers. Short bones, such as those of the carpus and tarsus, are supplied by multiple nutrient arteries, which often enter the bone close together. The nutrient artery does not branch until after it enters the medullary cavity, where it divides into branches to supply the vessels of the marrow and the cortex.

1. The metaphysis receives its blood supply from branches of the nutrient artery and (more importantly) from the metaphyseal vessels, which originate from the arterial anastomoses around the joints (circulus vasculosus articuli). The metaphyseal arteries enter the bone through various nutrient foramina and then form anastomoses with branches of the nutrient artery. These anastomoses are not of functional importance unless the diaphyseal circulation is interrupted.
2. Epiphyseal arteries, like metaphyseal arteries, stem from the periarticular anastomoses. They usually enter the epiphysis through a non-articular area.
3. Periosteal arteries are small vessels which enter the periosteum at the attachments of the intermuscular septa and penetrate the bones in the same regions. The periosteal blood supply is reinforced by vessels entering the areas for the attachment of muscles. The blood supply of the bone cortex is primarily provided by the periosteum. The long and flat bones possess different microvascular organization. In the long bones, the capillaries form a rich vascular, shunt-like network close to the main supplying vessels, but run parallel to the axis of the bone at the remote sites. If the flat bones are thinner than 0.4 mm, only a periosteal and a dural network are present. In these areas, the bone layers are interconnected via large vessels which are independent of these networks. In the thicker areas, the structure shares the characteristics of long bones (possessing both periostal and endosteal regions).
4. The arteries of the medullary cavity (the nutrient and metaphyseal arteries) supply a network of sinusoids, which is the vascular lattice of the bone marrow. The capillaries that lie in the canals at the centers of the osteonal systems are supplied by branches of

the nutrient and metaphyseal arteries, although (as noted above) there is also a small supply from the periosteal arteries.

1.3. Methods of examination of the periosteal microcirculation

The microarchitecture of the periosteum can be analyzed by static methods (standard histology, India ink and gelatin techniques) or by dynamic approaches, such as the quantification of the blood flow via the colored microbead technique or by intravital microscopy. Although the microbead method provides information on the blood volume of the tissue mass of many organs, the peculiar size and anatomical conditions of the periosteum make this method relevant. In contrast, intravital microscopy (both fluorescent and nonfluorescent) permits *in vivo* examinations of the microcirculation, providing a possibility for quantification of its alterations in well-defined structures. Accordingly, microcirculatory disturbances of the periosteum and (with some limitations) of the junctions can be analyzed in rats and mice in clinically relevant models using intravital videomicroscopy.

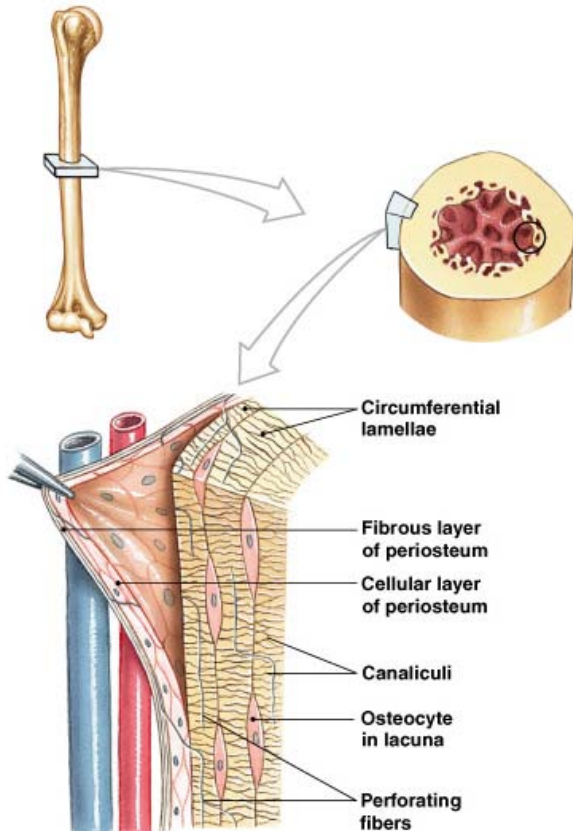

Figure 1. The vasculature of the periosteum and the outer (fibrous) and inner (cellular) layers (sec. Hooper *et al.* 1987.)

1.4. Clinical causes of periosteal damage

The rapid technical development and the widespread motorization cannot be followed by the sufficient expansion in size and quality of the road network, and therefore the occurrence of severe traumatological injuries has escalated in recent decades. Most traumatological interventions are performed under reduced blood flow conditions, elicited by the tourniquet method. These interventions *per se* cause a cascade of whole limb hypoperfusion-reperfusion. Traumatological injuries include multiple and open fractures and large bone defects. Apart from the bones, the periosteum can also be damaged which can lead to different degrees of bone healing disorders (delayed bone healing, pseudoarthrosis or sequester formation).

Apart from fractures, bone defects can also develop because of the radical resection of tumors. During such reconstructions, autotransplantation via vessel anastomoses is frequently used since the survival and incorporation of the transferred bone can be greatly enhanced by these interventions. The avascular bone necrosis of the femur head was earlier treated by applying vascular bone flaps, but this approach has now been completely replaced by the development of more suitable prosthesis techniques. Most of the autotransplantion and free flap procedures cause I-R injury of the bone.

Bone devascularization due to the impaired periosteal perfusion following fractures with severe soft tissue trauma has been proposed to precede and underlie perturbed bone healing. The pathogenetic influence of trauma-induced cellular and microvascular changes in the periosteum is highlighted by the clinical observation that extensive soft tissue injury and periosteal stripping typically precede delayed fracture repair and frequently result in a non-union or manifest pseudarthrosis (Gustilo *et al.* 1984, 1990, Esterhai *et al.* 1991, Kowalski *et al.* 1996, Utvag *et al.* 1998).

The aim of the present studies was to examine the I-R-induced microcirculatory reactions in the periosteum in clinically relevant animal models.

1.5. The ischemia-reperfusion injury

I-R initiates a cascade of pathophysiological events which in turn enhance local and remote tissue injury. Organ hypoperfusion and reperfusion generate a local inflammatory environment that primes circulating leukocytes, which provoke distant organ injury (Moore *et al.* 1994). In addition, capillary “no-flow” (as a result of capillary plugging) with prolonged ischemia and “no-reflow” (as a result of endothelial cell swelling and microcirculatory

occlusion by leukocytes) may *per se* initiate neutrophil activation (Barroso-Aranda *et al.* 1988).

With severe blood flow deficits and an impaired oxygen consumption, oxidative phosphorylation and metabolic functions are deranged (Menguy *et al.* 1974, Martin *et al.* 1987, Sodeyama *et al.* 1992). These alterations in local blood flow may also make the organs susceptible to I-R injury after resuscitation. Damage to many organs, such as intestinal reperfusion injury, appears to be mediated in part by leukocyte-derived oxygen free radicals (Parks *et al.* 1983, Hernandez *et al.* 1987) and can result from the accumulation of toxic oxygen radicals generated by xanthine oxidase in the tissues themselves. During the ischemic phase, xanthine dehydrogenase, an enzyme found in many cell types, undergoes irreversible conversion to xanthine oxidase, which, on the reestablishment of perfusion, forms the superoxide anion from hypoxanthine and molecular oxygen. Oxygen radicals have been implicated in several toxic pathways, including damage to cellular lipids, proteins, and DNA (Freeman *et al.* 1982, Powell *et al.* 1992).

As opposed to other organs, periosteal changes in response to I-R are not nearly so well characterized. The few reported studies of the periosteal microcirculation have focused on the effects of a flow reduction or soft tissue trauma-induced local microcirculatory reactions (Rucker *et al.* 2001, 2003, Menger *et al.* 2003, Schaser *et al.* 2003). For this reason, we set out to characterize the postischemic periosteal microcirculatory reactions in order to create a model of clinically applied interventions, such as bone autotransplantation and tourniquet-induced circulatory reactions.

1.5.1. The role of leukocytes

Investigations utilizing intravital microscopy have demonstrated that the recruitment of inflammatory cells into the perivascular tissue involves a complex cascade mechanism. The adhesion process consists of several steps, beginning with the rolling of polymorphonuclear leukocytes (PMN) on the endothelial surface of the postcapillary venules until they have slowed down to such a degree that they stick to the endothelium. At this point, the leukocytes are sequestered from the main vascular flow, and firm adherence to the endothelial cells may follow. Subsequently, the leukocytes pass an intercellular junction between the endothelial cells and reaches the abluminal side.

