Local and remote microcirculatory effects of transient ischemia of the lower extremities in rats

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LIST OF FULL PAPERS RELATING TO THE SUBJECT OF THE THESIS

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II. Szabó A, Hartmann P, Varga R, Jánvári K, Lendvai Z, Szalai I, Gomez I, Varga G, Greksa F, Németh I, Rázga Z, Keresztes M, **Garab D**, Boros M. Periosteal microcirculatory action of chronic estrogen supplementation in osteoporotic rats challenged with tourniquet ischemia. *Life Sci.* 2011; 88(3-4):156-162 **(IF: 2.451)**

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LIST OF ABSTRACTS RELATING TO THE SUBJECT OF THE THESIS

IV. **Garab D**, Bodnár D, Nyíri S, Pócs L, Janovszky A, Boros M, Szabó A. Periosteal microcirculatory effects of limb ischemic preconditioning in ovariectomized rats. *Eur Surg Res.* 2014; 52:pp 130

LIST OF ABBREVIATIONS

AD-SoS	amplitude-dependent speed of sound
ALT	alanine-aminotransferase
ANOVA	analysis of variance
AST	aspartate-aminotransferase
E2	17β-estradiol
ELISA	enzyme-linked immunosorbent assay
HMGB1	high-mobility group protein B1
H_2O_2	hydrogen peroxide
CGRP	calcitonin gene-related peptide
ICAM-1	intercellular adhesion molecule-1
IPC	ischemic preconditioning
IL	interleukin
IR	ischemia-reperfusion
IVM	intravital videomicroscopy
LDH	lactate-dehydrogenase
MPO	myeloperoxidase
NO	nitric oxide
NOS	nitric oxide synthase
NOX	NADPH oxidase
O2C	"oxygen to see" (modified lightguide spectrophotometry)
OVX	ovariectomy
PAF	platelet activating factor
PMN	polymorphonuclear leukocyte
RBCV	red blood cell velocity
rHb	tissue hemoglobin content
rIPC	remote ischemic preconditioning
ROS	reactive oxygen species
S_{O2}	tissue oxygen saturation
TNF-α	tumor necrosis factor-alpha
XOR	xanthine oxidoreductase
VCAM-1	vascular adhesion molecule-1

CONTENTS

SUMMARY

Tourniquet-induced limb ischemia is a generally applied maneuver in orthopedic-trauma practice which brings about marked local and systemic inflammatory effects. The affected tissue undergoes typical biochemical and microcirculatory changes of ischemia-reperfusion (IR) injury, primarily the periosteal microcirculation being affected in this process. The potential negative clinical outcome may include perturbed bone healing, delayed union or pseudoarthrosis formation. Since a tourniquet applied around the limb causes IR of an appreciably high amount of tissue, venous effluent or afferent neurogenic signals of the affected limb bring about further systemic inflammatory reactions. On the other hand, the metabolites released after limb IR may be involved in the protection of remote (or inter-organ) ischemic preconditioning (IPC), a phenomenon in which transient, brief periods of ischemia followed by short intervals of reperfusion afford protection against subsequent ischemia by increasing the ischemic tolerance of the tissues of remote organs.

The major aim of our study was to examine the local and remote microcirculatory effects of transient lower limb ischemia. In this context, our first objective was to observe the effects of osteoporosis and the consequences of estrogen therapy on the local periosteal microcirculatory reactions in a clinically relevant time frame in rats subjected to ovariectomy (OVX). The results showed that OVX does not predispose the periosteal microcirculation to enhanced IR-induced inflammatory complications such as local leukocyte activation and inflammatory cytokine release, whereas estrogen therapy has a pronounced ameliorating effect in the periosteal microcirculation. Further, we demonstrated that the beneficial microcirculatory effects of local IPC are lost after OVX, suggesting that endogenous estrogen plays a potential role in the protection provided by IPC.

In the second major part of the studies, the potential impact of IPC elicited at the limb on the postischemic liver injury (remote IPC) was examined from several aspects of inflammatory activation on liver IR injury, with special emphasis on the free radical-producing nicotinamide adenine dinucleotide phosphate-oxidases (NOXs). The data revealed that remote IPC reverses a hepatic microcirculatory perfusion deficit and inflammatory reactions, restores tissue oxygenation and reduces proinflammatory cytokine and necroenzyme levels. These beneficial effects are associated with a reduced NOX2 expression, suggesting a potential role of NOX2 in the mechanism of protection provided by remote IPC.

1. INTRODUCTION

1.1. Local biochemical and microcirculatory manifestations of ischemia-reperfusion (IR) injury

Re-establishment of the blood flow after tissue ischemia leads to a controversial circulatory phenomenon usually termed IR injury. Temporary ischemia *per se* initiates harmful processes in the endothelial cells and the surrounding tissues because of the severely compromised blood flow and the oxygen and energy deficits of the affected tissues. Although reestablishment of the circulation is essential for survival, it usually worsens the tissue damage caused by the ischemic phase alone (Nishida T 1987, Reynolds JM 1989).

An IR injury is characterized by various detrimental local and remote effects. The local reaction is closely linked to the nearly immediate production of reactive oxygen species (ROS), a phenomenon known as the oxidative burst. This intracellular biochemical reaction is based on sequential univalent reductions of molecular oxygen, leading to the generation of superoxide radicals, and then to hydrogen peroxide (H_2O_2) and hydroxyl radicals. One of the major sources of superoxide generation is the enzyme xanthine oxidoreductase (XOR). The oxidase form of XOR is irreversibly converted from xanthine dehydrogenase during ischemia, which generates superoxide anions from hypoxanthine and molecular oxygen, basically from the ATP content of the cells (McCord JM 1982, Kurose I 1994a). Another important source of ROS formation is nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase, NOX), a membrane-bound enzyme complex found in the plasma membrane and in the phagosomal membranes of polymorphonuclear neutrophil leukocytes (PMNs) (Rybicka JM 2010).

At the microcirculatory level, these reactions are exacerbated by inhibition of the biological activity of nitric oxide (NO), a highly effective vasodilator molecule with important anti-adhesive characteristics (Gaboury J 1993). A variety of cells, including vascular endothelial cells, synthesize NO from the guanido group of L-arginine, and the inhibition of NO synthesis evokes the recruitment of adherent PMNs (Kubes P 1991, 1993). The inactivation of NO by its reaction with superoxide causes peroxynitrite formation, which exerts further detrimental effects (Beckman JS 1996). Ultimately, the oxygen- and nitrogen-centered radicals damage cellular components such as DNA, membrane lipids and proteins (Freeman BA 1982, Powell SR 1992).

The subcellular biochemical changes are closely linked to deteriorations in the microcirculation. ROS and peroxynitrite cause impaired endothelium-dependent vasodilation and increased microvascular permeability (Björk J 1982, Parks DA 1983, Beckman JS 1996). Furthermore, tissue damage leads to an enhanced release of proinflammatory cytokines (e.g. interleukins (ILs), platelet activating factor (PAF) and tumor necrosis-factor alpha (TNF-α)), which results in an increased expression of adhesion molecules responsible for the interactions between endothelial cells and PMNs (Pohlman TH 1986, Sugama Y 1992). Moreover, ROS formation directly induces the adhesion of PMNs in the postcapillary venules (Suzuki M 1989, 1991), together with platelet−leukocyte adhesion and platelet aggregation (Iuliano L 1994, Leo R 1997). Finally, the recruited PMNs transmigrate from the endothelial surface of the lumen to the surrounding tissues across the postcapillary venules. Activated PMNs secrete further ROS, enhancing the inflammatory reactions in the microcirculation (Grisham MB 1986, Wankowicz Z 1988).

It is well established that leukocyte−endothelial cell interactions are decisive events among the complications of IR injury, because the extent of tissue damage and the number of recruited PMNs are interrelated phenomena (Harris AG 1996). As a consequence of an oxidative burst, the damaged endothelial cells produce various chemotactic factors (e.g. PAF, leukotriene B_4 and chemokines), which facilitate the movement of leukocytes toward the affected site of inflammation (Kurose I 1994b, Wanner GA 1996). The recruitment process involves at least two different stages, an initial low-affinity binding and a later high-affinity interaction that results in firm adhesion. The first step is “rolling” or tethering and momentary halting of the movement of PMNs along the vessel wall. It is mediated by cell surface glycoproteins known as selectins. The L-selectins are expressed constitutively on the leukocyte surface, but the binding affinity increases via activation of the cell (Li X 1998). The P- and E-selectins are expressed on the endothelial cell surface. The P-selectins are constitutively stored in a preformed state in the Weibel-Palade bodies of the endothelial cells, and translocated to the outer cell membrane within a few minutes after stimulation by thrombin, histamine or ROS (Lorant DE 1991). E-selectin appears on the endothelial surface 1-2 hours following inflammatory stimulation by endotoxin and proinflammatory cytokines (e.g. IL-1, TNF-α) (Chamoun F 2000). The next step in this process is the firm adhesion or “sticking” of the PMNs to the inflamed endothelium via integrins. For strong adhesive interactions, PMNs shed L-selectins and increase the expression of ß2 integrins (CD11a,b,c/CD18)

(Springer TA 1990), especially CD11b/CD18, which is induced by IR injury (Kurose I 1994b, Koike K 1995). At the endothelial site, the receptors of different integrins are to be found (intercellular adhesion molecules-1 and -2 (ICAM-1 and ICAM-2), vascular adhesion molecule-1 (VCAM-1) and platelet endothelial cell adhesion molecule-1), which are members of the immunoglobulin supergene family of cell surface molecules (Williams AF 1988). ICAM-1, abundantly present on the endothelium as the main receptor for ß2 integrins (Diamond MS 1990), is upregulated in response to cytokine stimulation (Groves 1992; Gaboury JP 1995). Last, but not least, the diapedesis of PMNs to the inflamed tissues occurs through tight junctions between the adjacent endothelial cells (Ginzberg 2001).

Swelling of the endothelial cells and edema formation resulting from the plasma leakage into the surrounding tissues through postcapillary venules (Mazzoni MC 1989, Gute DC 1998) are also typical manifestation of IR injury and, as a consequence of plasma loss, the blood is concentrated (Menger MD 1997). All these events may lead to the "no-reflow" phenomenon, the occlusion of capillaries by the plugging of blood cells at the time of reperfusion (Menger MD 1992).

1.2. Distant effects of ischemia

Apart from its local inflammatory effects, the ischemia of certain organs causes inflammatory reactions at distant anatomical sites. Generally speaking, inflammatory mediators released as a consequence of the reperfusion of any organ may activate endothelial cells in remote organs (that are not exposed to the initial ischemic insult), this resembling the phenomenon of the systemic inflammatory response syndrome (Carden DL 2000). In a classical experiment, plasma taken from a rat previously subjected to limb ischemia caused the oxidative burst of naive PMNs *in vitro* (Goldman G 1990), suggesting the role of released humoral factors in this reaction. Intestinal ischemia induces simultaneous increases in intestinal and lung phospholipase A2 activities and PMN accumulation (Koike K 1992), which appear to be also mediated by XOR (Koike K 1993). In another study, IR-induced lung injury appeared to be mediated by complement C5a (Fleming SD 2003).

Several studies have been conducted that involved limb IR. It was shown that limb IR causes systemic inflammatory reactions (Szabó A 2009) and hepatic injury via reactions mediated by circulating proinflammatory cytokines (e.g. TNF-α, PAF, IL-1β and IL-6) and activated PMNs (Lawlor DK 1999). Limb ischemia also brings about marked microvascular permeability and hemorrhage in the affected muscle and in the

remote lung, in association with simultaneous TNF-α and IL-1 release (Seekamp A 1993). Hepatic injury may occur as a remote consequence of limb IR. The damage is characterized by hepatic PMN recruitment via Kupffer cell-dependent activation, and it has been suggested that PMNs are the common link between the two organs (Vega VL 1999).

1.3. Clinical aspects of limb ischemia

Most clinical cases of limb ischemia result from chronic peripheral vascular diseases caused by atherosclerosis or diabetes, whereas acute cases are mostly due to arterial thrombosis, embolism, or less frequently trauma or tumors. Acute iatrogenic limb IR occurs during vascular surgery and orthopedic-trauma practice. In the former cases, subrenal clamping of the aorta may cause severe local tissue (primarily muscle) hypoxia, which may further lead to myonephropathic-metabolic syndrome.

In orthopedic-trauma surgery, transient limb ischemia achieved with a tourniquet (wrapping a band around the extremity) is used primarily for emergency or surgical indications. It is frequently applied during elective orthopedic interventions, when the procedures (e.g. autotransplantion or free flap transfer*)* require a bloodless operative field. The advantages of a tourniquet include a clear operating field, reduced overall blood loss and decreased risk of microemboli at the time of release (Sharma JP 2012). This manuever, however, leads to ischemia of the affected extremity, with the subsequent detrimental effects relating to the inevitable reperfusion phase and consequent iatrogenic IR injury. Along with the targeted bone and surrounding soft tissues, the periosteum is also affected in this process. Apart from important nutritive functions and vessel and nerve content, the periosteum plays an important role in the bone metabolism, mostly via its osteoprogenitor cell content, which is necessary for proper bone healing. The pivotal role of the periosteum is underlined by clinical observations that periosteal damage leads to perturbed bone healing as delayed union or pseudoarthrosis formation (Gustilo RB 1990, Esterhai JL 1991, Utvag SE 1998). Although transient limb ischemia can certainly cause microvascular disturbances in the periosteum (Wolfárd A 2002), the previous studies were mainly focused on the consequences of flow reduction and soft tissue trauma-induced local microcirculatory reactions (Rucker M 2001, Menger MD 2003, Schaser KD 2003), and the microcirculatory reactions in response to IR injury have not been well characterized.

1.4. Ischemic preconditioning (IPC)

1.4.1. The phenomenon of local IPC

IPC is a method aimed at increasing the ischemic tolerance of the tissues. During IPC, transient, brief periods of ischemia are followed by short intervals of reperfusion, affording protection against subsequent prolonged and deleterious ischemia. This approach was first described in connection with the heart (Murry CE 1986), but was later demonstrated in many organs (Gho BC 1996, Cheung MM 2006). Ischemic postconditioning was also recently described during the reperfusion period after a sustained ischemic episode (Tsang A 2004), but this topic is not included in the present thesis. The protection afforded by IPC involves the action of several endogenous vasodilator mediators, including NO, adenosine and carbon monoxide (for reviews, see Walsch SR 2007, Tapuria N 2008). One important aspect of IPC is the microcirculatory reaction with protective consequences in the postischemic vasculature (Koti RS 2002, Mallick IH 2005, Starr A 2008). IPC moderates the oxidative burst by decreasing the intracellular production of ROS (Quarrie R 2012) and also extracellular ROS from adherent leukocytes or liver Kupffer cells (Tejima K 2004). IPC downregulates the expression of adhesion molecules responsible for PMN adhesion and transmigration, and hence it alleviates tissue damage (Duda M 2006). As ATP is metabolized rapidly in consequence of an oxygen deficiency during ischemia, adenosine production is increased. The enhanced adenosine release evoked by IPC stimulates endothelial nitric oxide synthase (NOS), leading to NO release, improving the microcirculation and tissue oxygenation (Peralta C 1997). Moreover, IPC activates various intracellular protein kinases (e.g. PKC, p38 MAPK) mediating the gene transcription of proteins that have protective roles in IR (Mitchell MB 1995, Nakano A 2000, Yellon DM 2003). Neurogenic components of this protection have also been demonstrated (Chai W 2006). Human studies have revealed the reduction of postischemic organ injuries, for instance in the liver, where IPC is elicited by intermittent portal inflow occlusion during liver resection (Clavien PA 2000). In experimental settings, IPC exerts protection by affecting the adenosine- and cAMP-dependent pathways, the modulation of intracellular redox homeostasis and the functional activity of the mitochondria (Arai M 2000, Lee WY 2005, Rolo AP 2009).

1.4.2. Remote IPC (rIPC)

It has also been demonstrated that IPC has similar favorable effects when it is brought about on other (even distant) organs remote from the one exposed to the major IR

insult. This phenomenon is called remote ischemic preconditioning (rIPC) or interorgan preconditioning. The first observation, which demonstrated intraorgan preconditioning in the heart (Przyklenk K 1993), was followed by a study presenting a classical rIPC setup when IR of the kidney limited the infarct size of the myocardium (McClanahan TB 1993). Remote effects of IPC were later seen in many other organs, such as the intestine (Wang YP 2002), the kidney (Takaoka A 1999) and the limb (Kharbanda RK 2002). Although the mechanism of rIPC has been investigated intensively, certain biochemical details are still not known. Messenger molecules (e.g. NO, adenosine, bradykinin, opioids, calcitonin gene-related peptide (CGRP), PKC, TNF-α, NFκB, IL-1β and IL-6) taking part in the transmission of protection from the site of IPC to the distant organ have been identified (Küntscher MV 2002, Brzozowski T 2004, Hu S 2012). Within this complex system, humoral and neurogenic pathways may overlap (Wei D 2012). Eventually, rIPC prevents oxidative stress, reduces the permeability and retains the membrane integrity of the mitochondria, and conserves the ATP level of cells in the target organ (Dong HL 2010, Cao Y 2011, Mansour Z 2012). As concerns the liver, the effects of rIPC included ameliorations in aminotransferase levels, improvements in hepatic blood flow and peripheral oxygen saturation and hepatic venous nitrate/nitrite levels (Kanoria S 2006) being additionally mediated by heme oxygenase-1 (Lai IR 2006).

1.4.3. IPC during orthopedic-trauma surgery: mechanisms and clinical applications

The IPC of the extremities has numerous clinical advantages as compared with other organs. IPC is relatively easy to perform on limbs or arms, and can be carried out noninvasively. The intervention affects a relatively large tissue mass, and the extent of the exerted defensive mechanism is proportional to it, which means a robust protective signal against the subsequent IR insult. Indeed, previous studies have demonstrated favorable local microcirculatory effects during limb IPC (Szabó A 2009) and a potential neurogenic component of IPC has also been described (Hartmann P 2011), showing that tissue hypoxia and inflammatory mediators produced during IR stimulate transient receptor potential vanilloid receptor-1 expressed by C-afferent neurons, which have a crucial role in the preventive process. This effect may be mediated by the release of neuropeptides such as CGRP, one of the most powerful endogenous vasodilators. Furthermore, rIPC involving IR through the use of a lower extremity was shown to exert marked therapeutic benefit at the heart (Zitta K 2014), the lung (Harkin DW 2002), the kidney (Park KM 2001) and the liver (Kanoria S 2006).

1.4.4. The potential role NOXs in ROS-mediated tissue injury and IPC

Some of the beneficial distant effects of IPC are mediated by changes in gene expression of the cellular redox homeostasis, bringing about a lower degree of ROS in the endothelial compartment (Koti RS 2002, Mallick IH 2005). Signaling processes catalyzed by oxygen and nitrogen radical-producing enzymes, including neuronal and endothelial NOS and XOR (Abu-Amara M 2011, Yuan HJ 2012), have been demonstrated in the action of IPC, and a number of additional data have also established the decisive roles of NOXs (Tejima K 2007, Wang Q 2007). NOXs are members of a family of ROS-generating enzymes, producing superoxide via electron transfer from the reduced NADPH to molecular oxygen (Babior BM 2002, Lambeth JD 2004, Anilkumar N 2009). NOXs are particularly involved in redox signaling because, among many intracellular sources of superoxide, these are the only known enzymes whose primary function is ROS generation (Lambeth JD 2004). For a long time, NOX-linked superoxide generation was an oddity of phagocytic cells such as PMNs and macrophages (termed NOX2/gp91phox). With variation in the catalytic subunits, the NOXs comprise 7 family members (NOX1 to NOX5 and DUOX1 and DUOX2), which exhibit tissue-specific differences in their baseline expression (Bedard K 2007). In the case of the liver parenchyma, NOX2 and NOX 4 proteins have been found in the hepatocytes; NOX2 predominates in the Kuppfer cells, while NOX4 is more abundant in the microvessels (Bengtsson SH 2003, Ellmark SHM 2005). The expression of NOX4 is at least 20-fold greater than that of NOX2 in the endothelial cells (Sorescu D 2002), while the expression of NOX2 cannot be detected in the vascular smooth muscle cells (Görlach A 2000, Lassègue B 2001).

NOXs are specifically activated by many stimuli known to cause an endothelial dysfunction (Anilkumar N 2009), and previous studies have provided evidence of elevated mRNA levels of both NOX2 and NOX4 in response to a liver IR injury (Marden JJ 2008). Moreover, the mortality rate due to hepatic ischemia was reduced in NOX2-deficient mice (Harada H 2004) and the role of the phagocytic form of NOX in Kupffer cells has been demonstrated after preconditioning with a chemical agent that induces hypoxia (Tejima K 2007). Collectively, these data suggest that influencing NOX4 (derived from hepatocytes and/or vascular cells) and NOX2 (produced by phagocytic PMNs and/or Kuppfer cells) may contribute to the protective mechanism of remote IPC. It can additionally be hypothesized that the effects of rIPC linked to an alleviated inflammatory reaction in the postischemic hepatic microcirculation are associated with NOX2 and NOX4 activation.

1.5. Traumatological aspects of osteoporosis

1.5.1. Pathogenesis and clinical symptoms of osteoporosis. Possible role of the periosteum in the pathogenesis of osteoporosis

Osteoporosis is regarded as the most common metabolic bone disease, affecting approximately 750 million people worldwide, with a further tendency to increase in the elderly in both developed and developing countries (Schuiling KD 2011). Every other woman and every fifth man aged 50 years or older will suffer an osteoporotic fracture during their remaining lifetime (Lippuner K 2012).

Osteoporosis originates from a disturbance of the normal bone turnover, where the bone resorption is increased or the bone formation is decreased (or both components are affected) (Raisz LG 2005). The process is characterized by a decrease in bone mineral density, a deterioration of the bone microarchitecture, and progressive weakening of the trabeculae. The gradual loss of bone density and strength results in an increased risk of fractures.

Osteoporosis is generally classified as either primary or secondary. Primary osteoporosis is associated with postmenopausal women and aging. In women, an estrogen deficiency causes several metabolic changes, among them the release of proinflammatory cytokines, such as TNF-α, IL-1 and IL-6, leading to bone resorption (Kitazawa R 1994). The age-related osteoporotic changes stem from a low-level and impaired intake of calcium, the compensatory rise of parathyroid hormone, and a decrease in the absorption of vitamin D (Geusens P 2011, Ebeling PR 2014, Li GL 2014).

Secondary osteoporosis affects both women and men at any age. It may be a consequence of various diseases (e.g. leukemia, multiple myeloma or Gaucher's disease), endocrine disorders (e.g. hyperprolactinemia or thyroid diseases) or the association of these pathogenic conditions; different medications too may be related to osteoporosis, including heparin therapy, lithium and glucocorticoids (Gennari C 1998).

Several early studies provided convincing evidence that an imbalance between bone resorption and formation can be suppressed or even prevented by restitution of the pre-menopausal estrogen levels in females (Christiansen C 1982). The effector cells of these processes originate from mesenchymal osteoprogenitor cells found in the periosteum and endosteum, with the predominant role of the periosteum (Brighton CT 1992). Furthermore, the experimental and clinical evidence points to an important function for the periosteum not only in the pathogenesis of osteoporosis, but also in the effects of different anti-osteoporotic approaches (Allen MR 2004). Since the effector cells of bone

remodeling originate mainly from the periosteum, this structure plays a decisive role not only in the pathogenesis of osteoporosis, but also during successful bone healing.

1.5.2. Inflammatory complications related to estrogen loss and replacement

The systemic and local consequences of an endogenous estrogen loss and exogenous replacement in the circulatory system are ambiguous. The incidence of septic and inflammatory complications is significantly lower in many trauma-hemorrhage conditions in females than in males (Choudhry MA 2005, Sperry JL 2008), and short-term estrogen pretreatment confers significant protection from IR injury and leukocyte activation in males (Burkhardt M 2008). However, other lines of evidence support the notion that estrogen supplementation may increase the risk of intravascular clotting complications in post-menopausal women (Cushman M 2002). Although the underlying mechanistic details are still unclear and the concept is debated (Seelig MS 2004, Canonico M 2008), it is obvious that the skeletal and anti-inflammatory benefits of hormone replacement therapies should be carefully weighed against the possible circulatory side-effects. Given this background, it is important to note that the consequences of chronic estrogen depletion and repletion on the human bone circulation are still unclear. Under compromised flow conditions, the periosteal microcirculation may be a good indicator of the perfusion changes of the whole bone (Kolodny A 1923), but the microcirculatory effects of osteoporosis in this tissue layer are also largely unknown. It has been shown that the patency of the periosteal microcirculation is of particular importance in models of tourniquet application and soft tissue trauma (Zhang L 2003, Varga R 2008). In these cases, both the bone and the periosteum undergo microvascular events reflected by perfusion insufficiencies and severe antigen-independent inflammatory reactions. These processes are mainly initiated by the increased adhesion of PMNs to the microvascular endothelium (rolling and then firm adhesion), followed by their migration to and accumulation in the perivascular tissues (Wolfárd A 2002).

As the prevalence of skeletal injuries increases after the menopause, our studies were designed to determine whether hormonal replacement therapy might be of microcirculatory benefit in this subset of the osteoporotic population. Ovariectomy (OVX) in the rat is a well-established animal model for osteoporosis research, as it shares many similarities with the human disease. These include an increased rate of bone turnover (Wronski TJ 1986), and an initial rapid phase of bone loss and similar skeletal responses to treatment with estrogen, calcitonin, bisphosphonates and many other agents (Fleisch H 1993, Allen MR 2004).

2. MAIN GOALS OF THE STUDIES

The major aim of our study was to examine the local and remote microcirculatory effects of transient lower limb ischemia. We directly observed the periosteal and hepatic microcirculations by intravital microscopy in order to assess the responses to a local IR challenge elicited by tourniquet ischemia.

- In this context, our primary objective was to observe the effects of osteoporosis and the consequences of estrogen therapy in a clinically relevant time frame in rats. To this end, we first determined whether chronic treatment with estrogen influences the OVX-triggered local periosteal microcirculatory reactions. We additionally hypothesized that the periosteal microcirculation in osteoporotic rats would be more sensitive to the detrimental consequences of transient limb ischemia than that in estrogen-treated, age-matching controls. Further we wished to establish whether the microcirculatory protection provided by limb IPC is also effective in an osteopenic situation (elicited by OVX).
- IR injury of the liver has an increasing clinical impact, and remote limb IPC can be a modality through which to overcome a postischemic hepatic injury. Our aim was to investigate the effects of rIPC on the microcirculatory consequences and the underlying molecular mechanisms in the postischemic liver. In this respect, we hypothesized that the changes in NADPH-oxidase expression may contribute to the efficacy of limb IPC.

3. MATERIALS AND METHODS

The experiments were performed in 3 major studies: (1) the microcirculatory effects of chronic estrogen deprivation (elicited by OVX) and estrogen supplementation were examined on the postischemic microcirculatory inflammatory changes in the tibial periosteum after tourniquet ischemia; (2) The effects of limb IPC on the local tibial periosteal microcirculatory consequences were examined in OVX rats; and (3) the effects of rIPC (evoked by limb ischemia) on the postischemic microcirculatory reactions of liver ischemia were assessed in male rats.

3.1. Animals

All studies were carried out on Sprague–Dawley rats housed in an environmentally controlled room with a 12-h light–dark cycle, and kept on commercial rat chow (Charles River, Wilmington, MA, USA) and tap water *ad libitum*. The experimental protocol was approved by the local animal rights protection authorities and followed EC Directive 86/609/EEC and the National Institutes of Health guidelines for the care and use of laboratory animals and was approved by the Animal Welfare Committee of the University of Szeged (#CSI/01/144/2013).

3.2. Experimental design and protocols

Study 1

3-month-old female animals (weighing 200–210 g) were ovariectomized (Group 1, OVX, n = 12), or sham-operated (Group 2, Sham, n = 6). Five months later (at the age of 8 months), chronic estrogen therapy (Sims NA 1996) was initiated 5 days/week with 20 $\mu g\ kg^{-1}$ subcutaneous 17β-estradiol (E2, Sigma, St. Louis, MO, USA) in 6 animals of the OVX group (Group 1a). The E2 substitution was continued weekly until the end of the experiments (see time scheme in Figure 1). The remaining OVX (Group 1b) and Sham animals received the vehicle for E2 (100% ethanol diluted in corn oil) in the same volume. Body weight changes and the development of osteoporosis were continuously followed in the proximal tibiae by means of ultrasonic densitometry (see later). Eleven months after OVX, the animals were subjected to a 60-min complete hindlimb ischemia followed by a 180-min reperfusion period. Limb ischemia was induced by applying a tourniquet around the thigh and a miniclip on the femoral artery. Control hindlimb IR was conducted on another group of 3-month-old female rats (Group 3, n = 6). In this group the microcirculatory consequences of limb IR alone were investigated by intravital videomicroscopy (IVM). After a 30-min stabilization period, the baseline cardiovascular

and microhemodynamic parameters were determined (baseline; −60 min). The periosteal microcirculation was observed every 60 min during the 180-min reperfusion period.

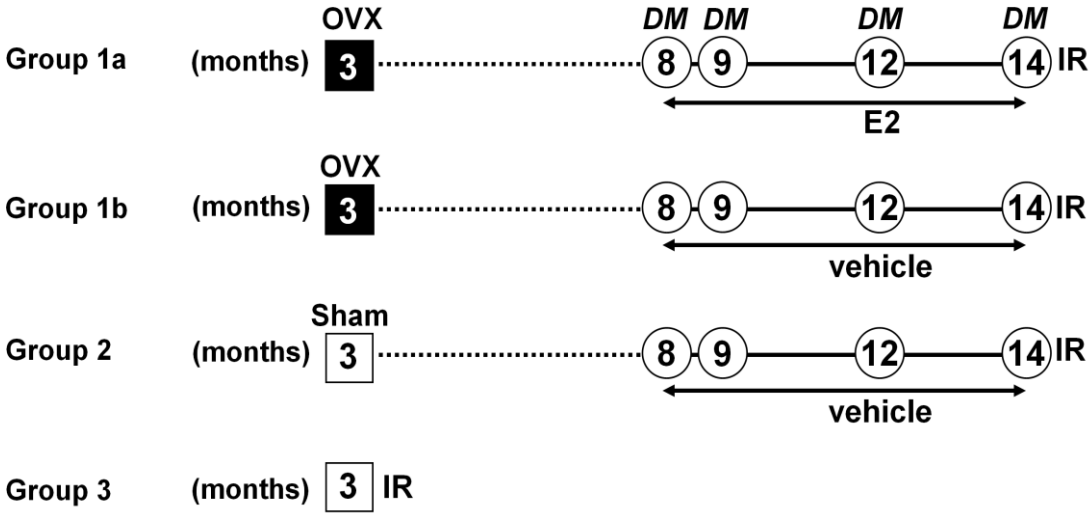

Figure 1. The time sequence of surgical interventions, treatments and measurements in Study 1. 3-month-old animals were ovariectomized (Group 1, OVX), or sham-operated (Group 2, Sham). Five months later (at the age of 8 months), chronic estrogen therapy was initiated 5 days/week with 20 μg kg^{-1} sc 17β-estradiol (E2) in 6 animals of the OVX group (Group 1a). The remaining OVX (Group 1b) and Sham animals received vehicle. Densitometric analysis was performed at the marked months of age (*DM*). At the end of the protocol, 60 min of tourniqet ischemia followed by 180 min of reperfusion (IR) was elicited and IVM recordings were performed.

In further groups, (Groups 4a, 4b, 5 and 6), identical protocols (OVX or Sham±IR, n = 7-10) were performed to detect changes in the proinflammatory cytokine TNF-α concentrations in the plasma and in the expressions of adhesion molecules known to play a role in the process of PMN adhesion to the endothelium. This was necessary because the fluorescent dyes used for IVM interfere with the flow-cytometry technique, and blood samples for the assessment of adhesion molecule expressions were therefore obtained separately. Blood samples from the carotid artery were taken at baseline and during the reperfusion period for the detection of changes in the plasma concentrations of TNF-α and in the expression of the adhesion molecule CD11b. TNF-α levels were determined on plasma samples taken at the 60th min of reperfusion. In a pilot study, the dynamics of the CD11b expression changes in response to limb IR were characterized; significant elevations were not found earlier than 120 or 180 min in the reperfusion period. Accordingly, this time frame was selected for flow cytometric evaluations of blood

samples (see later). Tissue biopsies were also taken for the detection of tissue ICAM-1 changes by immunohistochemistry (see later).

Study 2

In this study, the effects of limb IPC on the local tibial periosteal microcirculatory consequences were examined in OVX rats. To this end, OVX was performed on 3-month-old (weighing 200–210 g) female rats (Group 1, n = 16) while others were sham-operated (Group 2, n = 16). Two months later, 60 min of tourniqet ischemia followed by 180 min of reperfusion was performed in 8 animals of the OVX group (Group 1a, OVX+IR) and 8 animals in the Sham group (Group 2a, Sham+IR). Two further groups were also subjected to 2 cycles of 10 min of limb IPC/10 min of reperfusion (Group 1b, OVX+IPC+IR, n = 8; Group 2b, Sham+IPC+IR, n = 8) (see scheme in Figure 2).

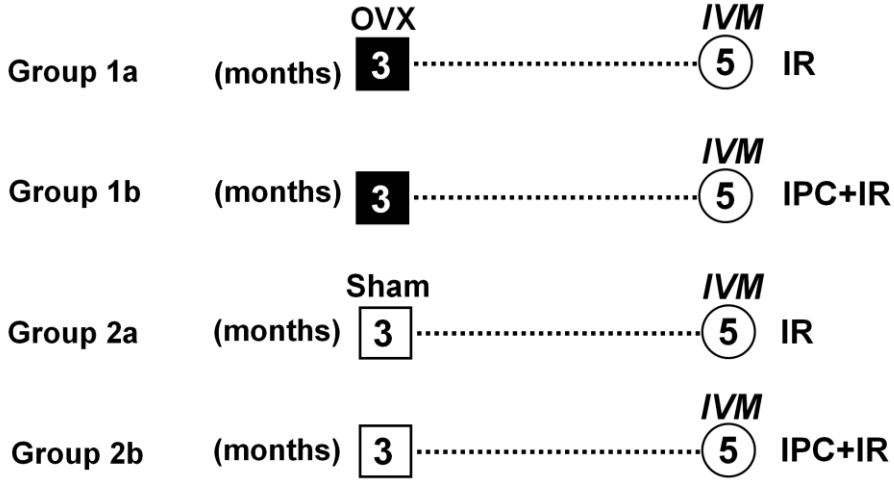

Figure 2. Experimental protocol in Study 2. 3-month-old animals were ovariectomized (Group 1, OVX), or sham-operated (Group 2, Sham). Two months later, 60 min of tourniqet ischemia followed by 180 min of reperfusion was performed in 8 animals of the OVX group (Group 1a, OVX+IR) and 8 animals in the sham-operated group (Group 2a, Sham+IR). Further two groups were also subjected to 2 cycles of 10 min of limb IPC / 10 min of reperfusion (Group 1b, OVX+IPC+IR; Group 2b, Sham+IPC+IR)

CD11b expression on the PMN leukocytes was determined in identical experimental groups, but in a separate experimental series, where blood samples were taken from the carotid artery at baseline and at 120 and 180 min of reperfusion (n = 6 or 7).