Three families of leukocyte-endothelial adhesion molecules have been identified: the selectins, the immunoglobulin gene superfamily, and the integrins. The selectin family comprises three proteins, designated by the prefixes L (leukocyte), P (platelet), and E

(endothelial). This is a class of cell adhesion molecules which mediate leukocyte rolling on the endothelium. P-selectin (CD62P), which is stored in the Weibel-Palade bodies of the endothelial cells, is rapidly mobilized to the plasma membrane in response to proinflammatory mediators such as thrombin or histamine (Bonfanti *et al.* 1989, Lorant *et al.* 1991). L-selectin (CD62L) is expressed on most types of leukocytes and is shed from the cell membrane by proteolytic cleavage after cellular activation. E-selectin (CD62E), which is not expressed on the endothelial cell membrane under basal conditions, is synthesized after stimulation by inflammatory mediators such as tumor necrosis factor-α (TNF-α) and endotoxin (Eppihimer *et al.* 1996). After the leukocyte has been arrested, integrins are activated by chemokines, chemoattractants and cytokines. During the transmigration process, a vascular dysfunction may occur due to the inappropriate release of oxidants, proteases and other potent mediators of the activated leukocytes.

1.6. Endothelial cell-derived vasoactive mediators in microvascular homeostasis and pathologies

1.6.1. Nitric oxide

Leukocyte reactions are linked by close ties to the actual state of the endothelial lining. Normal microvascular perfusion requires a stable environment; the cells must be maintained in a quiescent state, and intravascular cell adhesion must be regulated. Many of these homeostatic functions are served by the vascular endothelium, which acts as a local integrator of paracrine and autocrine signals. In this respect, one major factor responsible for vascular homeostasis is nitric oxide (NO), synthesized by the constitutively expressed endothelial enzyme NO synthase (eNOS or NOS 1), which oxidizes L-arginine, yielding NO and L-citrulline as products. The bioactivity of NO is particularly sensitive to oxidative stress as superoxide combines readily with NO in a diffusion-limited reaction to form peroxynitrite anion and its protonated form, peroxynitrous acid (Kissner *et al.* 1997). Unlike other intercellular messengers, NO does not bind to receptors, and its effects are transient and local. Under stress conditions, therefore, the critical balance between vasoconstrictors and vasodilators may be disrupted very quickly. Indeed, it has been suggested that an early endothelial dysfunction is probably a joint result of a decreased NO production and the generation of reactive oxygen species (Yokoyama *et al.* 1996, Lefer *et al.* 1999). As endogenous NO generation may be reduced by 90% in postischemic tissues, the loss of NO acts as the trigger mechanism (endothelial trigger), and this event becomes aggravated by the

involvement of leukocytes (i.e. the leukocyte amplification phase). The role of NO in I-R-induced damage was substantiated by the findings that the nonspecific inhibition of NO biosynthesis mimics the microvascular alterations (i.e. leukocyte adhesion and endothelial barrier disruption) observed after I-R (Lefer *et al.* 1999), even in the absence of additional inflammatory stimulation, and that this effect was reversed by L-arginine and other exogenous sources of NO (Kubes *et al.* 1993). Similar observations have been made in several tissues, suggesting that this is a universal phenomenon throughout the microcirculation, and this established the role of NO as a general modulator of the adhesive interactions between cells that may participate in the acute inflammatory response.

1.6.2. The endothelins. The role of endothelin-1 in ischemia-reperfusion injury

There is a growing body of evidence, that the release of endothelium-derived, potent vasoactive peptides, the endothelins (ETs), plays a crucial role in the development of I-R processes. The ETs embrace a family of 21-amino acid peptides produced by the endothelial cells. Three active isoforms (ET-1, ET-2 and ET-3) and two specific receptors for ET, the ET-A receptor and the ET-B receptor, have been identified and cloned. The ET-A receptor mediates vasoconstriction and has a high affinity for ET-1, whereas the ET-B receptor mediates vasoconstriction (ET-B_2) and vasodilation (ET-B_1 subtype), and has equal affinities for ET-1 and ET-3 (Clozel *et al.* 1992, Sumner *et al.* 1992, Shetty *et al.* 1993). Under physiologic conditions ET is produced predominantly by the endothelium, but in pathophysiological states other cells, such as leukocytes, macrophages, smooth muscle cells, cardiomyocytes and mesangial cells, can also be the source of ET release (Ehrenreich *et al.* 1992). ET production is regulated by both rheological and chemical factors, such as pulsatile stretch, shear stress and pH (Wesson *et al.* 1998). Hypoxia is considered one of the main stimuli for ET synthesis (Rakugi *et al.* 1990). Cytokines, adhesion molecules or vasoactive agents also stimulate ET production (Bodin *et al.* 1995). ETs not only mediate long-lasting vasoconstriction, but contribute to the induction of leukocyte and mast cell (MC) activation as well.

Although the exact pathomechanism of a reperfusion-induced microvascular dysfunction is still unclear, a number of recent data suggest that endothelium-derived vasoconstrictor ET peptides may play a decisive role in the sequence of I-R-related events via activation of the ET-A receptors (Rubanyi *et al.* 1994, Schlichting *et al.* 1995). It has been demonstrated that an upregulated ET-1 release induces vasoconstriction in various experimental and clinical pathologies induced by hypoxia or ischemia (Rubanyi *et al.* 1994).

Furthermore, it has been shown that ET-1 may cause endothelial cell-leukocyte interactions in the microcirculation *in vivo* (Boros *et al.* 1998). ET receptors are present on the vascular smooth muscle in bones (Filep *et al.* 1992), and the ET-A receptor subtype mediates vasoconstriction in the bone microcirculation (Coessens *et al.* 1996). However, the role of ET-1 in the bone microcirculation is still not clarified. Although exogenous ET-1 is a potent constrictor of isolated bone microvessels, it has also been shown that endogenous ET-1 does not actively regulate bone blood flow *in vivo* (Fleming *et al.* 2001).

In this study, we performed experiments to collect data on the microvascular alterations that occur in the tibial periosteum during complete hindlimb I-R in the rat. To investigate this question further, we administered specific ET-A receptor antagonist treatment during the reperfusion phase. We present evidence that anti-ET-A receptor treatment significantly affects both the sequence of leukocyte-endothelial interactions and the detrimental microhemodynamic changes in the periosteal microcirculation in an acute I-R situation.

1.7. Endogenous protective mechanisms against oxido-reductive stress. The potential role of endogenous phosphatidylcholine

Phosphatidylcholine (PC) is the most common and essential membrane-forming agent in the body. It has been shown, however, that I-R is associated with physical membrane defects which result in PC degradation and the exhaustion of endogenous PC sources (Jones *et al.* 1989, Gross *et al.* 1992, Bruhl *et al.* 2004). This observation suggests that PC supplementation may be beneficial in various diseases. This is supported by the notion that ischemic preconditioning restores the membrane stability with the simultaneous prevention of phospholipid degradation (Bruhl *et al.* 2004).

Stress induces the phospolipase D-catalyzed hydrolysis of membrane PC. This reaction leads to the endogenous production of phosphatidic acid and choline. Choline is a potent anti-inflammatory agent and is actively transported into the epithelial cells (Kuehl *et al.* 1957). Choline could form part of a defense mechanism which may operate against oxido-reductive stress in biological systems (Ghyczy *et al.* 2003). Furthermore, PC is taken up by phagocytic cells and accumulates in inflamed tissues (Cleland *et al.* 1979). *In vitro* studies have demonstrated that PC may protect against the membrane damage caused by bile salts (Martin *et al.* 1981, el-Hariri *et al.* 1992). Further, *in vivo* studies have revealed that choline is an essential nutrient for humans, and a choline deficiency may result in hepatic steatosis (Zeisel *et al.* 1991, Buchman *et al.* 1995). PC provides protection against many chemical

toxin-induced pathological conditions, and especially liver damage (Kidd *et al.* 1996).

In vitro and *in vivo* experiments have proved that topical PC protects the intestinal mucosa physically against the injurious actions of bile salts by forming less toxic mixed micelles (Barrios *et al.* 2000). Nevertheless, the experimental results and clinical experience suggest that PC could function as an active substance under certain *in vivo* conditions. The therapeutic effect of dietary PC in preventing esophageal strictures due to alkali-induced esophageal burns has been demonstrated in rats (Demirbilek *et al.* 2002), and parenteral PC and lyso-PC prolonged survival in experimental sepsis models (Drobnik *et al.* 2003, Yan *et al.* 2004).