Study 3

In this study, the microcirculatory and biochemical effects of rIPC (evoked by limb ischemia) on the postischemic inflammatory reactions of liver ischemia were assessed in two major experimental series in male rats.

In the first series, we evaluated the microcirculatory consequences of partial hepatic ischemia by using the noninvasive modified spectrometric O2C method (O2C system, see later). In one group, the hepatic microcirculatory responses to 60-min complete ischemia followed by a 180-min reperfusion period were examined (IR group, n = 6). After recording of the baseline microcirculatory variables (t = -100 min), ischemia was induced in the median and left hepatic lobes. The occlusions were then released (t = 0 min), and the microcirculation in the affected lobes was observed via O2C at t = 60, 120 and 180 min in the reperfusion phase. In another group, two cycles of 10-min complete hindlimb ischemia and 10-min reperfusion were used as a preconditioning trigger before the induction of liver ischemia (rIPC+IR group, n = 6). The animals in a third group were subjected to the same surgical procedures, except for the induction of liver or limb ischemia (Sham group, n = 6). Blood samples for biochemical determinations were taken at t = 0, 60, 120 and 180 min of the experiments. Tissue biopsies for enzyme activity and Western-blot analyses were taken at the end of the experiments. Tissue biopsies were stored at -80 ^{o}C, and plasma samples at -20 ^{o}C until later analysis.

In the second series of experiments, the groups (n = 6 each) and the protocols were identical with those in the first series, with the exception that the microcirculation in the affected liver lobes was investigated by means of IVM (see later) at t = 60 min in the reperfusion phase.

3.3. Quantitative ultrasound bone densitometry

Animals were anesthetized with an intraperitoneal combination of ketamine and xylazine (25 mg kg^{-1} and 75 mg kg^{-1}, respectively) and bone density measurements were performed at the tibia and the tail with a DBM-Sonic 1200 (IGEA, Carpi, Italy) ultrasonic bone densitometry device in Study 1. The changes in the average of the amplitude-dependent speed of sound (AD-SoS) were determined (Joly J 1999). After calibration, the AD-SoS values of the soft tissues (the muscle and skin above the thigh) were determined, and the system deducted this value from the bone density. The AD-SoS values were calculated via a computer program and the average of 5 measurements was used at each time point. Twenty-one weeks after OVX, statistically significant density alterations were observed in the proximal tibia, and this location was therefore used for further quantitative ultrasound measurements at the ages of 8, 9, 12 and 14 months.

3.4. Surgical procedures

Surgical procedure of OVX

Under ketamine–xylazine anesthesia (as discussed earlier), a median laparotomy was performed under sterile conditions. The connection of the Fallopian tubes was cut between hemostats, the ovaries were removed, and the stumps were then ligated. Thereafter, the abdomen was filled with warm sterile physiological saline and the abdominal wall was closed in two layers. Sham-operated animals underwent identical procedures except that the Fallopian tubes and ovaries remained intact.

Preparation of animals before IVM

IVM examinations of the tibial periosteum (Study 1 and 2) were performed under sodium pentobarbital (45 mg kg^{-1} ip) anesthesia and sustained with small supplementary intravenous doses when necessary. In the case of liver examinations, however, anesthesia was induced with a combination of 25 mg ml^{-1} (S)-ketamine (Ketanest; Parke Davis, Berlin, Germany) and 20 mg ml^{-1} xylazine (Rompun; Bayer, Leverkursen, Germany) in a ratio of 8:1, injected ip and sustained with small supplementary iv doses every 30 min. The right carotid artery and the jugular vein were cannulated for the measurement of mean arterial pressure and the administration of drugs and fluids, respectively. The animals were placed in a supine position on a heating pad to maintain the body temperature between 36 and 37 °C. Ringer's lactate was infused at a rate of 10 ml kg^{-1} h^{-1} during the experiments. The trachea was cannulated to facilitate respiration.

Preparation of the tibial periosteum for IVM measurements in rats

The medial/anterior surface of the left tibia was exposed by means of an atraumatic surgical technique where the skin above the anterior tibia was dissected, the anterior gracilis muscle was cut completely through with microscissors, and the connective tissue covering the tibial periosteum was carefully removed via an atraumatic microsurgical approach (under a Zeiss 6× magnification operating microscope) (Varga R 2008).

Preparation of the liver for IVM measurements in rats

After midline laparotomy and bilateral subcostal incisions, the liver was carefully freed from all ligamentous attachments and was exposed, and the left branches of the portal vein and the hepatic artery were mobilized. Complete ischemia of the median and left hepatic lobes was achieved by clamping the left lateral branches of the hepatic artery and the portal vein with a microsurgical clip for 60 min (Taniguchi M 2007). After the ischemic period, the clips were removed and the wound was temporarily covered with water-impermeable foil.

3.5. Microcirculatory measurements

IVM - technical setup

The right hindlimb with the exposed tibial periosteum (in Studies 1 and 2) or the exteriorized posterior surface of the left liver lobe (Study 3) was positioned horizontally on an adjustable stage for examination of the microcirculation by IVM (Zeiss Axiotech Vario 100HD microscope, 100W HBO mercury lamp, Acroplan 20× water immersion objective, Carl Zeiss GmbH, Jena, Germany). Microcirculation was visualized with fluorescein isothiocyanate (Sigma, St. Louis, MO, USA)-labeled erythrocytes (0.2 ml iv), whereas leukocytes were labeled with an iv injection of rhodamine 6G (Sigma, St. Louis, MO, USA, 0.2%, 0.1 ml iv). The microscopic images were recorded with a charge-coupled device video camera (Teli CS8320Bi, Toshiba Teli Corporation, Osaka, Japan) attached to an S-VHS video recorder (Panasonic AG-MD 830, Matsushita Electric Industrial Co., Tokyo, Japan) and a personal computer.

IVM - 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). As for the periosteum, leukocyte–endothelial cell interactions were analyzed within 5 postcapillary venules (diameter between 11 and 20 μm) per animal. In case of the liver, leukocyte adherence was assessed in the postsinusoid venules (diameter between 20 and 40 μm). 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 reported 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 given as the number of cells/vessel circumference in mm.

Modified lightguide spectrophotometry (O2C)

In Study 3, the O2C system (LEA Medizintechnik, Gieβen, Germany) was used for noninvasive and online examinations of the microcirculation; this allows the simultaneous recording of tissue oxygen saturation (S_{O2} percentage, absolute value), tissue hemoglobin (rHb, AU), capillary blood flow (AU) and capillary blood flow velocity (RBCV, AU). The O2C device combines white light spectroscopy with laser-Doppler measurement in one flat probe. To prevent the influence of regional heterogeneity and temporal blood flow variations, measurements were performed at three

predetermined locations on the liver surface for 30 s each with an ambient light correction before measurement (Schreinemachers MC 2009).

3.6. Immune labeling and flow cytometric analysis of adhesion molecule CD11b expression of PMNs

The surface expression of CD11b on the peripheral blood PMNs was determined through flow-cytometric analysis of whole blood in duplicate (Varga R 2008). 100 μl of whole blood was incubated with 20 μl of (50 μg ml^{-1}) mouse anti-rat monoclonal antibody (BD Pharmingen, San Jose, CA, USA) for 20 min. Negative controls were obtained by omitting the monoclonal antibody. The cells were then washed twice in Hanks buffer and centrifuged at 13,000 rpm for 5 min, and the resuspended pellet was incubated with fluorescein isothiocyanate-conjugated polyclonal rabbit anti-mouse immunoglobulin (10 μg ml^{-1}; DAKO Cytomation, Glostrup, Denmark; 20-μl aliquots of reagents to 180-μl aliquots of blood cells). The cells were again washed twice, and the erythrocytes were lysed with a Lysing kit (Biodesign, Saco, ME, USA), after which the cells were washed twice again (6000 rpm, 5 min) and resuspended in 200 μl of Hanks buffer. Computer-assisted FACStar Plus Becton-Dickinson equipment was used for cytometry; the granulocytes were gated on the basis of their characteristic forward and sidescatter features. Generally 10,000 events per sample were collected and recorded; the percentages of labeled (activated) granulocytes (relative to the overall marker-bearing cells) and the mean fluorescence intensity (average marker density) were calculated.

3.7. Determination of plasma TNF-α and high-mobility group protein B1 (HMGB1) levels

Blood samples (0.5 ml) were taken from the carotid artery into precooled EDTA-containing polypropylene tubes, centrifuged at 1000 g for 30 min at 4 °C, and then stored at -70 °C until assay. Proinflammatory cytokine TNF-α and HMGB1 concentrations were determined in plasma samples with commercially available enzyme-linked immunosorbent assays (Quantikine Ultrasensitive ELISA kit for rat TNF-α; Biomedica Hungaria Kft, Hungary and Shino-Test Corporation ELISA kit for HMGB1; Kanagawa, Japan).

3.8. Liver transaminase release determined in plasma samples

Blood samples withdrawn from the carotid artery were analyzed for aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) and lactate-dehydrogenase (LDH) by standard photometric procedures (Vitros 250 analyzer, Ortho-Clinical Diagnostics, Raritan, NJ, USA).

3.9. Liver 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 24,000 g and the supernatant was loaded into centrifugal concentrator tubes. The activity of XOR in the ultrafiltered supernatant was determined by fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total XOR) or absence of the electron acceptor methylene blue (XOR activity) (Beckman JS 1989)

3.10. Liver myeloperoxidase (MPO) activity

Tissue MPO activity was measured in liver biopsies by the method of Kuebler WM (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 24,000 g. 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. Western blot analysis of liver NOX2 and NOX4 protein expression

Liver samples were homogenized and then lyzed with RIPA buffer (Santa Cruz Biotech). Protein extracts (20 μg of total protein) were heated at 95 °C for 10 min, then placed in ice to cool, electrophoresed in 4–15% gradient sodium dodecyl sulfate–polyacrylamide gels, and transferred onto nitrocellulose membranes. Membranes were blocked with TRIS-buffered saline (TBS) and 5% skim milk at room temperature for 1 h prior to overnight incubation at 4 °C with primary antibodies against gp91phox (NOX2) (1:2000 dilution; Epitomics, Burlingame, CA, USA), and NOX4 (1:2000 dilution; Epitomics, Burlingame, CA, USA). After washing with TBS-Tween 20, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugate-corresponding secondary antibodies (anti-rabbit, (1:2500 dilution; Promega, Madison, WI, USA). The membranes were then developed with the SuperSignal West Pico horseradish peroxidase substrate kit (Pierce, Rockford, IL, USA) and the intensities of protein bands were quantitated and photographed on a Lumi-Imiger™ (Roche-Diagnostics, Boehringer Mannheim, Germany) image station. For the control of sample loading and protein transfer, the membranes were stripped and reprobed with β-actin antibody (1:1000 dilution; Sigma-Aldrich, St. Louis, MO, USA).

3.12. Detection of tissue ICAM-1 by immunohistochemistry

At the end of the experiments, both the limb subjected to IR and the contralateral one were removed and the tibias and the surrounding muscles were fixed in 4% phosphate-buffered formalin for 2–3 days in *Study 1*. The tissues were then decalcified with an electrophoretic apparatus (for 2×4 h), using a special decalcifying solution (Sakura TDE30; Sakura Finetek Corp. Torrance, CA, USA). The samples were embedded in paraffin, and 4-μm sections were placed on silanized slides. After conventional methods of dewaxing and rehydration (initiated in xylene, followed by decreasing concentrations of ethanol and methanol), tissue endogenous peroxidase was blocked with a mixture of methanol and 1% H_2O_2 for 5 min, and the nonspecific tissue antigens with conventional 2.8% cow milk. For ICAM-1 immunohistochemistry, 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, Glostrup, 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 200× magnification. During the semiquantitative analysis, periosteal and intramuscular vessels were evaluated separately and the percentage of ICAM-1-positive vessels was calculated. The samples were allotted to one or other of the following semiquantitative categories (scores 1–8) (Table 1).

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

Table 1. Semiquantitative scoring system for the analysis of ICAM-1-positive vessels from periosteal samples processed for immunohistochemistry

3.13. Statistical analyses

All data are expressed as means ± standard error of the mean (SEM). Data analysis was performed with the SigmaStat statistical software (Jandel Corporation, San Rafael, CA, USA). For microcirculatory variables and bone density data, differences within and between groups were analyzed by analysis of variance (ANOVA) followed by the Bonferroni test. Changes in microcirculatory parameters and liver enzyme activities between groups and within groups were analyzed by two-way ANOVA or two-way RM ANOVA, followed by the Bonferroni test. For the evaluation of biochemical assays and ELISA data, changes in variables between groups were analyzed by ANOVA on ranks, followed by the Holm-Sidak test. Western blot data were analyzed with non-normal distribution by the Mann-Whitney test. P values < 0.05 were regarded as significant.

4. RESULTS

4.1. Effects of OVX and chronic estrogen therapy on bone density and postischemic tibial periosteal inflammatory reactions

As shown by bone densitometry on the proximal tibia (Study 1), osteoporosis had developed by 21 weeks after bilateral OVX. The AD-SoS was significantly lower than that for the Sham animals (1674 ± 33 ms^{-1} vs 1850 ± 101 ms^{-1}, respectively). The OVX-induced osteopenia was completely restored by E2 therapy (Figure 3).

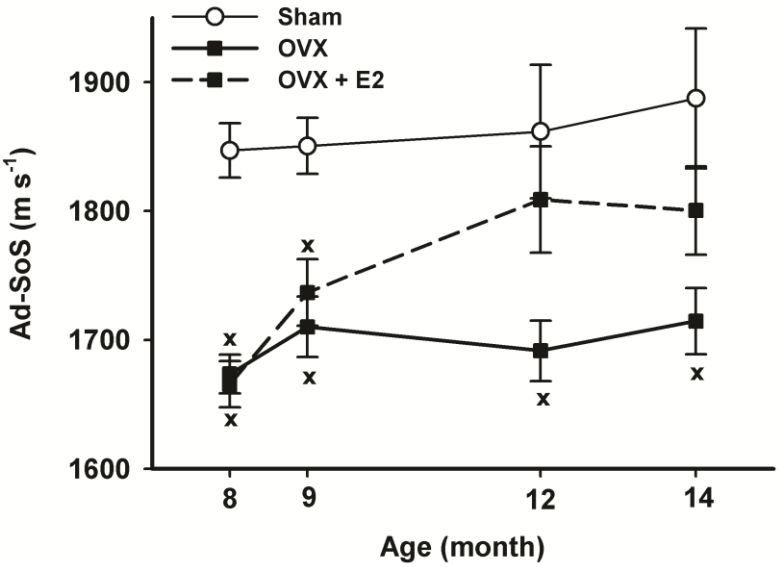

Figure 3. Bone density changes in the proximal part of the tibia in Sham animals or OVX animals treated with E2 (OVX+E2) or vehicle (OVX). Data are expressed as Ad-SoS and presented as means ± SEM ANOVA was followed by the Bonferroni test. ^{X} $P < 0.05$ vs Sham group.

The number of firmly adherent (sticking) leukocytes was significantly increased in the postcapillary venules of the tibial periosteum in the 3-month-old controls, the Sham rats and the OVX animals at the end of the reperfusion phase (120 and 180 min). This phenomenon was nearly completely prevented by chronic E2 supplementation in the OVX animals (Figure 4).

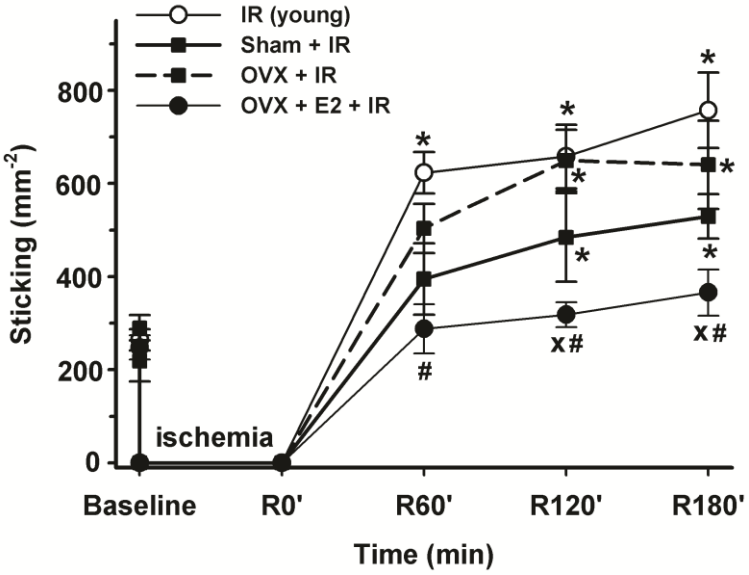

Figure 4. Secondary leukocyte-endothelial cell interactions (sticking) in the postcapillary venules of the tibial periosteum after 60 min of limb ischemia followed by 180 min of reperfusion (IR). Values in Sham+IR, OVX+E2+IR or OVX+IR animals were compared with those in 3-month-old (young) female rats (IR). Data are presented as means ± SEM. ANOVA was followed by the Bonferroni test. $^{*}P < 0.05$ vs baseline, $^{X}P < 0.05$ vs OVX+IR group, $^{\#}P < 0.05$ vs IR group.