It is widely believed that the biological efficacy of PC depends on the fatty acid moiety (Lieber *et al.* 1997). In contrast, some studies have revealed that the protective role of PC is independent of fatty acids, and it may be assumed that the active principle is choline. Phospholipase-D is activated by almost all stress factors resulting in the release of phospholipid metabolites, and several of these factors could be of importance in stress-induced defense reactions (Exton *et al.* 1999). Indeed, it has been shown that PC metabolites might relieve a potentially dangerous increase in the ratio of $NADH/NAD^+$ (reductive stress), a predisposing cause of oxidative damage (Ghyczy *et al.* 2003). This reaction sequence could explain the still incompletely understood, essential role of choline in the diet, and its preventive efficacy in a number of experimentally induced pathologies associated with a redox imbalance. It may be assumed that the endogenous pool of these metabolites may become exhausted during exogenous provocation, and that an exogenous supply might help to replenish and strengthen the endogenous protective mechanism.

Endogenous PC influences several pathways of the bone physiology, including the induction of bone formation (Han *et al.* 2003), the modulation of resorption (Kwak *et al.* 2004) and calcification (Bonucci *et al.* 1997). Due to the degradation of PC, the liberated choline can play an important protective role during intracellular redox imbalances (Ghyczy *et al.* 2003). Exogenous PC likewise exerts beneficial effects during ischemia (Duan *et al.* 1990), but its role in I-R-related microvascular changes is as yet undefined. For this reason, we set out to modulate the limb I-R-induced microcirculatory alteration by PC supplementation. The results suggest a marked therapeutic benefit of PC during the course of postischemic microcirculatory events in the limb.

2. GOALS

The main goals of the present studies were:

- to elucidate the microcirculatory alterations caused by limb ischemia-reperfusion. This included examinations of the efficacy of tissue perfusion, primary and secondary leukocyte-endothelial cell interactions, the tissue sequestration of leukocytes and the degranulation of mast cells;
- to clarify the role of endogenous endothelin in postischemic microvascular injury of the tibial periosteum by using two structurally unrelated inhibitors of the endothelin-A receptor;
- to investigate the effects of phosphatidylcholine supplementation on the above microcirculatory and tissue reactions.

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. Surgical procedures

The experiments were performed in two main series on male Wistar rats (average weight 300±35 g) that were housed in an environmentally controlled room with a 12-h light-dark cycle, they were deprived of food but not water 12 h before the experiments. The rats were anesthetized with sodium pentobarbital (45 mg kg^{-1} ip), 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, 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 (Figure 2).

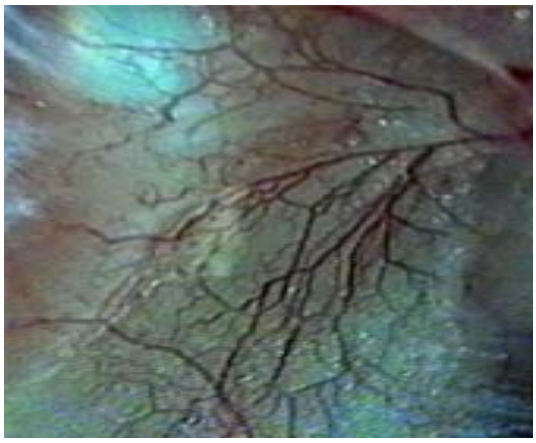

Figure 2. Microvasculature of the tibial periosteum under an operation microscope.

3.2. Experimental protocol

After a 30-min stabilization period, the baseline cardiovascular and microhemodynamic parameters were determined (baseline; t = -60 min). In the first series of experiments, the role of endogenous endothelin was examined. The animals were allotted into one or other of 4 experimental groups. The first group (n = 4) served as sham-operated controls to exclude microcirculatory changes related solely to the anesthesia and surgery. In group 2 (n = 5), complete hindlimb ischemia was induced by clamping the femoral artery with an atraumatic vascular miniclip (Mehdorn clip; Aesculap AG, Germany) and placing a tourniquet around the femur, immediately after the occlusion of the vessel. After ischemia for 60 min, the tourniquet and the artery clip were removed, and the reperfusion was observed for 180 min. In group 3 (n = 5), the experimental protocol was identical to that described above, except that the animals were treated with the specific ET-A receptor antagonist ETR-p1/fl peptide (VLNLCALSVDRYRAVASWRVI; Kurabo Ltd., Osaka, Japan) in a dose of 0.25 mg kg^{-1} (100 nmol kg^{-1}) at the beginning of reperfusion. ETR-p1/fl peptide is an antisense-homology box-derived compound with strong ET-A receptor inhibitor potency both *in vitro* and *in vivo* (Baranyi *et al.* 1998, Boros *et al.* 1998, Massberg *et al.* 1998). Previously, it was shown that the peptide significantly reduces the constrictor effect of ET-1 in isolated vessels and inhibits ET-1-induced Ca^{++} influx and cell proliferation (Baranyi *et al.* 1998). The ETR-p1/fl peptide was infused iv into the systemic circulation during 5 min (I-R+ETR-p1/fl group). An additional group of animals (n = 4) received another type of ET-A receptor antagonist, BQ 610 (100 nmol kg^{-1}, Sigma Chemical) iv at the beginning of reperfusion (I-R+BQ 610 group). The periosteal microcirculation was observed hourly during the 180-min reperfusion period.

In the second series of experiments, the effects of exogenous PC on the I-R-related microcirculatory disturbances of the periosteum and the neighboring muscles were examined. In this series, two groups of rats were subjected to complete hindlimb ischemia. Group 1 (n=7) was treated with the vehicle for PC, while Group 2 (n=6) received PC in a dose of 50 mg/kg iv for 10 min, starting 10 min after the beginning of reperfusion. The 5.0% PC solution (soybean lecithin, MW: 785, Phospholipon 90, Phospholipid GmbH, Cologne, Germany) was freshly prepared according to the description of the manufacturer. Further two groups served as sham-operated, vehicle- or PC-treated controls (n=7 and n=6, respectively). At the end of the experiments, muscle biopsies (m. gracilis anterior) for biochemical and histological examinations were taken from the operated and contralateral hindlimbs.

3.3. Intravital videomicroscopy

The right hindlimb with the exposed tibia was positioned horizontally on an adjustable stage and superfused with 37 ^{o}C saline. The microcirculation of the distal tibia was visualized by intravital microscopy (Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury lamp, Acroplan 20x water immersion objective), using fluorescein isothiocyanate (Sigma Chemicals, USA)-labeled erythrocytes (Ruh *et al.* 1998) (0.2 ml iv) for red blood cell staining (Figure 3), and rhodamine-6G staining (Sigma, St. Louis, MO, 0.2%, 0.1 ml iv) for leukocytes (Figure 4). The microscopic images were recorded with a charge-coupled device videocamera (AVT HORN-BC 12) attached to an S-VHS videorecorder (Panasonic AG-MD 830) and a personal computer.

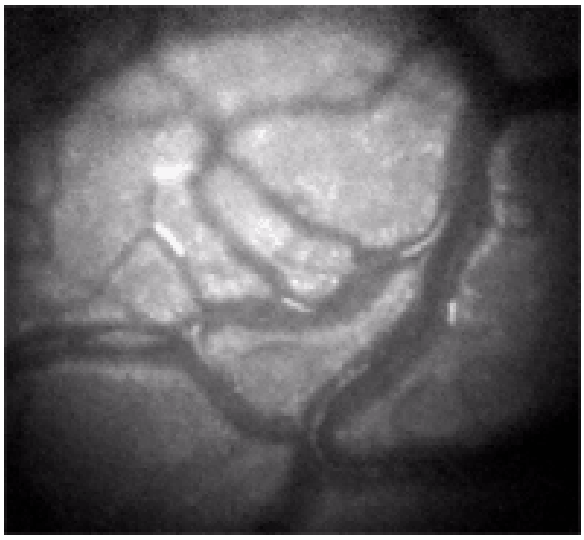

Figure 3.
Periosteal microcirculation under the intravital microscope (red blood cell staining)