An increased surface expression of adhesion molecule CD11b was observed after 120 and 180 min of reperfusion; no major differences could be detected between the findings for the OVX and the Sham or the 3-month-old rats (Figure 5). Chronic E2 administration did not influence this parameter.

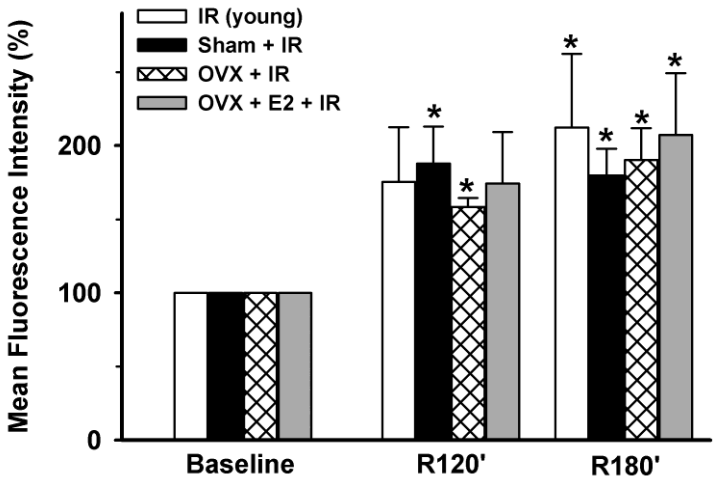

Figure 5. Changes in expression of the CD11b adhesion molecule on the surface of PMNs in response to 60 min of limb ischemia followed by 180 min of reperfusion. Values for Sham+IR, OVX+E2+IR or OVX+IR animals were compared with those for 3-month-old (young) female rats (IR). Data are presented as means ± SEM. $^{*}P < 0.05$ vs baseline.

The plasma TNF-α had reached similar concentrations (young animals: 21.24 ± 3.30 pg ml^{-1}; Sham: 22.16 ± 5.79 pg ml^{-1}; OVX: 19.84 ± 1.35 pg ml^{-1}) by the 60th min of reperfusion. E2 treatment, however, resulted in a significantly lower TNF-α value (OVX+E2: 11.14 ± 1.19 pg ml^{-1}) as compared with that for the OVX-challenged rats ($P < 0.05$). No TNF-α was detected in control samples taken before the ischemic insult.

As assessed by immunohistochemical analysis (Figure 6), the tissue ICAM-1 density in the vessels of the periosteum was significantly higher in the limbs subjected to IR (young animals: 1.67 ± 0.67; Sham: 1.83 ± 0.60; OVX: 2.17 ± 0.48; OVX+E2: 2.0 ± 0.52) than in the intact contralateral limbs in all the experimental groups (young animals: 0.33 ± 0.21; Sham: 0.50 ± 0.22; OVX: 0.67 ± 0.21, OVX+E2: 0.50 ± 0.22). The intensity of the IR-induced ICAM-1-positive reaction was only moderate in the muscle tissue, and the data for the 3-month-old controls, the Sham, the OVX and the OVX+E2 animals were also similar.

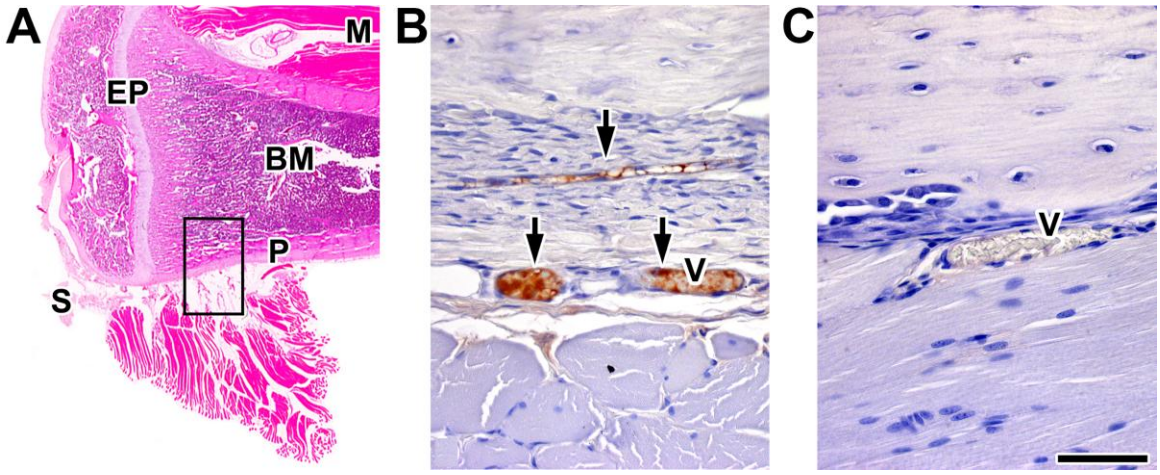

Figure 6. Representative longitudinal section of the rat tibia surrounded by soft tissues (stained with ICAM-1 antibody plus hematoxylin). Left panel (**A**): tibial epiphysis (EP), bone marrow (BM), knee-joint synovia (S), muscle (M) and tibial periosteum (P) are indicated. Middle panel: positive staining for ICAM-1 (arrows) was found in the periosteal venules (V) after IR (**B**), but not in the contralateral (non-IR) limbs (**C**). Bar represents 50 μm.

4.2. Effects of local IPC on the postischemic tibial periosteal microcirculatory inflammatory reactions in the presence of estrogen depletion

As compared with the baseline values, the numbers of primary PMN–endothelial interactions (termed rolling) were increased at all examined timepoints (120 and 180 min) of reperfusion after limb IR in all groups in the postcapillary venules of the tibial periosteum (Figure 7A). When limb IR was combined with local IPC in the

Sham+IPC+IR group, significantly lower rolling values were observed than when only limb IR was induced (Sham+IR group). In the OVX animals, however, the reduction caused by IPC was significantly lower (OVX+IPC+IR group). Secondary leukocyte-endothelial cell interactions (sticking) were similar to those seen at rolling; no effect of IPC was evidenced in the OVX+IPC+IR group (Figure 7B).

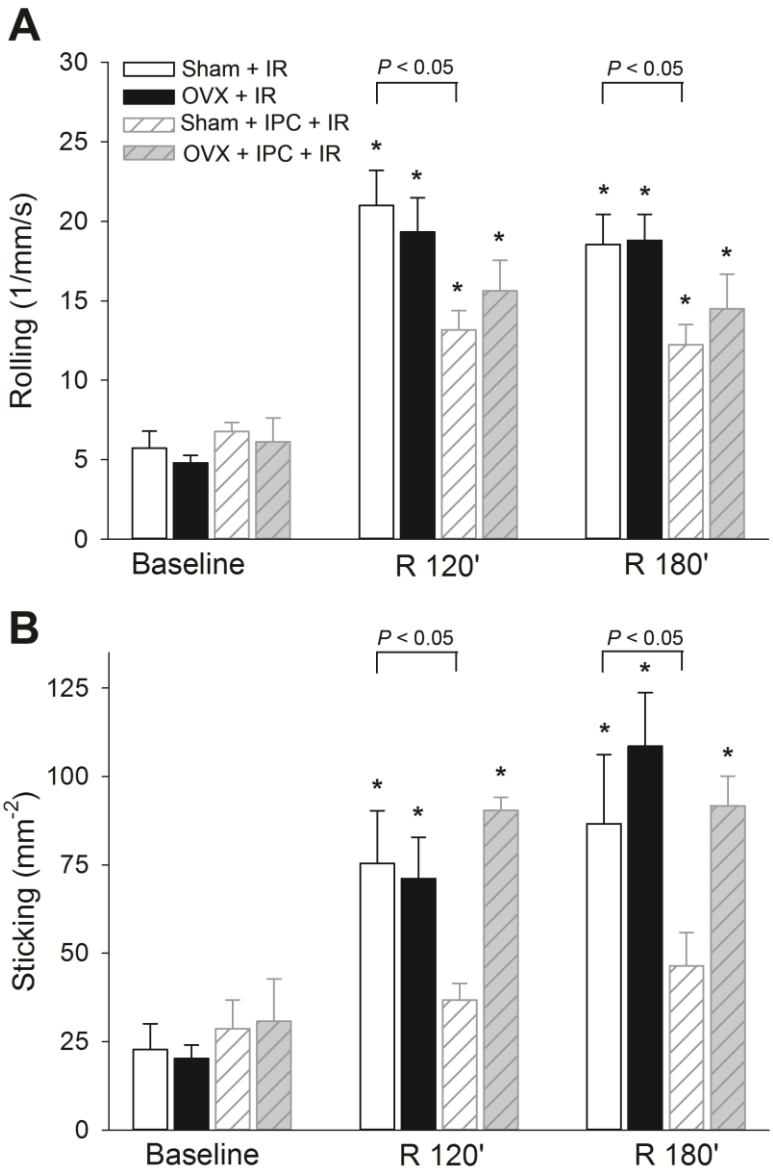

Figure 7. Primary (rolling) and secondary (sticking) leukocyte–endothelial cell interactions in the postcapillary venules of the tibial periosteum after 60 min of limb ischemia. Values for the Sham+IR and OVX+IR rats were compared with those for the Sham+IPC+IR and OVX+IPC+IR animals. Data are presented as means ± SEM. Two-way RM ANOVA was followed by the Bonferroni test. $^{*}P < 0.05$ vs baseline.

CD11b expression changes used as measures of PMN activation showed markedly enhanced values during reperfusion (120 and 180 min) in response to the limb IR insult in

both groups (Figure 8). Neither OVX+IR nor OVX+IPC caused attenuation of this parameter.

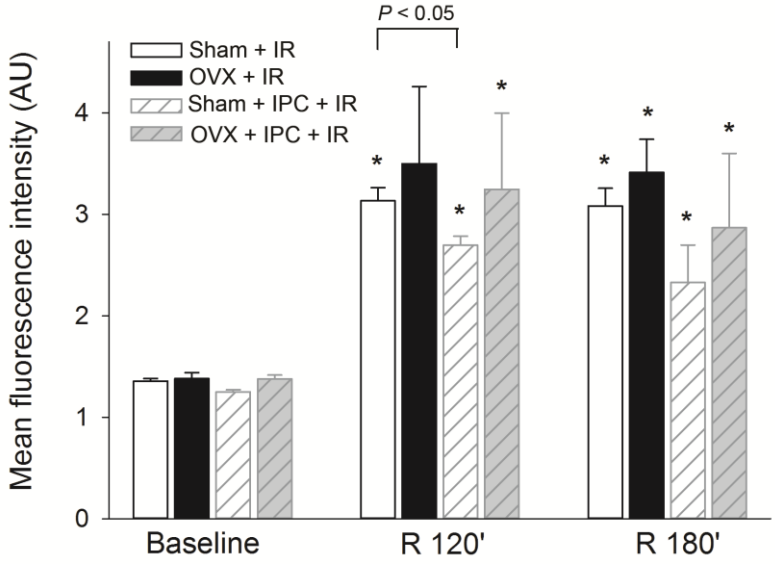

Figure 8. Expression changes of the CD11b adhesion molecule on the surface of PMNs after 60 min of limb ischemia, followed by 180 min of reperfusion. Values for Sham+IR and OVX+IR rats were compared with those for Sham+IPC+IR and OVX+IPC+IR animals. Data are presented as means ± SEM. Two-way RM ANOVA was followed by the Bonferroni test. $^{*}P < 0.05$ vs baseline.

4.3. Effects of rIPC on inflammatory consequences of partial liver IR

Hepatic microcirculatory dysfunction

The microhemodynamic parameters, capillary blood flow, RBCV, S_{O2} and rHb were assessed simultaneously and on-line in the left liver lobes (Figure 9). Reperfusion after 60-min ischemia was not associated with significant changes in intrahepatic blood flow (Figure 9A) as compared with the Sham group. When IR was preceded by rIPC, however, the postischemic hepatic blood flow was significantly higher than the pre-ischemic values throughout the examination period. The RBCV in the IR group was significantly lower during reperfusion in comparison with the pre-ischemic values, and no recovery was observed during the examination period (Figure 9B). rIPC, however, reversed the RBCV changes to the level measured in the Sham group. Taken together, the flow and velocity changes caused by the 60-min partial ischemia were manifested in deteriorated levels of tissue S_{O2} and rHb, which were restored by the rIPC protocol (Figure 9C,D).

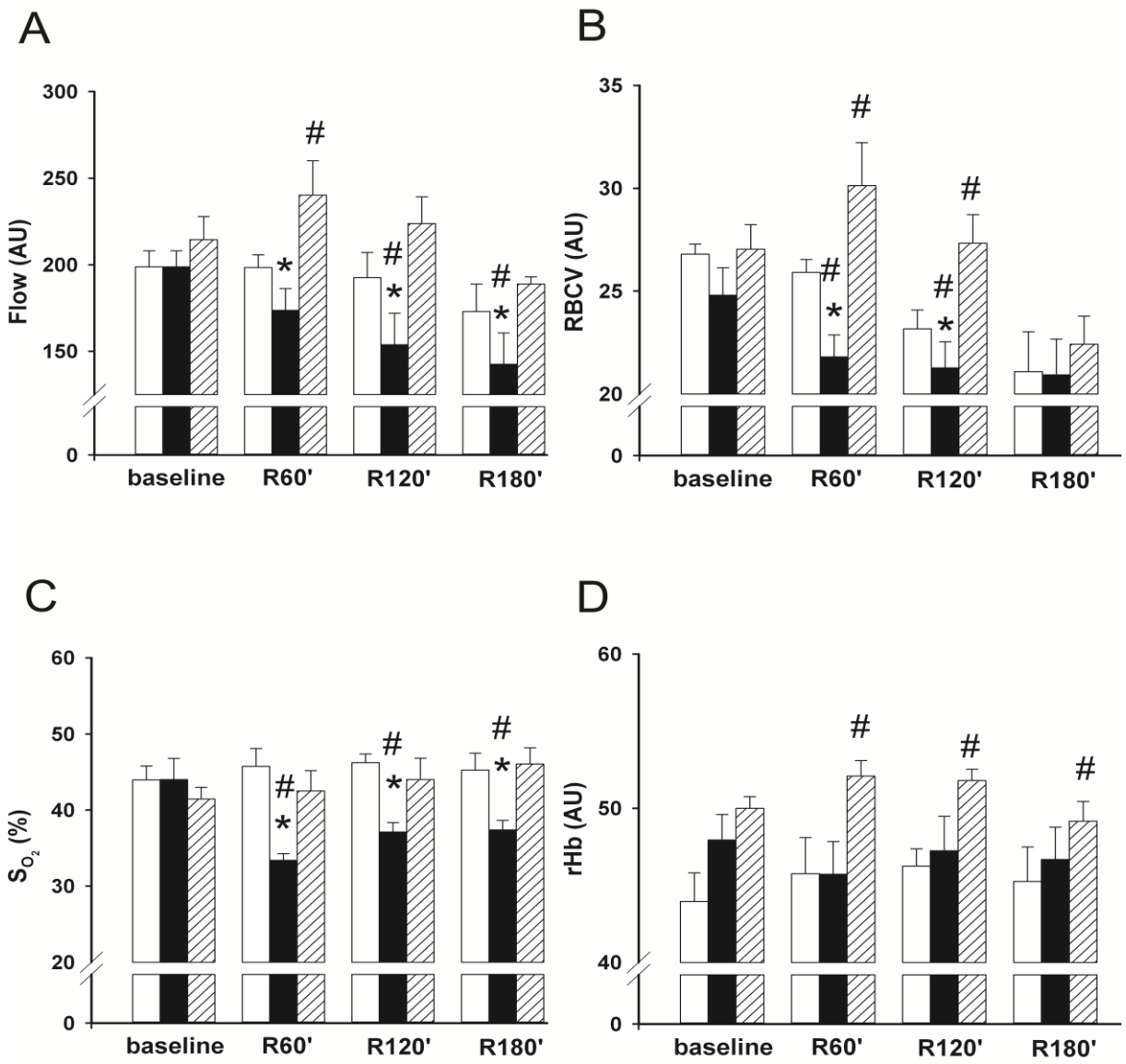

Figure 9. The animals were sham-operated (Sham group, white columns) or subjected to 60 min partial hepatic ischemia followed by 180 min of reperfusion (IR group, black columns), or two cycles of 10-min complete hindlimb ischemia followed by a 10-min reperfusion period were applied as a preconditioning trigger before liver ischemia (rIPC+IR group, striated columns). Capillary blood flow, RBCV and rHb are given in arbitrary units (AU) (Figure 9A,B,D), and S_{O2} in percentage (%) (Figure 9C). Data are presented as means ± SEM. Two-way RM ANOVA was followed by the Bonferroni test. $^{*}P < 0.05$ vs baseline; $^{\#}P < 0.05$ vs Sham group.

Due to the lack of selectin molecules in the post-sinusoidal endothelium, "classical rolling" could not be observed, but the number of PMNs exhibiting the rolling phenomenon could nevertheless be determined in the central venules. Liver IR was accompanied by an approximately 4-fold increase in the number of rolling leukocytes relative to that of the Sham animals (Figure 10A). In accordance with this, the number of sticking cells also displayed a significant increase in response to liver IR (Figure 10B). rIPC resulted in significant improvements in both forms of cell-to-cell interactions.

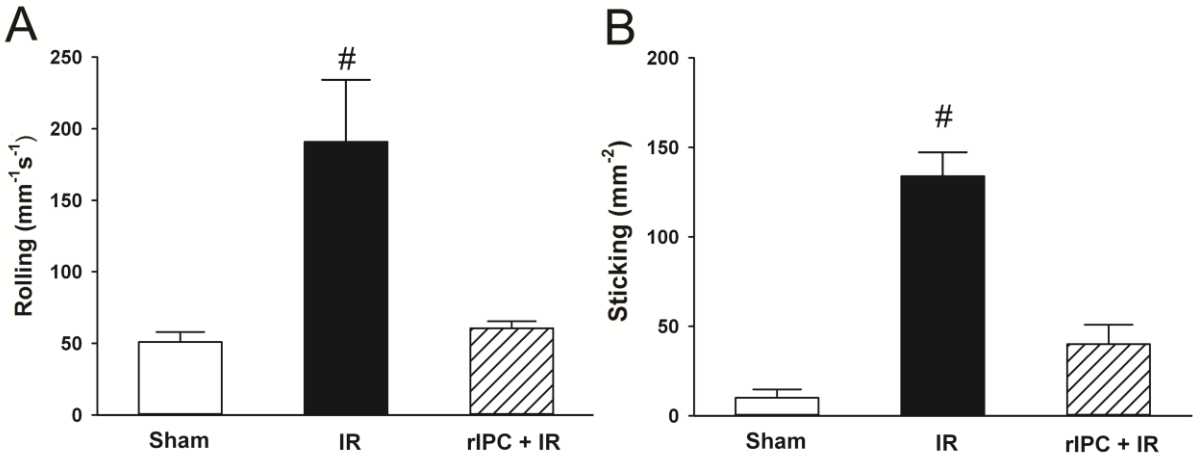

Figure 10. Changes in PMN–endothelial interactions at 60 min of reperfusion. Numbers of rolling (A) and sticking (B) leukocytes in Sham, IR and rIPC+IR groups. Data are presented as means ± SEM. ANOVA was followed by the Holm-Sidak test. $^{\#}P < 0.05$ vs Sham group.

Systemic inflammatory changes

Following 60-min partial liver ischemia, significantly increased HMGB1 and TNF-α levels were observed at 180 min of the reperfusion period (Figure 11 A,B). The IR-induced elevations of the plasma HMGB1 and TNF-α were effectively attenuated by rIPC (Figure 11A,B).

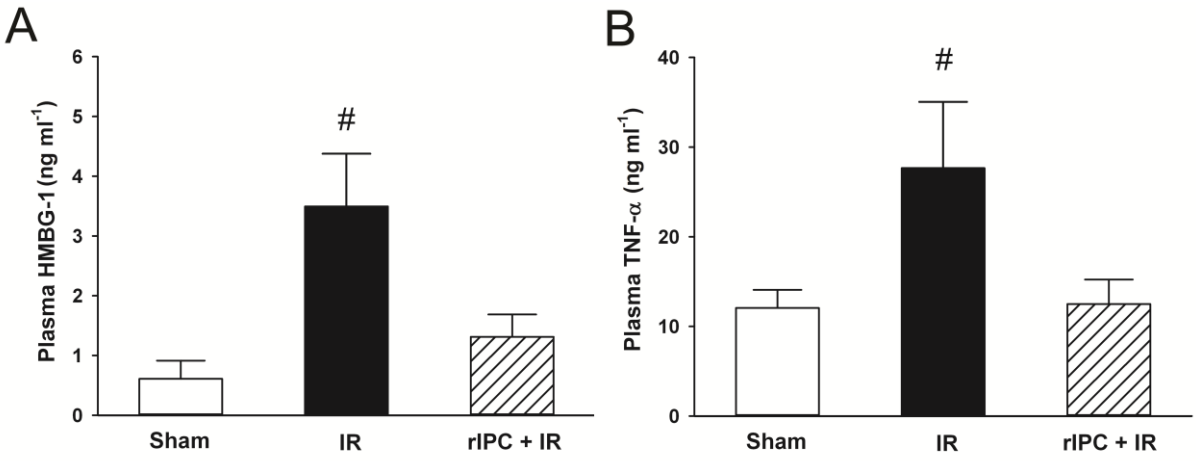

Figure 11. Plasma HMGB1 (A) and TNF-α (B) levels at the end of reperfusion (180 min) in the Sham, IR and rIPC+IR groups. Data are presented as means ± SEM. ANOVA was followed by the Holm-Sidak test. $^{\#}P < 0.05$ vs Sham group.

Inflammatory enzyme (MPO and XOR) levels

The PMN deposition analyzed via the MPO activity was increased significantly 180 min after the ischemia, together with the XOR activity, which was elevated approximately 2-fold as compared with the Sham group. rIPC applied prior to the partial

liver IR insult reduced the MPO and XOR activities almost to the Sham levels (Figure 12A,B).

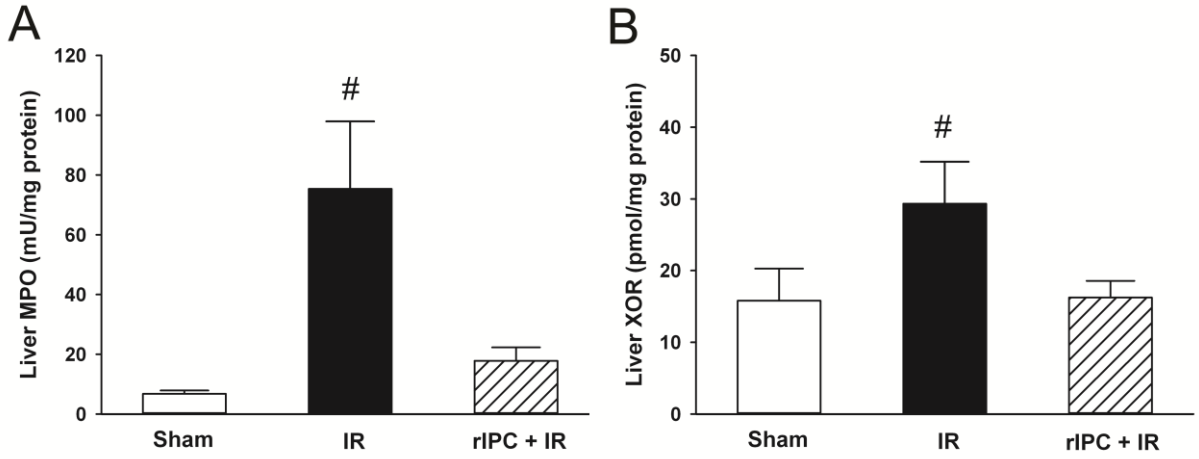

Figure 12. Hepatic MPO (A) and XOR (B) activities 180 min after ischemia in the Sham, partial liver IR and rIPC+IR groups. Data are presented as means ± SEM. ANOVA was followed by the Holm-Sidak test. $^{\#}$ *P* <0.05 vs Sham group.

NOX2 and NOX4 protein expression

Western blot analysis of the NOX2 and NOX4 proteins revealed significant increases after partial liver IR in comparison with the Sham animals. The application of rIPC before IR decreased the expression of NOX2 significantly (Figure 13A), but did not affect the level of NOX4 expression (Figure 13B).

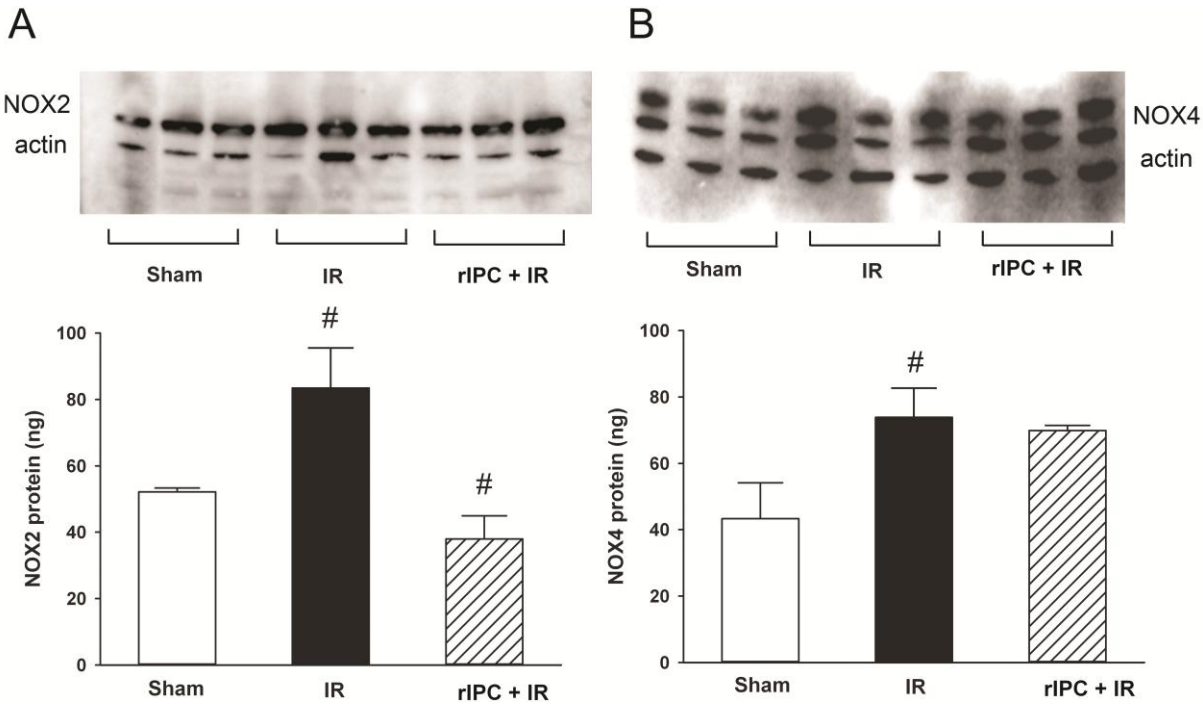

Figure 13. Hepatic expressions of NOX2 (A) and NOX4 (B) proteins at 180 min of reperfusion in the Sham, IR and rIPC+IR animals. Data are presented as medians ± SD, analyzed with non-normal distribution by the Mann-Whitney test. $^{\#}P < 0.05$ vs Sham group.

Functional hepatic changes

The animals in the Sham group displayed minimally increased necroenzyme levels throughout the experimental protocol as compared with the baseline values. In comparison, the IR group exhibited significantly higher AST, ALT and LDH activities during the reperfusion period, indicating significant functional damage. When the rIPC protocol preceded liver IR (rIPC group), the plasma levels of the necroenzymes were significantly reduced in comparison with the IR group (Figure 14).

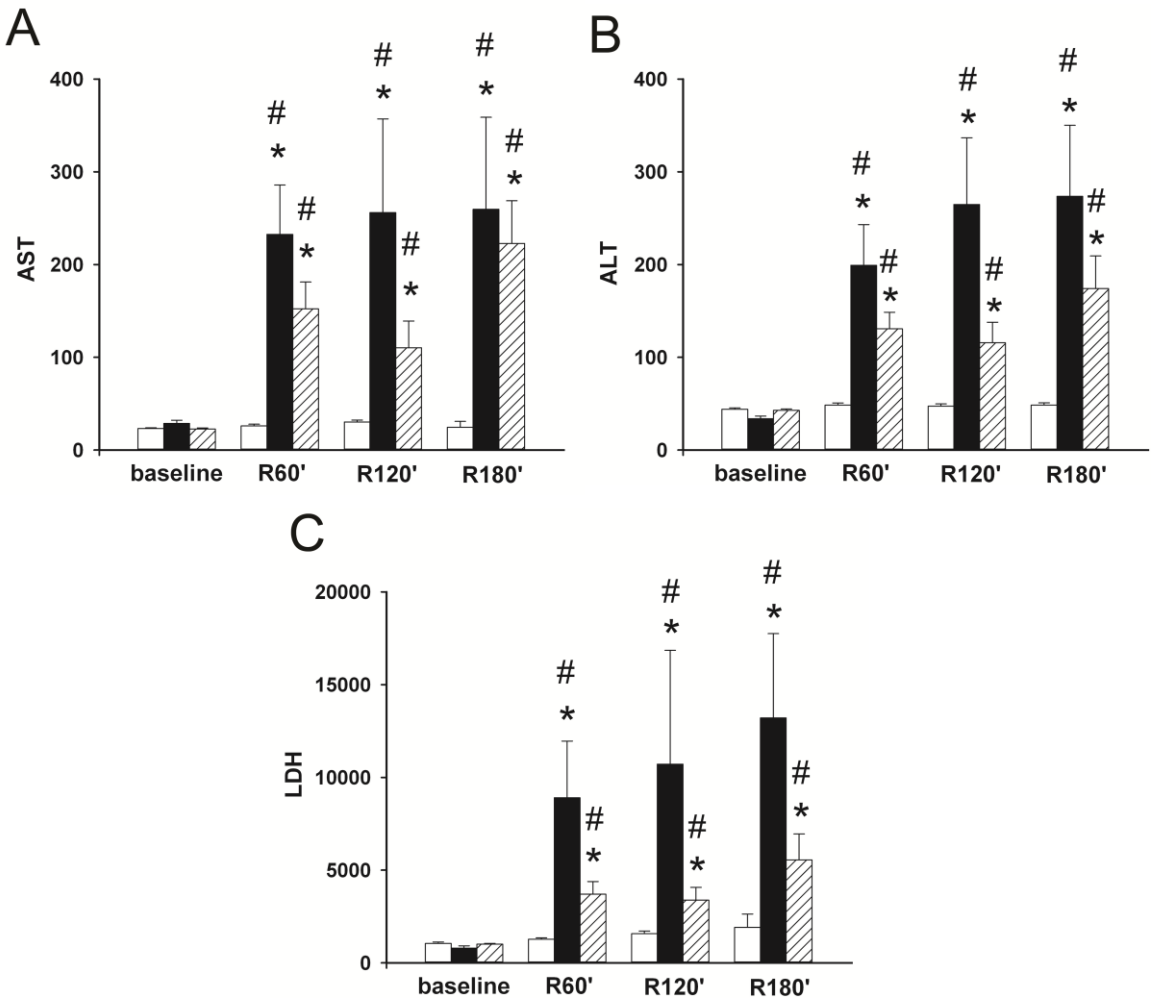

Figure 14. Time courses of plasma AST (A), ALT (B) and LDH (C) changes. Values are given in UL^{-1}. The animals were sham-operated (Sham group, white columns) or subjected to 60-min partial hepatic ischemia followed by 180-min reperfusion (IR group, black columns), or two cycles of 10-min complete hindlimb ischemia followed by a 10-min reperfusion applied as a preconditioning trigger before liver ischemia (rIPC+IR group, striated columns). Data are presented as means ± SEM. Two-way RM ANOVA was followed by the Bonferroni test. $^{*}P < 0.05$ vs baseline; $^{\#}P < 0.05$ vs Sham group.

5. DISCUSSION

5.1. Effects of OVX and estrogen supplementation on local postischemic periosteal microcirculatory injury

Females are more resistant to circulatory shock and inflammation than males, but this gender-related anti-inflammatory protection is lost during the menopause or after OVX (Angele MK 2000, Wichmann MW 2000). As the incidence of traumas and osteoporotic fractures steadily rises in the elderly population (Gullberg B 1997), the question arises of whether osteoporotic women are more susceptible to postoperative or post-traumatic inflammatory bone complications than postmenopausal women receiving estrogen replacement therapy. In our first study, we set out to answer this question by employing a chronic rat model of osteoporosis where the consequences of a long-term female hormone deficiency can be adequately estimated (Tan Z 2003). The results revealed that osteoporosis reproducibly evolved after OVX, and the reversing efficacy of E2 therapy on osteopenia, together with the periosteal microcirculatory status of the tibia, could subsequently be evaluated in the long term. The data also showed, however, that the postischemic periosteal microcirculatory PMN recruitment and the expressions of the adhesion molecules CD11b and ICAM-1 undergo similar changes in this condition, irrespective of the age or the endogenous estrogen status of the animals. Thus, the primary message of our study is that a chronic estrogen deficit does not change the magnitude of the PMN-associated microcirculatory menace in the event of transient ischemia of the osteoporotic limb.

IVM allows direct observation and quantitative analysis of the microcirculation of the exposed tissues. With this technique, the consequences of an estrogen deficit and estrogen replacement have already been demonstrated in certain organs (Santizo RA 2000, Watanabe Y 2001), but never in the periosteum, though the protecting and feeding functions of the periosteum are hallmarked by the fact that restoration of the periosteal microcirculation guarantees bone survival even in an environment with a limited blood supply (Berggren A 1982). Additionally, osteoporosis has been found to develop on the basis of an imbalance between bone resorption and formation, the effector cells being derived from mesenchymal osteoprogenitor cells located in the periosteum and endosteum, the former displaying predominance in this respect (Brighton CT 1992). Furthermore, the experimental and clinical evidence suggests an important role of the periosteum not only in the pathogenesis of osteoporosis itself, but also in the effects of

different anti-osteoporotic approaches (Allen MR 2004). The periosteal region of the cortical bones exhibits a better anti-fracture efficacy than that of the endosteal region and it is proposed that the major target of different bone-reinforcing therapies is the periosteal site (Ferretti JL 1995). The periosteum also undergoes structural changes during the course of the development of osteoporosis (Fan W 2010). Together with the nutritive functions of the periosteum, these observations underline the importance of the periosteum in the pathogenesis of different disorders that affect the bone metabolism.