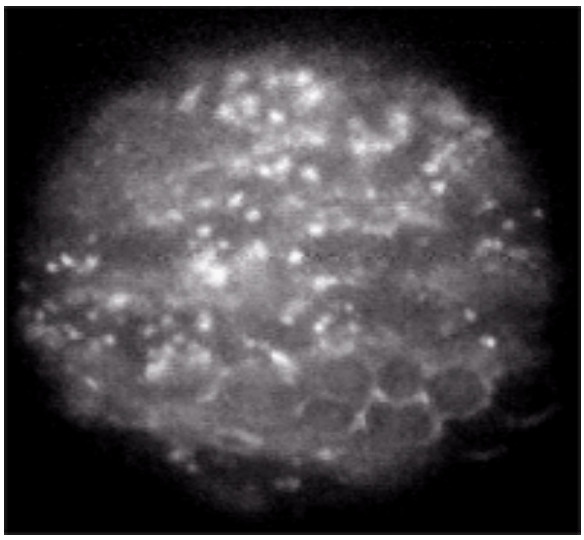

Figure 4.
PMN staining

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). Periosteal capillaries were located according to the description of Menger *et al.* (1997). The functional capillary density (FCD), i.e. the length of the perfused nutritive capillaries per observation area (cm^{-1}), and the red blood cell velocity (RBCV, $\mu m\ s^{-1}$) were 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 between 15 and 25 μm: Study 1; and between 11 and 20 μm: Study 2) 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. Rolling leukocytes were defined as cells moving at a velocity less than 40% of that of the erythrocytes in the centerline of the microvessel, and are given as a percentage of the number of nonadherent leukocytes passing through the observed vessel segment within 30 s.

3.5. Histological analysis

Samples of muscle biopsies were fixed in ice-cold Carnoy's fixative, embedded in paraffin, sectioned (6 μm) and stained with hematoxylin - eosin, acidic toluidine blue (pH 0.5) or alcian blue - safranin O (pH 0.4) (Szabó *et al.* 1997). MCs were counted in coded sections in 10 fields at an optical magnification of 400. Loss of intracellular granules, and stained material dispersed diffusely within the lamina propria, were taken as evidence of MC degranulation.

3.6. Biochemical analyses

The tissue myeloperoxidase (MPO) activity, as a marker of tissue leukocyte infiltration, was measured in muscle biopsies by the method of Kuebler *et al.* (1996). Briefly, samples were initially homogenized in 0.02 M potassium phosphate buffer at pH 7.4 containing protease inhibitor, and centrifuged at 20,000g for 20 min. The pellet was resuspended in 0.05 M potassium phosphate buffer at pH 6.0 containing 0.5% hexadecylammonium bromide. The suspension was sonicated, frozen-thawed 3 times and centrifuged again at 20,000 g for 20 min. The supernatant was then heated at 60 ^{o}C for 60 min to facilitate the recovery of MPO. An aliquot of supernatant was mixed with a solution of 1.6 mM tetramethylbenzidine and 0.002% hydrogen peroxide. The activity was measured spectrophotometrically as the change in absorbance at 650 nm at 37 ^{o}C. Results are expressed as units of MPO activity per gram of wet tissue.

3.7. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Nonparametric methods were used. Friedman repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline were assessed by Dunn's method. Differences

between groups were analyzed by Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In the Figures and Table, median values and 75th and 25th percentiles are given. *P* values <0.05 were considered significant.

4. RESULTS

In both experimental series, the baseline values of MAP did not differ significantly in the different groups, and there was no significant change in MAP in the sham-operated control group during the experimental period. In all I-R groups, a moderate decrease in MAP was observed in the first few minutes of reperfusion, but thereafter MAP stabilized at the control level (data not shown). Intravital microscopy revealed homogenous microvascular perfusion in the periosteum in all groups under the baseline conditions.

4.1. Effect of ET on the postischemic periosteal microcirculatory changes

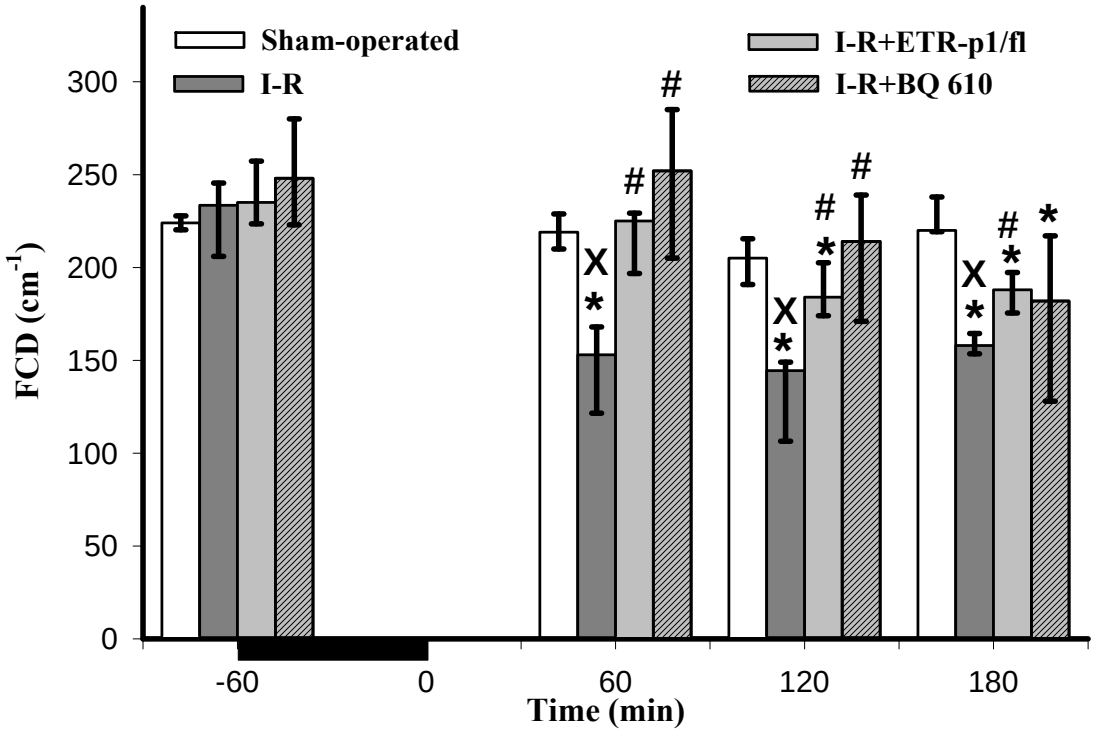

Figure 5. Functional capillary density (FDC) (cm^{-1}) of the periosteum in the sham-operated group (empty columns), the I-R group (dark-gray columns), the I-R+ETR-p1/fl group (gray columns), and the I-R+BQ-610 group (hatched columns). * $P < 0.05$ within the groups, as compared with the

preischemic value (X, $P < 0.05$) between the sham-operated and the I-R group, and # $P < 0.05$ between the I-R and the treated groups. Kruskal-Wallis one-way analysis of variance on ranks and Friedman test.

In the first experimental series in the sham-operated group, no significant change in periosteal FCD was observed (Figure 5). The FCD of the periosteum decreased significantly during reperfusion in the I-R group from 235 cm^{-1} to 153, 144 and 158 cm^{-1} at 1, 2 and 3 h, respectively. ET-A receptor inhibition significantly attenuated the reduction in FCD (225, 184 and 188 cm^{-1} after ETR-p1/fl peptide and 252, 214 and 182 cm^{-1} after BQ-610 at matching time points, respectively).