In our experimental protocol IR induced considerable increases in the periosteal leukocyte–endothelial interactions during the reperfusion phase, but these changes were similar in the aged OVX, the Sham-OVX, and the young animals. This observation adds a new aspect to the previous finding of enhanced PMN adhesion in the venules of the femur in the short run, i.e. 2 weeks after OVX in unstressed animals (Kasiyaphat A 2008). Similarly, an early enhancement of PMN infiltration has been demonstrated in the muscle of OVX rats after IR (Stupka N 2001). In fact, there is currently no consensus on the magnitude or pathologic role of leukocyte adhesion in non-skeletal tissues after OVX. OVX has been reported to enhance PMN accumulation in the brain of resting animals (Santizo RA 2000), but other data indicated that OVX does not influence the PMN adherence in the brain tissue after transient ischemia (Xu HL 2004). We suggest that these differences in baseline leukocyte adhesion might be explained by the different timelines. Use of a standardized experimental setup with matching sex, age and bone density conditions would solve this question.

The microcirculatory patency is influenced by functional and morphological changes in the microvasculature. As concerns the structural aspects, it has been shown that OVX leads to a reduced blood flow in the bone (Kapitola J 1995) and a lower total capillary density in the heart (Jesmin S 2002) and brain (Jesmin S 2003), a phenomenon probably related to the well-known effects of estrogen on angiogenesis. In our chronic OVX rat model, the periosteal vessel density was not affected by OVX or E2 replacement therapy. It is important to note that these changes were followed in the proximal tibial periosteum, where venules are the predominant vessel type. The postcapillary venules are predilectory sites for PMN–endothelial interactions, and thus a higher density may predispose to local inflammatory complications. The particularly high venular density of the periosteal microarchitecture may account, at least in part, for a tissue-specific response.

Although relatively little information is available on the effects of chronic OVX itself, there have been numerous experimental studies on the microcirculatory changes of

hormone substitution (e.g. E2). Since estradiol is protective in many forms of injury, it is possible that E2 is protective independently of the presence of OVX. This view is supported by findings where a single dose of E2 given hours or even minutes before reperfusion ameliorates PMN-related processes in different organs (e.g. the heart, liver and retina) (Nonaka A 2000, Booth EA 2003, Burkhardt M 2008). It can be assumed that acute E2 administration could also be beneficial in reducing leukocyte trafficking in our model. Nonetheless, E2 has been shown to reduce leukocyte accumulation, infarct size (Jeanes HL 2006) and ROS production (Florian M 2004) in OVX rats; PMN adherence and infiltration, for instance, can be effectively prevented by estrogen supplementation after OVX (Stupka N 2001).

The mechanism of action by which E2 influences PMN–endothelial interactions is not known in detail. It has been suggested that E2 reduces the expression of endothelium-derived adhesion factors such as P-selectin, VCAM-1 and ICAM-1 (Miller AP 2004). Interestingly, some of these molecules also have a role in bone development, since the CD11/CD18a complex and ICAM-1 have been shown to influence the osteoclast function (Kurachi T 1993). It is hypothesized that an estrogen deficiency results in increased ICAM-1 expression on osteoclast precursors, which may contribute to the bone loss following the menopause or OVX (Gao Y 2000). The smaller diameter of the medullary resistance arterioles in OVX rats further suggests that endogenous estrogen exerts a significant dilator influence within the bone circulation (Soukhova-O'Hare G 2005). We investigated the changes in expression of CD11b, a key determinant of PMN-mediated injuries (Hentzen ER 2000). Upon activation, CD11b (the αM part of the CD11b-CD18 complex) is transferred to the cell surface from preformed intracellular pools (Jones DH 1988) and, as opposed to the constitutive CD11a, its expression is increased several-fold in response to IR stimuli. The considerably increased CD11b expression implies that complete limb ischemia leads to severe systemic inflammatory consequences in this model. The local expression of ICAM-1, the endothelial counterpart of CD11b, was also enhanced in the postischemic periosteum, where the PMN–endothelial interactions were simultaneously and visibly increased. These reactions were not influenced by E2 repletion, which suggests that E2 exerts its anti-adhesive effect independently of CD11b or ICAM-1. The mechanism of how estrogen can modulate *in vivo* PMN reactions demands further attention, but our results are in agreement with those of *in vitro* studies, where OVX did not influence the CD11b expression of the PMNs (Deitch EA 2006). There is evidence of a cellular sexual dimorphism in the activation of PMNs, implying

that PMNs from females respond to trauma and humoral stimuli to a lower extent than do those from males (Deitch EA 2006). Further, estrogen directly modulates the expression of the neuronal isoform of NOS in PMNs (García-Durán M 1999). In our study, E2 treatment clearly reduced the IR-induced secondary PMN–endothelial interactions (i.e. sticking), but this potentially protective effect was not mediated by a mechanism involving tissue ICAM-1 expression changes. Although an increased PMN-derived NO production might reduce PMN adhesion, this reaction is ICAM-1-dependent (Dal Secco D 2006). Overall, the above findings may implicate that chronic estrogen supplementation modulates PMN activation directly, through a novel, specific, estrogen-sensitive pathway.

In our rat model, OVX was followed by definite diagnostic signs of osteoporosis 21 weeks later. In this condition, the postischemic periosteal microcirculatory complications were not aggravated further relative to the non-OVX age-matched controls. Similar changes were observed in TNF-α release, providing further evidence that OVX itself does not modify the inflammatory complications, but E2 supplementation greatly reduces this reaction. It has also been demonstrated that E2 inhibits TNF-α gene transcription via the beta estrogen receptors (Srivastava S 1999) and via the TNF-α-mediated increases in the expressions of adhesion molecules and chemoattractants (Xing D 2007). A downregulation of NO synthesis by TNF-α may also contribute to the mechanisms of these microcirculatory reactions (Yoshizumi M 1993). Our data indicating the positive effect of prolonged E2 substitution on the PMN reactions and TNF-α release, however, point to another clinical implication of our findings: ovarial hormone deprivation supplemented with estrogen therapy (apart from the well-known positive effect in reducing the risk of osteoporotic fractures) affords marked protection against inflammatory mediator release.

5.2. Influence of local IPC on limb IR injury in intact and OVX female rats

In the second study, we examined a somewhat shorter term of OVX, which is sufficient to evoke a chronic estrogen deficit (Iwaniec UT 2001, Xu HL 2001), but does not cause osteopenia in rats. Simarly to our previous results with male rats, the enhanced periosteal primary PMN–endothelial interactions evoked by the 60-min IR insult of the lower extremity could be ameliorated effectively by local IPC. This protection, however, was only incomplete in the OVX animals, as some attenuation could be evidenced in the case of leukocyte rolling, but firm adhesion was not affected. Similarly, the CD11b expression of PMNs did not change after IPC. Accordingly, the protection against

postischemic inflammatory complications in the periosteum is lost in an estrogen-depleted situation. It is therefore reasonable to assume that endogenous estrogen is involved in anti-inflammatory mechanisms provided by IPC in the periosteum.

As concerns the role of endogenous estrogen in the mechanism of IPC, only relatively few studies have been performed and rather conflicting data are available. Shinmura et al. (2008) demonstrated that cardiac ischemia has similar detrimental effects in OVX and control mice, showing that endogenous estrogen in itself is not cardioprotective. The positive effects of IPC were shown to be lost in postischemic hearts harvested from OVX rats, but this reaction could be reversed by E2 (Shinmura K 2008). Interestingly, the protection by IPC was present in OVX rabbit hearts (Sbarouni E 2006). No cardiac protection was afforded by IPC in OVX mice in another study and this could be reversed by E2 substitution (Song X 2003). Others showed that IPC in OVX animals could evoke only a partial postischemic recovery in contractile functions and blood flow, but E2 administration caused a significant improvement (Kolodgie FD 1997). Long- and short-term estrogen administration was shown to have different effects (Peng WJ 2004). The controversy behind these findings remains to be elucidated, but the potential role of differences between species and even organs can not be excluded. It is also a matter of debate why the actions of endogenous and exogenous estrogen are different (Cohen MV 2008). As regards the heart, OVX causes a marked decrease in the phosphorylation of protein kinase C epsilon- and phosphoinositide-dependent kinases, emphasizing the importance of estrogen as a transcription factor (Shinmura K 2008). The present study design did not permit an examination of the underlying mechanisms at a molecular level behind the missing anti-inflammatory protection in OVX animals. Further studies are also needed to clarify the impact of long-term E2 treatment in the postischemic injury of the periosteum in the presence of IPC.

5.3. Remote effects of limb IPC. Beneficial microcirculatory and biochemical effects on the consequences of partial liver IR

The liver plays a complex role in the metabolism of the body as it neutralizes and excretes toxins and drugs, synthesizes enzymes, proteins and glycogen and stores iron and vitamins. For an appropriate function, the liver requires a continuous blood supply to carry out the above processes with their high oxygen demand. Although the liver has good blood perfusion, the ischemic tolerance is low (González-Flecha B 1993). Thanks to the improvements in surgical techniques, complete exclusion of the hepatic blood supply (complete hepatic ischemia) during surgical interventions can be fully avoided, usually

being restricted to hardly manageable cases of bleeding or traumatological situations. To some degree, however, hepatic IR injury is inevitable in clinical practice during hepatic resection and liver transplantation (Huguet C 1992, Hunt CM 1998, Jaeschke H 1998, Teoh NC 2003, Datta G 2013). Along with the increasing need for and improving feasibility of transplantation, novel strategies targeting the attenuation of liver IR injuries may crucially affect the postoperative outcome and graft survival (Dulkanchainun TS 1998).

Extensive or prolonged hepatic ischemia could result in severe inflammation, which begins with Kupffer cell activation, inflammatory cytokine release and ROS production, and then, as a consequence of the oxidative burst, leads to microcirculatory failure (Schauer RJ 2001). Locally rising proinflammatory cytokine and chemokine concentrations recruit circulating PMNs. Adhesion and the following transmigration of PMNs into the perisinusoidal tissues further propagate inflammation (Hasegawa T 2005). In critical cases, postischemic tissue damage leads to hepatocellular apoptosis or necrosis (Gujral JS 2001). Moreover, this local inflammatory cascade can become generalized by leukocytes, causing a systemic inflammatory response syndrome (Lentsch AB 1999, Nastos C 2014) or even a multiorgan dysfunction syndrome (Işeri SO 2005).

Several studies have been performed to augment the tolerance of the liver and to attenuate the harmful effects of IR. Although numerous approaches have been used in experimental settings such as hypothermia (Kim YI 1996), gene therapy (Contreras JL 2004), pharmacological treatment and machine perfusion (Abu-Amara M 2009, Monbaliu D 2010) with various preservation fluids of the graft (Kotulski M 2003, Li L 2007) and surgically-induced pre- and postconditioning (Zhao ZQ 2003, Gurusamy KS 2008), only a few methods have been tested clinically. A reduction in postischemic liver injury was demonstrated after direct hepatic IPC elicited by intermittent portal inflow occlusion during liver resection in patients (Clavien PA 2000), but the potential drawbacks include an increased operating time and greater blood loss during reperfusion (Belghiti J 1999). Hence, rIPC may be a novel and simple method of protecting the liver without inflicting direct stress. One of the most reasonable approaches is when rIPC is elicited by limb ischemia. In such a study, many positive effects of rIPC were identified, including ameliorations in aminotransferase levels, improvements in hepatic blood flow and peripheral oxygen saturation and hepatic venous nitrate/nitrite levels (Kanoria S 2006). The role of heme oxygenase-1 has also been demonstrated (Lai IR 2006).

In our study, the complex microcirculatory and biochemical effects of rIPC by limb IR were examined with particular emphasis on the role of endogenous NOXs. The partial hepatic ischemia model was chosen in order to evaluate effects that are solely related to IR, excluding poorly tolerated mesenteric congestion with the concomitant mediator release and deterioration of the systemic hemodynamics (Vollmar B 1994). We earlier demonstrated the microcirculatory effects of IPC achieved by using two cycles of complete hindlimb ischemia on IR-induced periosteal microcirculatory derangement and systemic inflammatory responses (Szabó A 2009). For a direct continuation of these studies, we first explored the consequences of warm hepatic IR affecting the left liver lobe. Changes in microcirculatory inflammatory reactions and in the efficacy of microcirculation were examined with fluorescence IVM and laser Doppler flowmetry supplemented with modified light spectrometry. Functional parameters and the expression of oxidant-generating intrahepatic enzymes were also assessed. Similarly to the beneficial local effects of local IPC, our study clearly demonstrated the microcirculatory benefits of remote limb IPC on IR-induced inflammatory responses in the liver. These microcirculatory changes were manifested in improvements in postischemic hepatic perfusion and oxygenation and reductions in PMN–endothelial interactions. The IR-related increases in the levels of necroenzymes and inflammatory cytokines were also ameliorated.

A major aim was to investigate the effects of rIPC on liver enzymes that generate ROS. The scope of this project was narrowed down to the most likely ROS-producing sources (i.e. XOR, MPO and NOXs) and we focused on the most relevant NOX isoforms, NOX2 and NOX4, whose roles in the mechanisms of IR damage or IPC have already been implicated. In our model, the expression of NOX2 was reduced by rIPC, but the enhanced NOX4 expression was not affected. Our findings are consistent with those of other research, which revealed that IPC inhibits the effects of mediators involved in the microcirculatory dysfunction, including ROS (Cutrn JC 2002, Jabs A 2010). The main cellular sources of ROS generation in the blood vessels are the NOXs in the smooth muscle cells (Griendling KK 2000), endothelial cells (Bayraktutan U 2000) and adventitial fibroblasts (Pagano PJ 1998). Among the NOX homologs, NOX4 has been reported to be the main isoform in the vascular cells (Bengtsson SH 2003, Ellmark SHM 2005), although the expression of NOX2 or even NOX1 has also been demonstrated. Enhanced NOX4 activity has been implicated in the development of various cardiovascular pathologies, because NOX-derived superoxide rapidly reduces the

bioavailability of endothelium-derived NO, thereby promoting vasoconstriction and enhancing the vascular resistance (Dusting GJ 1998, Zicha J 2001). Nevertheless, NOX4 within the vessel wall generates a low level of ROS continuously, even in the absence of extrinsic stimulation, and, unlike phagocytic NOXs, is involved in physiological vascular processes such as maintenance of the vascular tone, regulation of the endothelium-dependent vasodilator function, oxygen sensing and modulation of the redox-sensitive signaling pathways (Cave AC 2006, Dworakowski R 2008). Moreover, endothelial NOX4 acts as a constitutive endothelial generator of H_2O_2, which positively affects the vascular function, linked to signaling events that promote vasodilatation and cell protection (Dikalov SI 2008, Takac I 2011). The exact factors determining the action of NOX4 in producing superoxides or H_2O_2 are unclear, but in overexpression systems NOX4 releases predominantly H_2O_2 (Ray R 2011). Additionally, molecular mechanisms implicated in NOX4 vasoprotection includes the activation of endothelial NOS and the increased production of NO, and the increased expression of antioxidant systems such as nuclear factor erythroid 2-related factor 2 (Schröder K 2012). Indeed, a significant decrease in myocardial infarct size was recently observed in NOX2- and NOX1/NOX2-, but not in NOX4-deficient mice (Braunersreuther V 2013). Thus, data are accumulating concerning a Janus-faced mechanism by which NOX4 can protect or cause harm to the circulatory system (Schmidt HH 2012) and, if a defensive action of NOX4 activity is possible, it could be hypothesized that the potentially vasoprotective characteristics of NOX4 may, at least in part, explain the ineffectiveness of rIPC in reducing the IR-related NOX4 expression.

Another possibility is that the influence of IPC is not universally effective, in consequence of the different sensitivities of the various target genes and enzymes. rIPC provided significant, but incomplete protection, and the residual damage and IR-induced inflammatory activation could therefore well be linked to the unchanging NOX4 activity. In this sense, the effect of IPC does not extend to all homologs (at least not to NOX4). Recent findings suggest that, unlike other ROS-generating NOXs, the tightly-assembled active conformation of NOX4 cannot be disrupted by conventional means, because the membrane-bound subunit does not require interaction with the cytosolic subunits (Csányi G 2013).

Previous studies demonstrated that the beneficial effect of IPC extends to reducing the adherence of PMNs to the ischemic-reperfused sinusoidal endothelium, attenuating the PMN-mediated endothelial dysfunction, and limiting leukocyte accumulation in the

postischemic liver tissue (Yuan GJ 2005). In accordance with these observations, the influx of activated PMNs into the postsinusoidal venules was observed in the present model, and, as a result of rIPC, the extent of rolling and sticking of the leukocytes was significantly reduced in the central venules. The mechanisms underlying PMN trafficking after IR and rIPC can be explained in terms of several pathways leading to a modified adhesion molecule expression. The characteristic of the hepatic sinusoidal endothelium may contribute to leukocyte trapping: non-classical rolling and mainly VCAM-1-mediated strong adhesion occur in response to inflammation (Lee YS 2004). NOXs have been shown to be particularly involved in regulating VCAM-1 expression through TNF-α release (Cayatte AJ 2001). When applied *in vitro*, S17834, a flavonoid NOX inhibitor, directly repressed the vascular NOX activity, and it reduced TNF-α-stimulated VCAM, ICAM-1 and E-selectin expression *in vivo* (Cayatte AJ 2001). Further, the lower degree of ROS formation after rIPC partially produced by NOXs can also be linked to reduced adhesion molecule expression. Via mineral corticoid receptor activation, the aldosterone-induced adhesion molecule expression also involves activation of the NOXs (Hashikabe Y 2006). Moreover, LPS induces the expression of surface adhesion molecules through the activation of toll-like receptor 4, which has been shown to be involved in the NOX-dependent activation of NF-κB (Park HS 2006).