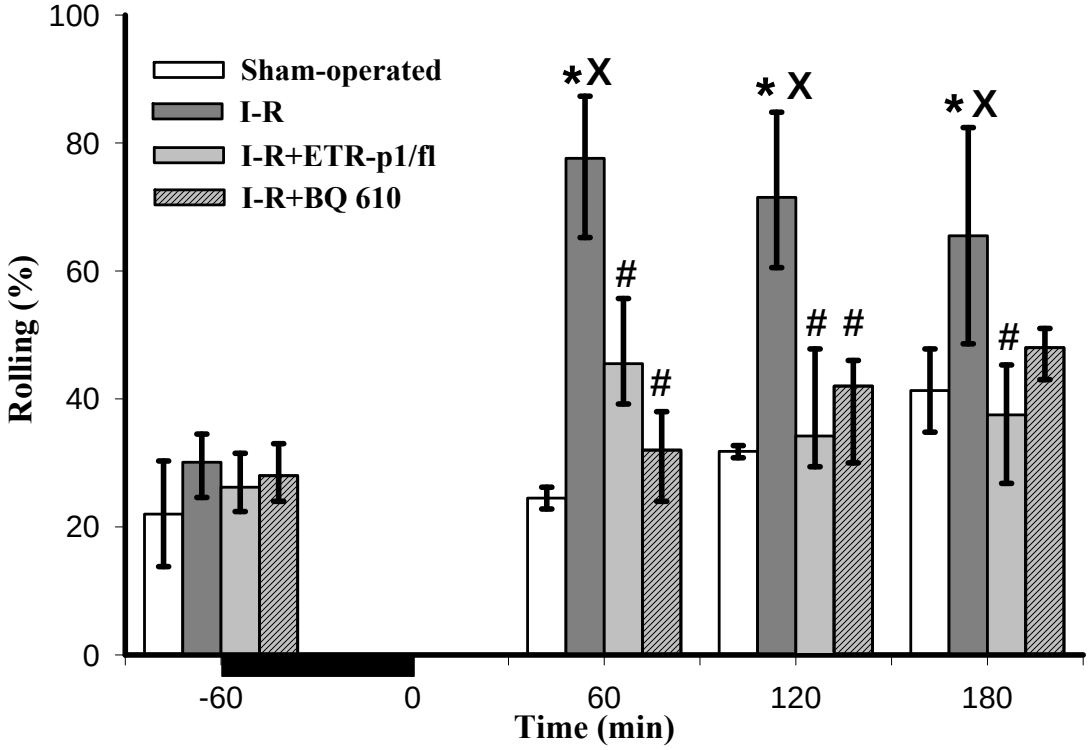

Figure 6. Percentage of rolling leukocytes in the postcapillary venules in the periosteum, in the sham-operated group (empty columns), the I-R group (dark-gray columns), the I-R+ETR-p1/fl group (gray columns), and the I-R+BQ-610 group (hatched columns). * $P < 0.05$ within the groups, as compared with the preischemic value (X, $P < 0.05$) between the sham-operated and the I-R group, and # $P < 0.05$ between the I-R and the treated groups. Kruskal-Wallis one-way analysis of variance on ranks and Friedman test.

A maximum of 30% of the nonadherent PMNs rolled along the endothelial lining of the postcapillary venules under the baseline conditions in the different groups (Figure 6). In

the sham-operated control group, there were no significant changes in the numbers of rolling and adherent PMNs at any of the observation points throughout the experiments (Figures 6 and 7). The 60-min ischemia and reperfusion was accompanied by a significant increase in leukocyte-endothelial cell interactions. Both the percentage of rolling cells and the number of adherent PMNs was increased as compared with the preischemic values or the values for the sham-operated group at matching time points (Figures 6 and 7). Both ET-A receptor antagonist treatments significantly reduced the PMN rolling and the number of adherent cells in the venules of the periosteum (45%, 34%, and 37% and 379, 515, and 393 mm^{-2} at 1, 2 and 3 h postreperfusion, respectively, after ETR-p1/fl treatment, and 32%, 42% and 48% and 275, 434, and 450 mm^{-2} at 1, 2 and 3 h postreperfusion, respectively, after BQ-610 treatment).

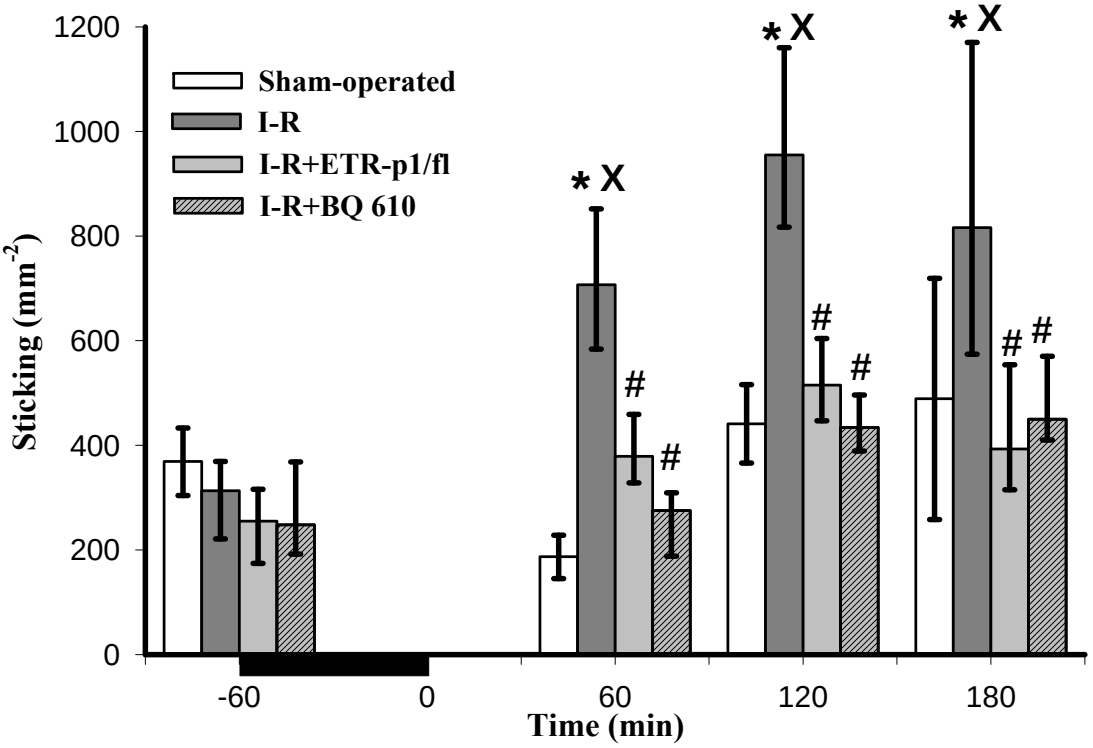

Figure 7. Number of adherent leukocytes (sticking, mm^{-2}) in the postcapillary venules in the periosteum, in the sham-operated group (empty columns), the I-R group (dark-gray columns), the I-R+ETR-p1/fl group (gray columns), and the I-R+BQ-610 group (hatched columns). * $P < 0.05$ within the groups, as compared with the preischemic value, X $P < 0.05$ between the sham-operated and the I-R-group, # $P < 0.05$ between the I-R and the I-R+ETR-p1/fl group. Kruskal-Wallis one-way analysis of variance on ranks and Friedman test.

4.2. Effects of PC on the microcirculatory, biochemical and histological alteration caused by limb I-R

In the second experimental series, the RBCV was similar in the different groups (median values ranging between 560 μm s^{-1} and 620 μm s^{-1}) and did not change over time in the sham-operated group (Table 1). I-R, however, led to a significantly decreased RBCV during the reperfusion period. The decrease was transient in the PC-treated animals, and the RBCV returned to the baseline during the later phase of the reperfusion.

In the sham-operated group, the periosteal FCD did not change significantly, but it decreased from a median value of 221 to 140 and 142 cm^{-1} on reperfusion for 60 min and 120 min, respectively (Table 1). In the PC-pretreated group, a transient FCD decrease was observed 60 min after the reperfusion was started, but in this group the I-R-induced capillary perfusion failure was alleviated.

TABLE 1

Groups	Parameters	Baseline	0 min	30 min	60 min	120 min	180 min
Sham	**RBCV (M)**	**620**	**601**	**663**	**598**	**577**	**597**
+	*(25p; 75p)*	*573; 635*	*534; 609*	*546; 729*	*580; 617*	*565; 592*	*575; 600*
vehicle	**FCD (M)**	**226**	**208**	**213**	**219**	**215**	**220**
	(25p; 75p)	*223; 229*	*197; 217*	*205; 216*	*212; 221*	*200; 225*	*218; 228*
Sham	**RBCV (M)**	**619**	**620**	**725**	**611**	**550**	**600**
+	*(25p; 75p)*	*565; 650*	*493; 664*	*667; 737*	*520; 741*	*530; 606*	*523; 689*
PC	**FCD (M)**	**214**	**211**	**209**	**217**	**209**	**221**
	(25p; 75p)	*207; 219*	*203; 221*	*204; 215*	*211; 223*	*191; 229*	*217; 227*
I-R	**RBCV (M)**	**560**	**327**x	**286**∗x	**278**∗x	**294**∗x	**230**∗x
+	*(25p; 75p)*	*533; 667*	*283; 439*	*261; 342*	*248; 370*	*239; 333*	*216; 301*
vehicle	**FCD (M)**	**221**	**147**∗x	**158**x	**140**∗x	**142**∗x	**161**x
	(25p; 75p)	*209; 233*	*141; 155*	*151;168*	*121; 153*	*139; 149*	*157; 167*
I-R	**RBCV (M)**	**586**	**372**∗x	**533**	**632**#	**572**	**568**
+	*(25p; 75p)*	*580; 650*	*311; 542*	*487; 600*	*555; 691*	*489; 591*	*476; 579*
PC	**FCD (M)**	**237**	**157**∗x	**177**	**192**	**202**	**210**
	(25p; 75p)	*194; 255*	*145; 160*	*170; 180*	*185; 210*	*195; 204*	*209; 212*

Table 1. Effects of phosphatidylcholine (PC) on ischemia-reperfusion (I-R)-induced red blood cell velocity (RBCV, μm s^{-1}) and functional capillary density (FCD, cm^{-1}) changes in the tibial periosteum in rats. M = median values; 25p and 75p = 25th and 75th percentiles, respectively. ∗ $P < 0.05$ vs the baseline; x $P < 0.05$ vs the corresponding sham-operated group; # $P < 0.05$ between the I-R groups. See the Materials and Methods for a description of the experiments.

In the sham-operated group, the numbers of rolling (Figure 8) and adherent leukocytes (Figure 9) did not change significantly throughout the experiments. In the vehicle-treated group, the proportion of rolling leukocytes increased from 20.8% at baseline to 34.4% and 40.0% after 120 min and 180 min of reperfusion, respectively (Figure 8), and significant increases were observed in the number of firmly adherent leukocytes at 120 min and 180 min of reperfusion (Figure 9). In the PC-pretreated animals, the elevations in the numbers of rolling and firmly adherent leukocytes were significantly lower than those in the control I-R group throughout the 180-min reperfusion period (Figures 8 and 9).

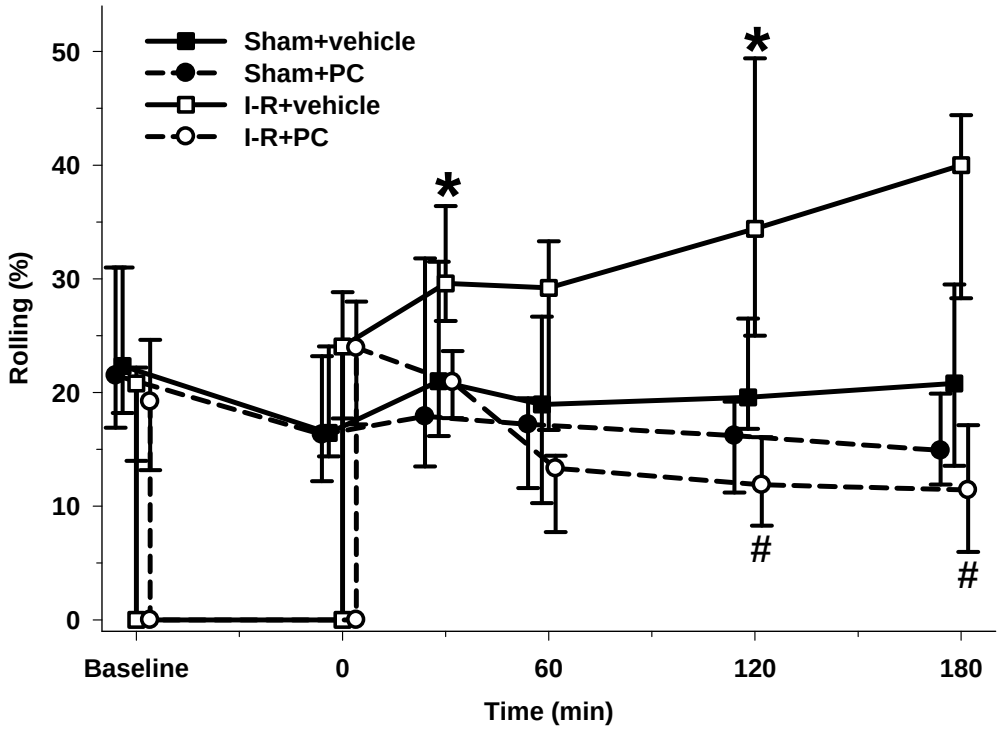

Figure 8. Primary leukocyte-endothelial cell interactions (rolling) in postcapillary venules of the tibial periosteum in the sham-operated controls (black rectangles), after 60-min ischemia and 180-min reperfusion in the animals receiving vehicle (empty rectangles) or an iv PC infusion (empty circles) in the second 10 min of reperfusion. Observations were made at baseline and after reperfusion for 0, 30, 60, 120 and 180 min. # $P < 0.05$ vs the I-R + vehicle group,. $P < 0.05$ vs the baseline values. Kruskal-Wallis one-way analysis of variance on ranks and Friedman test.

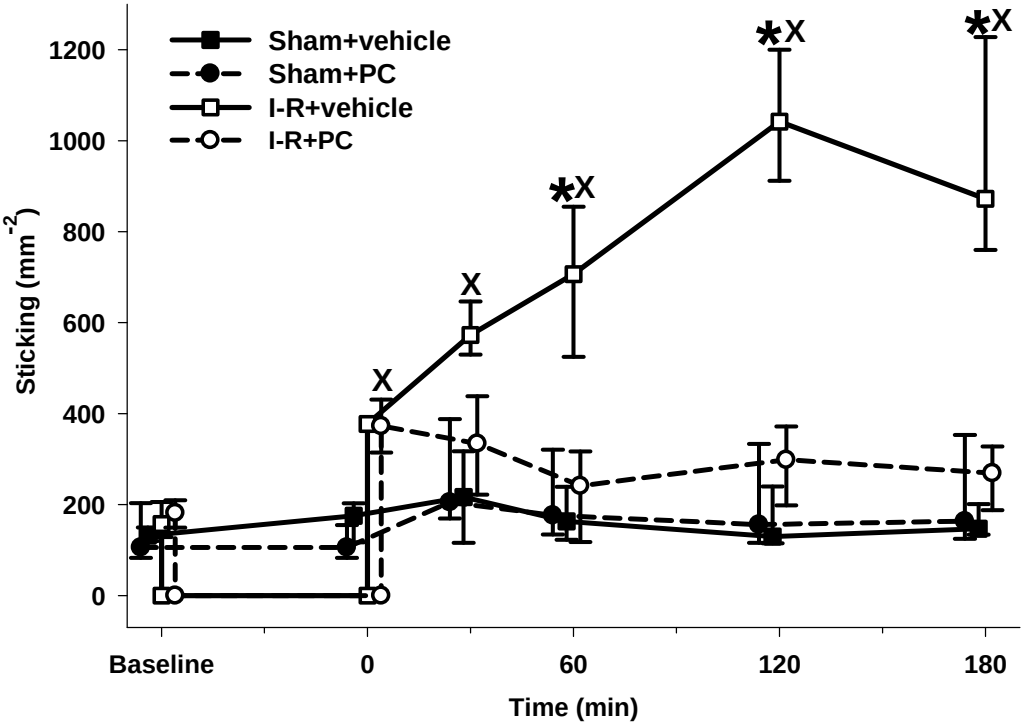

Figure 9. Secondary leukocyte-endothelial cell interactions (sticking) in postcapillary venules of the tibial periosteum in the sham-operated controls (black rectangles), after 60-min ischemia and 180-min reperfusion in the animals receiving vehicle (empty rectangles) or an iv PC infusion (empty circles) in the second 10 min of reperfusion. Observations were made at baseline and after reperfusion for 0, 30, 60, 120 and 180 min. # $P < 0.05$ vs the I-R + vehicle group,. $P < 0.05$ vs the baseline values. Kruskal-Wallis one-way analysis of variance on ranks and Friedman test.

In the vehicle-treated I-R group, the tissue MPO level was significantly increased approximately 3-fold as compared with that of the sham-operated animals and the contralateral nonischemic limb. In the PC-treated group, the MPO activity was significantly lower than in the vehicle-treated I-R group (Figure 10).