The detection of MPO activity, the commonly used index of PMN priming and activation (Yuan GJ 2005), further strengthened our IVM observations. In Study 3, the MPO activity correlated significantly with the number of PMNs visualized. This observation is in agreement with a previous report that accumulated PMNs correlated positively with the development of hepatocellular damage after IR (Yuan GJ 2005). Upon activation, the prototypic isoform NOX2 has been proven to be responsible for the superoxide generation of PMNs (Babior BM 1999). This isoform, also referred to as "respiratory burst oxidase", was first described in PMNs as the starting point of ROS production (Babior BM 1999). The effects of NOX2-derived ROS and proteases released from activated PMNs in promoting cell death in the liver have been widely documented (Yuan GJ 2005, De Minicis S 2010). In the present study, rIPC attenuated both arms of the PMN-related injury: it lowered the PMN priming/MPO activity and reduced the expression of NOX2. This is in accordance with previous observations where preconditioning was induced via NOX2 in the ischemic brain, myocardium and endothelial cells (Kawano T 2007, Milovanova T 2008, Thirunavukkarasu M 2012).

Cellular ROS (superoxide and H_2O_2) are now well recognized as playing an integral role in several growth factors and cytokine signal transduction pathways (Lander HM 1997) and serve as second messengers for cellular tyrosine kinase signaling (Goldstein BJ 2005). NOXs appear to be especially involved in redox signaling because they are the only enzymes (among the many intracellular sources of superoxide formation) whose primary function is to generate ROS (Lambeth JD 2004). The tissue specificity and difference in catalytic subunits offers an opportunity for the development of NOX inhibitors that do not compromise the essential physiological signaling and phagocytic functions carried out by ROS.

rIPC significantly attenuated systemic cytokine release. It has previously been shown that NOX-mediated ROS production has a major role in the ischemia-induced inflammatory responses involving activation of the NF-κB (Dworakowski R 2008). TNF-α and HMGB1 have a common NF-κB-dependent transcription; both are involved in inflammatory responses (Dworakowski R 2008, Kamo N 2013). Moreover, hepatocytes have been demonstrated to produce TNF-α in a NOX-dependent manner following hypoxia-reoxygenation and liver IR (Spencer NY 2013). The relationship between TNF-α and HMGB1 expression and ROS production is bidirectional: TNF-α and HMGB1 are involved in regulating the expression of cytokines and other mediators that participate in acute inflammatory responses, many of which are associated with the increased generation of ROS (Kamo N 2013). On the other hand, oxidative stress has been reported to upregulate the expression of cytokines that promote inflammatory responses during reperfusion. These antigen-independent responses interact and amplify each other, finally leading to impaired microhemodynamics, functional and structural cell damage, and remote or systemic inflammatory complications.

6. SUMMARY OF NEW FINDINGS

1. The periosteal microcirculatory consequences of tourniquet-induced ischemia were first quantified by IVM in an adequately long-term follow-up of osteoporosis. OVX does not predispose the periosteal microcirculation to enhanced IR-induced inflammatory complications such as local leukocyte activation and inflammatory cytokine release. The ameliorating effect of E2 therapy on the systemic and PMN-driven local periosteal inflammatory reactions is independent of its anti-osteoporotic effects.
2. Beneficial periosteal microcirculatory effects of local limb IPC are lost after OVX, suggesting that endogenous estrogen plays a potential role in the protection provided by IPC.
3. rIPC (elicited by limb ischemia) reverses the postischemic hepatic microcirculatory perfusion deficit and inflammatory reactions, restores tissue oxygenation and reduces proinflammatory cytokine and necroenzyme levels. These beneficial effects are associated with a reduced NOX2 expression, suggesting a potential role of NOX2 in the mechanism of protection provided by rIPC.

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9. ANNEX

I.

CASE REPORT

MICROCIRCULATORY CONSEQUENCES OF OSTEOPOROSIS

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REZUMAT

Introducere. Etiologia sindromului de osteoporoza senila este plurifactorial, dar nivelul scazut de estrogeni la femeile aflate in perimenopauza este clar asociat cu o pierdere accelerata de material osos. In cadrul studiului nostru experimental, au fost investigate consecintele osteoporozei si a terapiei cronice cu estrogeni asupra reactiilor inflamatorii de la nivelul microcirculatiei periostale si a expresiei receptorilor de estrogeni. **Material si metoda.** Dupa sase luni de la initierea terapiei de substitutie cu estrogeni cu 17β-estradiol (E2, 20 $\mu g^{-1}kg^{-1}zi^{-1}$) la sobolani Sprague-Dawley ovarectomizati (OVX), au fost examinate consecintele inflamatorii la nivel de microcirculatie la 60-min de ischemie totala a membrului posterior si dupa reperfuzie la 180-min. Modificarea expresiei receptorului de estrogen de la nivel leucocitar a fost de asemenea evaluata la o luna de la OVX sub tratament cu E2. **Rezultate:** In afara de inversarea efectelor osteopenice ale OVX, tratamentul amelioreaza semnificativ cresterea aderentei leucocitelor neutrofile indusa de reperfuzarea zonei ischemice la nivelul venulelor postcapilare de la nivelul periostului (vizualizat prin microscopie cu fluorescenta). Acest efect nu a fost asociat cu reducerea expresiei CD11b de la nivelul leucocitelor neutrofile (analiza FACS). OVX a determinat o scadere moderata a expresiei proteinei ER-beta de la nivelul receptorului de estrogen leucocitar, care tinde sa fie inversata de E2. Nivelele mRNA ER-alpha si ER-beta (RT-PCR) a leucocitelor si expresia proteinei ER-alpha (Western blotting) au fost sub limita de detectie. **Concluzii:** Suplimentarea cronica de estrogeni amelioreaza eficient osteoporoza si consecintele microcirculatorii inflamatorii ale ischemiei tranzitorii de la nivelul membrului. Se pare ca acest efect nu este mediat de leucocite CD11b sau de modificari ale expresiei receptorilor de estrogeni.

Cuvinte cheie: ovarectomie, ischemie-reperfuzie, neutrofil, microscopie in vivo, recptor de estrogen

ABSTRACT

Introduction. The etiology of senile osteoporosis syndrome is multifactorial, but the reduced estrogen levels in peri-menopausal women are clearly associated with an accelerated bone loss. In our experimental studies, the consequences of osteoporosis and chronic estrogen supplementation were investigated on the periosteal microcirculatory inflammatory reactions and estrogen receptor expressions. **Material and method.** Six months after the initiation of estrogen replacement therapy with 17β-estradiol (E2, 20 $\mu g^{-1}kg^{-1}day^{-1}$) in ovariectomized (OVX) Sprague-Dawley rats, the microcirculatory inflammatory consequences of 60-min total hindlimb ischemia followed by 180-min reperfusion were examined. Leukocyte estrogen receptor expression changes were also assessed 1 month after OVX plus E2 treatment. **Results.** Apart from reversing the osteopenic effects of OVX, E2 treatment significantly ameliorated the ischemia-reperfusion-induced elevation of neutrophil leukocyte adherence to the postcapillary venules of the periosteum (visualized by means of intravital fluorescence microscopy). This effect was not associated with a reduction of CD11b expression of the neutrophil leukocytes (FACS analysis). OVX caused a moderate decrease in leukocyte estrogen receptor (ER)-beta protein expression which tended to be reversed by E2. Leukocyte ER-alpha and ER-beta mRNA levels (RT-PCR) and the ER-alpha protein expression (Western blotting) were below the detection limit. **Conclusions.** Chronic estrogen supplementation efficiently ameliorates osteoporosis and the inflammatory microcirculatory consequences of transient limb ischemia. It appears that this effect is not mediated by leukocyte CD11b, or estrogen receptor expression changes.

Key words: ovariectomy, ischemia-reperfusion, neutrophil, intravital microscopy, estrogen receptor

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INTRODUCTION

Osteoporosis is the most common disease in the elderly population which progressively weakens bones and often leads to painful accidental fractures. The primary causative therapy is chronic estrogen supplementation which prevents or reverses osteopenic bone loss, and also exerts protection against different inflammatory processes[1]. Apart from their pivotal role in sexual development and reproduction, estrogens (in particular 17β-estradiol, E2) play a remarkable role in the modulation of the cardiovascular system. Some of circulatory effects of E2 are mediated by the

influencing of endothelial production of nitric oxide and prostacyclin[2]. Besides, E2 also promotes endothelial healing and angiogenesis.[3] Both acetylcholine-induced and flow-dependent vasodilation are preserved or potentiated by estrogen treatment.[4]

The role of estrogen receptors in the mediation of microcirculatory reactions is yet largely unknown. Estrogen actions are essentially mediated by two molecular targets: estrogen receptor-alpha (ER-alpha) and ER-beta. The impact of ER-beta has been addressed in vitro only, but a prominent role has been attributed to these receptors in the vascular biology[5] (for review see at Arnal JF et al.[6]). E2 causes vasodilation via ER-alpha receptors which effect is lost in ovariectomized (OVX) rats.[7] Besides, it has been shown that OVX leads to the down-regulation of ER-alpha expression in the vasculature.[8]

As the prevalence of skeletal injuries increase after menopause, our studies were designed to determine the potential microcirculatory benefit of E2 in a limb ischemia-reperfusion model of osteoporosis. An ovariectomized rat model with clinically relevant time frame was used which shares many similarities with the human disease[9]. Our aims were (1) to examine the consequences of osteoporosis on the postischemic periosteal microcirculatory reactions, (2) to evaluate the effects and the consequences of chronic estrogen on these microcirculatory reactions, and (3) to clarify whether the mechanisms of microcirculatory reactions provided by E2 are mediated by expression changes of ER-alpha or ER-beta.

MATERIALS AND METHODS

Animals and experimental design

The experiments were carried out on female Sprague-Dawley rats housed in an environmentally controlled room with a 12-h light-dark cycle, and kept on commercial rat chow (Charles River, Wilmington, MA, USA) and tap water ad libitum. The experimental protocol was approved by the local animal rights protection authorities and followed the EC Directive 86/609/EEC and the National Institutes of Health guidelines for the care and use of laboratory animals.

The experiments were performed in two series. In the first experimental series, 3-months-old animals (weighting 200-210 g) were ovariectomized (Group 1, OVX, n=12), or sham-operation was performed (Group 2, SH-OVX, n=12; see methods at Szabó *et al.*[9]). Five months later (at the age of 8 months), chronic estrogen therapy[10] was initiated 5 days per week with 20 μg kg^{-1} sc 17ß-estradiol (E2, (Sigma, St. Louis, MO, USA) in 6 animals of the OVX group (Group 1a, OVX+E2). The E2 substitution was continued weekly until the end of the experiments (see time scheme in Fig. 1).

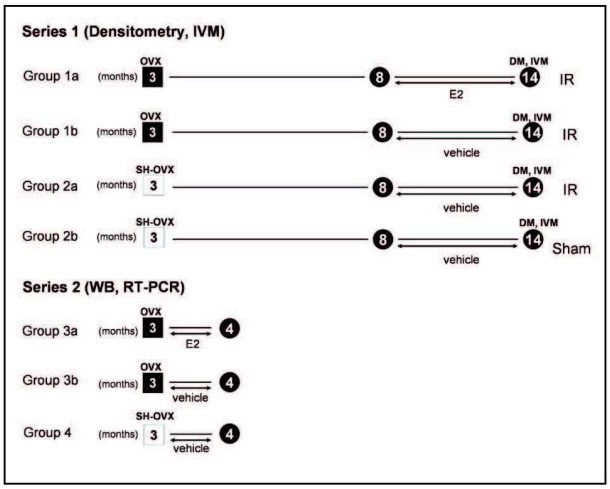

Figure 1. Experimental protocol. The time sequence of surgical interventions, treatments and measurements in Series 1 and Series 2. OVX=ovariectomy; SH-OVX= sham operation for ovariectomy; DM=densitometry; IR=ischemia-reperfusion; Sham=non-ischemic control; IVM=intravital microscopy, E2=17ß-estradiol treatment; WB=Western blotting; RT-PCR=real-time PCR.

The development of osteoporosis was detected by means of ultrasonic densitometry eleven months after OVX, and then microcirculatory consequences of a 60-min complete hindlimb ischemia followed by a 180-min reperfusion period (IR) were investigated using intravital video microscopy (IVM, in groups OVX+IR, OVX+E2+IR and SH-OVX+IR). Total limb ischemia was induced by applying a tourniquet around the thigh and placing a miniclip on the femoral artery. Six animals in the SH-OVX group served as non-ischemic controls SH-OVX+SH. Recordings of the tibial periosteal microcirculation were performed before ischemia and at the end of the 180-min reperfusion period.

A second series of experiments was performed to detect changes in expression of ER-alpha and beta in polymorphonuclear leukocytes (PMNs, by western blotting and real time-PCR). OVX was induced in 8 rats, 4 of which received 20 μg kg-1 E2 supplementation (Group 3a) (in the former dose), starting 2 days after the OVX, while the remaining 4 rats were injected with vehicle (Group 3b). Vehicle-treated sham-operated animals served as controls (Group 4; n=5). 28 days after the OVX, PMNs were isolated from these animals for the determination of ER-alpha and ER-beta protein contents with Western blot analysis and mRNA expressions.

Intravital video microscopy

At the age of 14 months of the animals, the periosteum of the medial surface of the right tibia was exposed under a Zeiss 6x magnification operating microscope, using an atraumatic surgical technique[11] using pentobarbital anesthesia (45 mg kg^{-1} ip). The right hindlimb with the exposed tibial periosteum was positioned horizontally on an adjustable stage, and the microcirculation of the proximal tibia was visualized by IVM (Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury lamp, Acroplan 20x water immersion objective, Carl Zeiss GmbH, Jena, Germany), using fluorescein isothiocyanate (Sigma, St. Louis, MO, USA)-labeled erythrocytes (0.2 ml iv) for red blood cell staining, and rhodamine 6G (Sigma, St. Louis, MO, USA, 0.2%, 0.1 ml iv) for PMN staining. The microscopic images were recorded with a charge-coupled device video camera (AVT HORN-BC 12, Aalen, Germany) attached to an S-VHS video recorder (Panasonic AG-MD 830, Matsushita Electric Industrial Co., Tokyo, Japan) and a personal computer.

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). Leukocyte-endothelial cell interactions were analyzed within 5 postcapillary venules (diameter between 11 and 20 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 reported 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 given as the number of cells/vessel circumference in mm.

Flow cytometric analysis of CD11b expression on leukocytes

The surface expression of CD11b on peripheral blood granulocytes was determined by whole blood flow-cytometric analysis (a modification of a literature method)[9]. CD11b is the M part of the CD11b-CD18 complex which binds to its endothelial counterpart ICAM-1 resulting in leukocyte adhesion to the venular endothelium (a process which can directly be observed with IVM.

Isolation of PMN leukocytes from the blood

PMN leukocytes were isolated by a method described by Russo-Racolante EMS et al..[12] The viability of the PMNs was found to be 80-95% (by the trypan blue exclusion test) and the purity of the suspension was >80% (with the May-Grünwald/Giemsa staining on cell smears). The cell number in 0.1 ml of suspension was in the range 3.5-5.8x10^6.

The suspension was divided into two portions. Fifty μl of suspension was frozen and later used for the detection of ER-alpha and ER-beta protein by Western blot analysis. The remaining suspension was dissolved in TRIzol buffer and frozen for the later evaluation of ER-alpha and ER-beta mRNA expression by real-time PCR.

Western blotting procedure

SDS-polyacrylamide gel electrophoresis was performed on 10% gels; samples were applied without prior heating. Membrane proteins were transferred onto nitrocellulose membrane (Amersham Hybond-C Extra). After quenching with 5% Blotto/Tween (5% milk powder in Tris-buffered saline containing 0.2% Tween 20) for 1.5 h, blots were incubated with the primary antibody overnight. The anti-estrogen receptor alpha rabbit polyclonal antibody (Thermo Fischer Scientific, Fremont, CA, USA) was used in a dilution of 1:150, while the dilution factor for the anti-estrogen receptor beta mouse monoclonal antibody (Leica Biosystems Newcastle Ltd, Newcastle, UK) was 1:50. As secondary antibodies, either HRP-conjugated goat anti-rabbit immunoglobulins (DAKO Glostrup, Denmark) or HRP-conjugated anti-mouse IgG (R&D Systems, Minneapolis, MN, USA) were employed at a dilution of 1:1000 for 1 h. All the antibodies were diluted in 1% Blotto/Tween (containing 1% milk powder), and the washing steps were performed with Tris-buffered saline containing 0.2% Tween 20. Labeled membrane proteins were visualized through a chemiluminescence reaction (Immobilon Western HRP substrate, Millipore, Billerica, MA, USA).

Real-time PCR

Total RNA was isolated from cell lysates with TRIzol™ reagent (Gibco, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA concentration was determined by measuring the A260 values. First-strand cDNA was synthetized from 1 mg of total RNA, using the iScriptTM cDNA Synthesis Kit from Bio-Rad (Hercules, CA, USA). Real-time RT-PCR experiments were performed to quantify the relative abundance of each mRNA by using the iCycler IQ Real-Time PCR machine of Bio-Rad. The Universal Probe Library (UPL) system (Roche, Basel, Switzerland) was used to design primers and probes (see Table 1). Real-time RT-PCR reactions were performed in the FastStart TaqMan Probe Master mix (Roche, Basel, Switzerland), following the

Table 1. Probes and primers used in the study

	Probe no.	Left primer	Right primer
18S	77, Cat. no. 04689003001	ctcaacacgggaaacctcac	cgctccaccaactaagaacg
ER-alpha	130, Cat. no. 04693663001	ttctttaagagaagcattcaaggac	tcttatcgatggtgcattgg
ER-beta	111, Cat. no. 04693442001	ggctgggccaagaaaatc	tctaagagccggacttggtc

manufacturer's instructions.