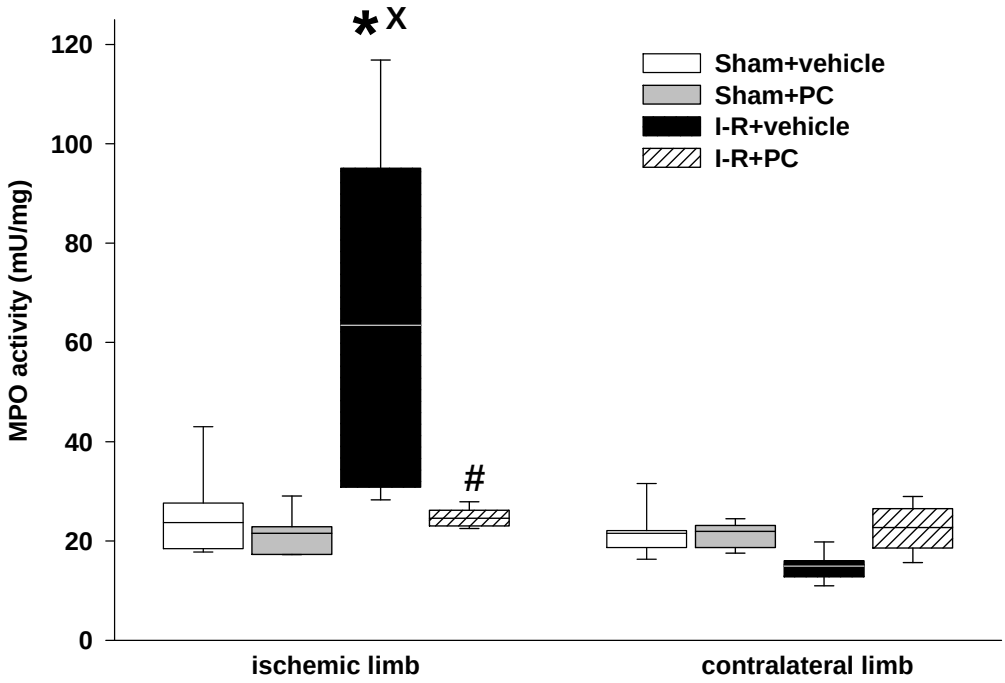

Figure 10. The muscle myeloperoxidase (MPO) activity was assessed at the end of the 240-min observation period in limbs subjected to sham operation (Sham) or to 60-min complete ischemia followed by 180-min reperfusion in the presence of vehicle (I-R + vehicle) or 50 mg kg^{-1} PC treatment (I-R + PC). The data are compared with those for the intact contralateral limbs. Median (thick line in the box), 25th percentile (bottom of the box), 75th percentile (top of the box), 5th and 95th percentiles (lower and upper whiskers, respectively) are indicated. # $P < 0.05$ vs the I-R + vehicle group, x $P < 0.05$ vs the sham-operated group,. $P < 0.05$ vs the contralateral limb. Kruskal-Wallis one-way analysis of variance on ranks and Friedman test.

In the sham-operated group or in the contralateral, nonischemic hindlimb, no significant increase in MC degranulation was observed in the muscle by the end of the observation period (Figure 11). I-R, however, caused a significant extent of MC degranulation. In these biopsies, the degree of MC degranulation was approximately 82.5%, whereas 7.6% degranulation was found in the contralateral limb. The PC pretreatment prevented the I-R-induced increase in MC degranulation (M = 20%, p25 = 18; p75 = 24), and the values were not significantly different from those for the sham-operated group or the samples from the contralateral limb.

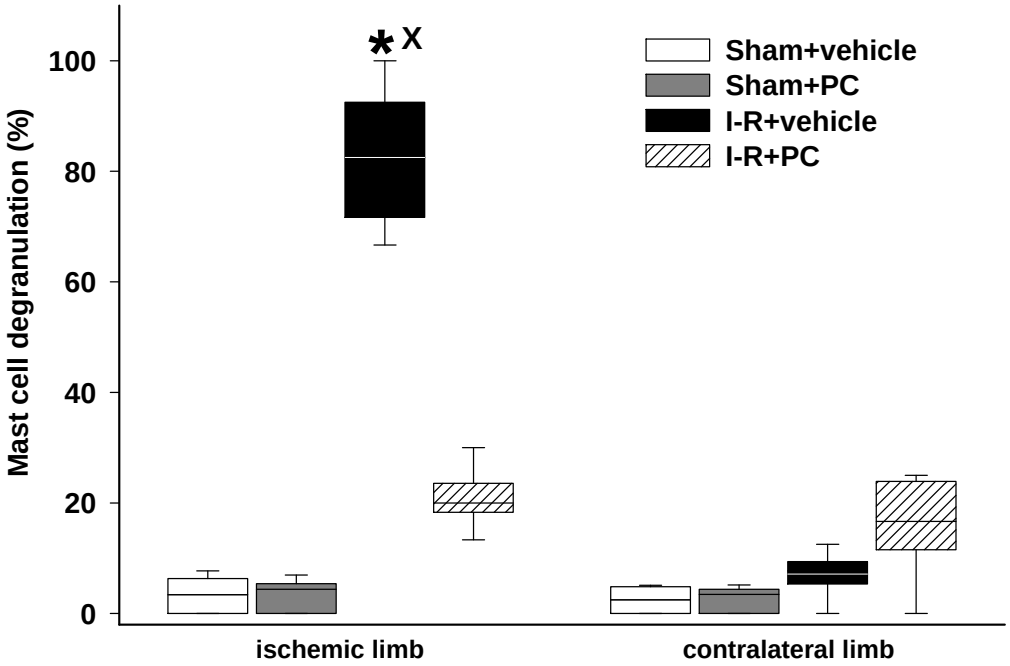

Figure 11. The changes in muscle mast cell (MC) degranulation (%) were assessed at the end of the 240-min observation period in limbs subjected to sham operation (Sham) or to 60-min complete ischemia followed by 180-min reperfusion in the presence of vehicle (I-R + vehicle) or 50 mg kg^{-1} PC treatment (I-R + PC). The data are compared with those for the intact contralateral limbs. Median (thick line in the box), 25th percentile (bottom of the box), 75th percentile (top of the box), 5th and 95th percentiles. # $P < 0.05$ vs the I-R + vehicle group, x $P < 0.05$ vs the sham-operated group, $P < 0.05$ vs the contralateral limb. Kruskal-Wallis one-way analysis of variance on ranks and Friedman test.

5. DISCUSSION

Open fractures of the extremities are often associated with delayed union or non-union, and the accompanying periosteal stripping may contribute significantly to the morbidity. Restoration of a compromised periosteal microcirculation is essential for infection prevention and the incorporation of microvascular bone grafts (Berggren *et al.* 1982). Despite meticulous microvascular surgical techniques and well-functioning feeding vascular anastomoses, ischemia and reperfusion may induce perfusion failure and severe tissue damage.

In the present experiments, we used a rat tibia model of a standardized ischemia-reperfusion challenge. Although the relationship of the bone microcirculation to the periosteum is poorly understood, several earlier studies have indicated that the blood supply of the bones depends mainly on the periosteal circulation (Berggren *et al.* 1982, de Saint-Georges *et al.* 1992). Accordingly, when a periosteal circulatory disturbance develops, it may influence the bone microcirculation too. Similarly, it has been recognized that maintenance of an adequate blood flow to the covering periosteal membrane is critical for the survival and function of the transplanted bone, and the microvascular blood supply is necessary for the further osteogenic and fibrogenic activity of the periosteum.

In our experimental study, a short period of ischemia induced a capillary perfusion failure, leukocyte-endothelial cell interactions in the periosteal microcirculation and significant MC degranulation in the adjacent muscle. Major components of this complex inflammatory reaction, including leukocyte-endothelial cell interactions in the periosteum, were effectively ameliorated by inhibition of the ET-A receptors and also by systemic PC supplementation.

Inefficacy of the microvasular perfusion was an accented indicator of injury in our model. During the reperfusion phase, the ratio of perfused capillaries decreased significantly, and thus a large proportion of the inflowing blood turned back into the venules without passing the capillaries. The reason for this shunt circulation may be precapillary vasoconstriction, but other reperfusion-related factors can also contribute to the reduction of FCD. The capillary no-reflow phenomenon may develop as a result of external compression induced by interstitial edema formation, or it may be due to intraluminal plug formation (Menger *et al.* 1997). In addition, we have observed increased leukocyte-endothelial cell interactions in the postcapillary venules. In the periosteal vessels with smaller diameters, the adherent and rolling white cells formed typical leukocyte plugs, thereby probably leading to obstruction of the venules. Since the enhanced leukocyte-endothelial cell interactions lead

eventually to leukocyte extravasation, this process plays an important role in reperfusion-associated late tissue injury too. It is recognized that neutrophils contribute significantly to I-R injury in many organs (Hernandez *et al.* 1987, Menger *et al.* 1997, Rucker *et al.* 1998). It has been shown that the depletion of circulating leukocytes does not *per se* counterbalance the consequences of the reduction in perfused capillaries in free-flap surgery, but the role of PMNs in causing an impaired capillary perfusion is nevertheless well established (Peter *et al.* 1998).

The mechanisms underlying I-R-induced microcirculatory disturbances are still a subject of debate, but a number of observations suggest that ET-A receptor activation is critically linked to the microcirculatory derangement in the reperfused tissues (Massberg *et al.* 1998, Wolfard *et al.* 1999). Indeed, Filep *et al.* have shown that ET-1 causes dose-dependent increases in vascular permeability through the activation of ET-A receptors as a consequence of disruption of the endothelial barrier (Filep *et al.* 1992). Here, warm I-R evoked a decrease in FCD and the accumulation of leukocytes in the rat tibial periosteum could be significantly influenced by antagonizing the ET-A receptor-mediated effects during reperfusion. ET-1 is one of the most powerful vasoconstrictor substances known to date; the vasoconstrictive effects are mediated predominantly via the ET-A receptors present on the vascular smooth muscle cells (Rubanyi *et al.* 1994). The ET-B receptors mediate vasodilation (ET-B1) and vasoconstriction (ET-B2) too, but the vasoconstrictor effect of ET-1 seems to be mediated only through the ET-A receptors in the bone (Coessens *et al.* 1996). ET-1 was first concluded to be a vasoactive protein, but more recently it has been considered to have widespread physiological functions that include regulation of the osteochondrogenic metabolism (Kitano *et al.* 1998, Stern *et al.* 1995). However, it has been shown that ischemia time-dependently increases ET-1 production, and this could lead to severe consequences in the bone microcirculation (Kato *et al.* 1998). The exact molecular mechanism of the pro-adhesive effect of ET-1 is still unclear; however, antibodies against P-selectin were recently found to reduce ET-induced leukocyte rolling in the rat (Sanz *et al.* 1999). On the other hand, it has been reported that ET-1 induces the expression of CD18 and CD11b adhesion molecules on the neutrophil surface and antibodies against CD18, E-selectin, and L-selectin are also able to inhibit ET-induced leukocyte adhesion (Lopez Farre *et al.* 1993, Zouki *et al.* 1999). Our present observations provide further support for the role of endogenous ET in the mediation of postischemic microvascular injuries.

The present study revealed not only an enhanced adherence of leukocytes to the periosteal postcapillary venules, but also an augmented tissue deposition of PMNs in the affected neighboring muscle. In sequence of methodological limitations, not the bone and the peritoneum, but the neighboring postischemic muscle could be subjected to histological evaluation. The results show that leukocyte accumulation in the postischemic periosteum and skeletal muscle was also accompanied by degranulation of the majority of MCs. The therapeutic intervention applied in the second study - the PC treatment - significantly ameliorated both alterations. In other studies, replenishment of the endogenous PC pool reduced tissue injury in the heart (Lieber *et al.* 1997, Bruhl *et al.* 2004). Although exogenous PC exerts protection in various experimental scenarios (Gabizon *et al.* 1986, Duan *et al.* 1990, Dunjic *et al.* 1993, Demirbilek *et al.* 2002, Yan *et al.* 2004), its mechanism of action is not fully understood. PC is taken up by phagocytic cells, and hence it accumulates in inflamed tissues (Cleland *et al.* 1979) and restores the mitochondrial function (Duan *et al.* 1990). In response to noxious stimuli, phospholipase-D is activated, which results in the release of phospholipid metabolites, several of which could be of importance in stress-induced defense reactions (Exton *et al.* 1999, Hansen *et al.* 2000). As such, it has been shown that PC metabolites may relieve a potentially dangerous increase in the NADH/NAD^+ ratio (reductive stress), a situation predisposing to oxidative damage (Ghyczy *et al.* 2001).

Our former observations lead us to believe that postischemic events of many tissue types, including that of the hindlimb, are complex and that the activation of MCs is a causative factor in this scenario (Boros *et al.* 1995, Szabó *et al.* 1997). Indeed, the protective action of PC supplementation was accompanied by a reduced degree of MC activation in the affected limb. Although MC activation has been associated to date with IgE activation, there is a growing body of evidence to suggest that MCs are depleted via oxidants, anaphylatoxins and bacterial products during I-R and are also extremely sensitive to very subtle changes in the surrounding milieu (Galli *et al.* 1993). Once activated, MCs generate and release newly-formed mediators and preformed granule-associated constituents with proinflammatory properties (Tannenbaum *et al.* 1980). Apart from these pathophysiological triggering effects, MC degranulation has been shown to result in the release of endothelium-derived factors such as ET (Boros *et al.* 1998). Therefore, it is conceivable that the ET-dependent inflammatory reaction is brought about the release of MC derived compounds. MCs undergo degranulation in response to compromised flow conditions and I-R injuries (Fawcett *et al.* 1951, Boros *et al.* 1995), and contribute to leukocyte sequestration (Thorlacius *et al.* 1994, Gaboury *et al.* 1995).

Although a wide range of protective functions have been attributed to MCs in the bone (mostly related to the mediation of early and late stages of bone healing) (Lindholm *et al.* 1967, 1970, Saffar *et al.* 1990), these cells also appear to play an effector role in I-R-related tissue injuries. MCs undergo degranulation upon reperfusion (Kanwar *et al.* 1994, Szabó *et al.* 1997), and it has been shown that granulocyte recruitment is closely related to MC activation, even after remote ischemia (Schmeling *et al.* 1989, Kubes *et al.* 1994). The enhanced adhesion of leukocytes is attributed to a triggering role of MC-derived mediators on the expression of several adhesion molecules (e.g. P-selectin, β2 integrin and ICAM-1) (Kubes *et al.* 1994, Gaboury *et al.* 1995). Similarly, it has been demonstrated that both PMNs and endothelial cells possess receptors for MC-derived proteases that directly modulate adhesion molecule expression and leukocyte-endothelial interactions (Shpacovitch *et al.* 2004, Meyer *et al.* 2005).

The present research protocol did not allow an assessment of MC degranulation in the periosteum itself, but only in the surrounding skeletal muscle. Systemic PC administration decreased both the leukocyte recruitment and the MC degranulation in this compartment, which strongly suggests that PC-induced MC stabilization was at least partially involved in the beneficial microcirculatory responses in the postischemic periosteum, too. This result is in line with our previous observation that PC pretreatment inhibited MC degranulation in a canine model of experimental esophagitis (Erős *et al.* 2006). However, the inhibition of leukocyte adherence, together with the improved microvascular flow, could also contribute to the overall tissue protection in the affected area during reperfusion. The exact mechanism of action of PC on MC degranulation remains to be established: more direct (e.g. *in vitro*) approaches are needed to define the effects of PC on MC reactions. The present studies do not allow an examination of the causal relationship between ET release and MC degranulation, but we think that PC supplementation exerts an effect on the final steps of this pathway. Further studies are warranted to elucidate the relative contribution of PC to endothelial cell membrane protection and to influence the mast cell stabilization and the PMN-mediated microvascular injury.

6. SUMMARY OF NEW FINDINGS

1. Our *in vivo* experiments permitted quantification of the microcirculatory alterations caused by limb I-R in a clinically relevant animal model.
2. The I-R injury was manifested in a deterioration of the efficacy of periosteal microvascular perfusion, the escalation of PMN-endothelial interactions and the activation and degranulation of the adjacent muscle mast cells.
3. Postischemic injury of the periosteum is accompanied by the release of vasoconstrictor mediators such as ET, which participates in the mediation of a perfusion deficit and microcirculatory inflammation. The inhibitors of ET-A receptors applied in this study greatly ameliorated these changes; hence, most of the detrimental actions of ET are mediated by ET-A receptors in the periosteum.
4. PC also exerted a marked alleviating effect in the above model. PC supplementation efficiently decreased the harmful consequences of limb I-R-induced microcirculatory perfusion failure and inflammatory reactions in the rat. Its action was also accompanied by reduced PMN sequestration and stabilization of MCs in the postischemic tissues. These data suggest a therapeutic potential for parenteral PC with a view to decreasing the harmful consequences of ischemia-induced tissue reactions.

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9. ANNEX