Statistical analysis

All data are expressed as means±standard error of the mean (SEM). Data analysis was performed with the SigmaStat statistical software (Jandel Corporation, San Rafael, CA, USA). Changes in variables within and between groups were analyzed by one-way ANOVA followed by the Bonferroni test. P values < 0.05 were considered statistically significant.

RESULTS

Bone mineral density changes

A decrease in bone density in the proximal portion of the tibias was observed 11 months after the ovariectomy (proving the development of osteopenia). This reaction could be reversed by the chronic E2 supplementation (Figure 2).

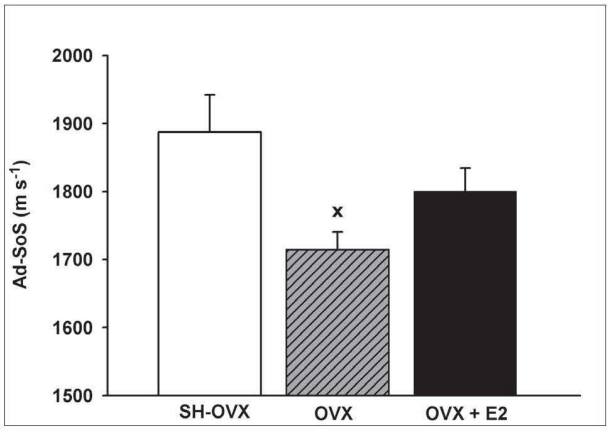

Figure 2. Bone density. Bone density changes in the proximal part of the tibia in sham-operated (SH-OVX), ovariectomized (OVX) or 17ß-estradiol-treated ovariectomized animals (OVX+E2) animals. Data are expressed as Ad-SoS and presented as means±SEM. ANOVA was followed by the Bonferroni test. X P<0.05 vs SH+OVX group.

Leukocyte-endothelial interactions

Using intravital microscopy, a significant increase in the number of firmly adherent leukocytes (as compared to the baseline values or data of the sham-operated SH-OVX animals) was observed in the OVX+IR group by the 180th min of reperfusion (Figure 3). No major differences, however, between the OVX+IR and OVX+SH+IR animals could be observed. These leukocyte-endothelial interactions were significantly ameliorated by E2 (see OVX+E2+IR group).

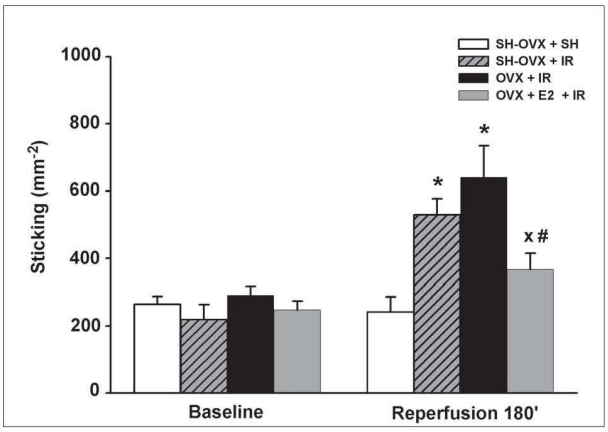

Figure 3. Leukocyte adherence. Firm adhesion (sticking) of neutrophil leukocytes in the postcapillary venules of the tibial periosteum in response to IR injury in sham-operated (SH-OVX+IR), ovariectomized (OVX+IR) or 17ß-estradiol-treated ovariectomized animals (OVX+E2+IR) animals in comparison with sham-operated animals without IR (SH-OVX+SH). Data are presented as means±SEM. ANOVA was followed by the Bonferroni test. * P<0.05 vs baseline, X P<0.05 vs OVX+IR group, # P<0.05 vs SH-OVX+IR group.

CD11b expression changes

The surface expression of adhesion molecule CD11b did not change significantly after sham operation, but the activation of the PMNs was increased approx. 1.8-fold in the OVX group subjected to IR (Figure 4). Similar increases were observed when SH-OVX animals or E2-treated OVX animals were subjected to IR.

Changes in ER-alpha and beta protein expression in the PMNs

On the basis of the available literature, a very low extent of ER expression was expected on the PMNs, hence relatively high amount of protein (75 µg/lane) was loaded during the Western blotting procedures.

Nevertheless, only low ER-alpha and ER-beta signals were detected, and we did not find statistically significant differences in ER-alpha expression within the different experimental groups (data no shown). ER-beta also afforded a very weak signal, which was weaker in the OVX animals in comparison with the Sham or OVX+E2 animals (Figure 5).

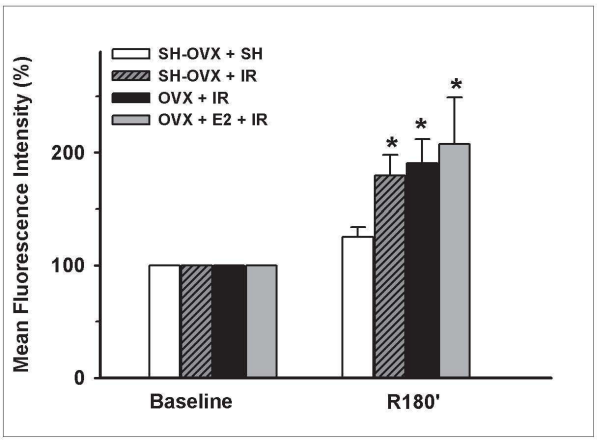

Figure 4. CD 11b expression. Changes in expression of the CD11b adhesion molecule on the surface appearance of PMN leukocytes in response to 60 min of ischemia followed by 180 min of reperfusion in sham-operated (SH-OVX+IR), ovariectomized (OVX+IR) or 17ß-estradiol-treated ovariectomized animals (OVX+E2+IR) animals in comparison with sham-operated animals without IR (SH-OVX+SH). Data are presented as means±SEM. * P<0.05 vs baseline.

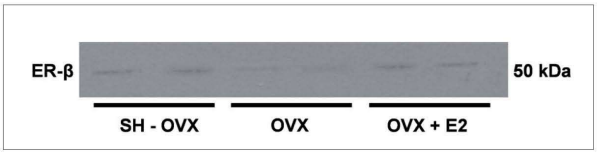

Figure 5. Estrogen receptor-beta protein expression. Representative Western blot demonstrating the expression of estrogen receptor beta (ERβ) in neutrophil leukocytes obtained from sham-operated (SH-OVX) or ovariectomized (OVX) or ovariectomized plus 17ß-estradiol treated (OVX+E2) animals.

Changes in ER-alpha and beta mRNA in the PMNs

The ER-alpha mRNA content of the samples did not reach the limit of detection (Figure 6A). The ER-beta mRNA was detected in most of samples of the Sham group, but never in the OVX groups, while in the OVX+E2 group the data were inconclusive (Figure 6B).

The possibility of technical failure could be excluded, since the expression of 18S RNA was very high and even in each cases, indicating that the amount and quality of the total RNA isolated from the cell lysates and the cDNA samples were satisfactorily high (Figure 6C).

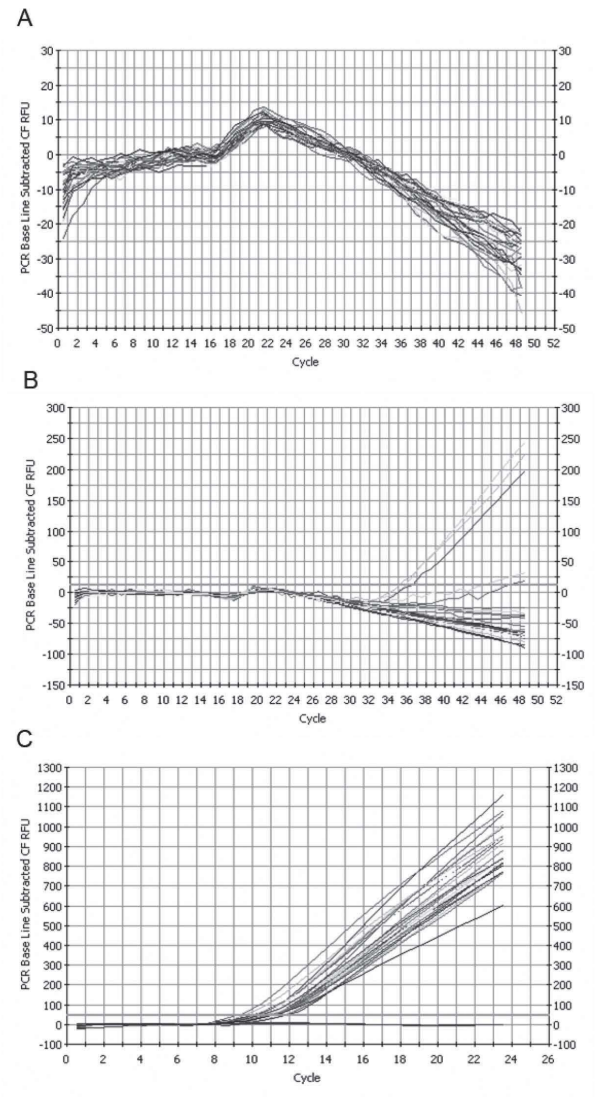

Figure 6. Estrogen receptor-alpha and beta mRNA expressions. Real-time PCR data for ER-alpha (A) and ER-beta (B) and 18S RNA signals of the samples (C).

DISCUSSION

The incidence of osteoporotic fractures remains to be high in the aging population.[13] So far it has not been clarified whether osteoporotic women are more prone to post-ischemic inflammatory bone complications than postmenopausal women receiving estrogen replacement therapy. In this study, we targeted this issue by applying an animal model of osteopenia combined with chronic estrogen replacement therapy. The periosteal microcirculatory consequences of tourniquet-caused ischemia were quantified by IVM after an adequately long-term follow-up of osteoporosis. The data show that osteoporosis or chronic estrogen deficit does not change the magnitude of the PMN-associated microcirculatory menace in

the event of transient ischemia of the osteoporotic limb. In this condition, however, chronic estrogen supplementation ameliorates bone demineralization and also decreases systemic and PMN-driven local periosteal inflammatory reactions. The protective, anti-inflammatory effects of E2 regarding the PMN-endothelial interactions were evident without influencing the expressions of the adhesion molecule CD11b which plays a role in the adhesion process of the PMNs to the venular endothelium. In our previous studies we have shown the alleviation of ischemia-reperfusion-induced activation of leukocyte-endothelial interactions after E2 treatment,[9] but not the effect on ICAM-1 expression, the endothelial adhesion molecule being responsible to a final stage of leukocyte-endothelial interactions. Hence we extended the examinations on the effects of E2 on estrogen receptor expression changes in the PMNs.

To date little is known about the role of ER receptors during the course of neutrophil-mediated microcirculatory inflammatory reactions. In vitro studies generally agree on the positive effect of E2 and the modulatory role of ER receptors regarding the outcome of leukocyte-driven inflammatory processes.[14] There are studies demonstrating that these reactions are dependent on ER-beta receptors.[5] Others emphasized the significance of a biological (transcription) modulator effect, because E2 can modify (via ER-alpha) the phosphorilation of genes being responsible for the transcription of adhesion molecules that play roles in the process of leukocyte-endothelial interactions.[15]

In our studies, the expression of both ER receptor mRNA in the PMNs was under the detection limit of RT-PCR. Only a few papers were published on this issue, and the data are controversial. Considerable species differences were detected and Yu *et al.* demonstrated tissue-specific expression of ER-alpha and beta.[16] Specifically, only the ER-beta protein was found in cows, but both subtypes could be traced at the mRNA level.[17] In a human study only ER-beta mRNA was found,[18] while in another study neither ER-alpha nor ER-beta mRNA were detected, though the proteins could be traced by Western blotting analysis.[19] The levels of ER proteins were similarly low in our studies. In humans, both ER-alpha and ER-beta are present on PMNs, and E2 enhances the expression of both proteins.[20]

CONCLUSION

In summary, chronic estrogen supplementation reduced the inflammatory complications caused by ischemia-reperfusion injury in the periosteal microcirculation, but the anti-inflammatory effects were mediated by mechanisms other than estrogen receptor expression changes on the circulating PMN leukocytes. As far as we are aware, this is the first report of leukocyte ER receptor expression in rats, and it is clear that further experiments are needed to improve the sensitivity of the methods to clearly define the potential role of estrogen receptors during the course of PMN-endothelial interactions.

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Periosteal microcirculatory action of chronic estrogen supplementation in osteoporotic rats challenged with tourniquet ischemia

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ABSTRACT

Aims: Transient ischemia of osteoporotic bones during elective orthopedic surgery or fracture repair carries risks for serious complications, and estrogen loss or replacement has a potential to influence ischemia–reperfusion-induced inflammatory activation. To clarify this, we investigated the periosteal inflammatory changes in a clinically relevant time frame in ovariectomized rats, an experimental model of postmenopausal bone loss. Furthermore, the effects of chronic estrogen supplementation on the postischemic local and systemic inflammatory reactions were assessed.

Main methods: Bilateral ovariectomy or sham operation was performed in 3-month-old female Sprague–Dawley rats. Five months later, estrogen replacement therapy with 17β-estradiol ($20\ \mu g^{-1}\ kg^{-1}\ day^{-1}$) or vehicle treatment was initiated. The microcirculatory inflammatory consequences of 60-min total hindlimb ischemia followed by 180-min reperfusion were examined 11 months after ovariectomy and were compared with those in 3-month-old animals.

Key findings: The osteoporosis that developed 5 months after ovariectomy was significantly ameliorated by estrogen replacement therapy. Both in ovariectomized and in non-ovariectomized animals, ischemia–reperfusion elevated the neutrophil adherence ~3-fold in the postcapillary venules of the periosteum (intravital microscopy), with an ~50–60% increase in intravascular neutrophil activation (CD11b; FACS analysis), an enhanced TNF-α release (ELISA) and periosteal expression of ICAM-1 (the endothelial ligand of CD11b; immunohistochemistry). Exogenous 17β-estradiol considerably reduced TNF-α release and the number of neutrophil–endothelial interactions in the periosteum, without affecting the CD11b and ICAM-1 expression changes.

Significance: Osteoporosis itself does not increase the magnitude of the limb ischemia–reperfusion-associated periosteal inflammatory reaction. Chronic estrogen supplementation, however, reverses osteoporosis and significantly ameliorates the microcirculatory consequences of transient ischemia.

Introduction

The etiology of senile osteoporosis syndrome is multifactorial, but the reduced estrogen levels in peri-menopausal women are clearly associated with an accelerated bone mineral density loss (Richelson et al., 1984). The structural bone deficit is thought to be attributable to enhanced resorption and simultaneously decreased bone formation, and several early studies provided convincing evidence that these changes can be suppressed or even prevented by restitution of the pre-menopausal estrogen levels (Christiansen et al., 1982).

The systemic and local consequences of endogenous estrogen loss and exogenous replacement in the circulatory system are more ambiguous. The incidence of septic, inflammatory complications is significantly lower in many trauma-hemorrhage conditions in females than in males (Sperry et al., 2008; Choudhry et al., 2005), and short-term estrogen pretreatment confers significant protection from ischemia–reperfusion (IR) injury and leukocyte activation in males (Burkhardt et al., 2008). However, other lines of evidence support the notion that estrogen supplementation may increase the risk of intravascular clotting complications in post-menopausal women (Cushman, 2002). Although the underlying mechanistic details are

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still unclear and the concept is debated (Seelig et al., 2004; Canonico et al., 2008), it is obvious that the skeletal and anti-inflammatory benefits of hormone replacement therapies should be carefully weighed against the possible circulatory side-effects. Given this background, it is important to note that the consequences of chronic estrogen depletion and repletion on the human bone circulation are still unclear. Under certain compromised flow conditions, the periosteal microcirculation may be a good indicator of the perfusion changes of the whole bone, but the microcirculatory effects of osteoporosis in this tissue layer are also largely unknown. The patency of the periosteal microcirculation is of particular importance in transient ischemic states such as traumas, fractures, soft tissue injuries or limb operations involving tourniquet application (Varga et al., 2008; Zhang et al., 2003). In these cases, both the bone and the periosteum undergo microvascular events reflected by perfusion insufficiencies and severe antigen-independent inflammatory reactions. These processes are mainly initiated by the increased adhesion of polymorphonuclear (PMN) granulocytes to the microvascular endothelium (rolling and then firm adhesion), followed by their migration to and accumulation in the perivascular tissues (Wolfárd et al., 2002).

As the prevalence of skeletal injuries increases after menopause, our studies were designed to determine whether hormonal replacement therapy might be of microcirculatory benefit in this subset of the osteoporotic population. The ovariectomized (OVX) rat is a well-established animal model for osteoporosis research, as it shares many similarities with the human disease. These include an increased rate of bone turnover (Wronski et al., 1986), and an initial rapid phase of bone loss and similar skeletal responses to treatment with estrogen, calcitonin, bisphosphonates and many other agents (Fleisch, 1993; Allen et al., 2004). In this context, our first objective was to observe the effects of osteoporosis and the consequences of estrogen therapy in a clinically relevant time frame. To this end, we first determined if chronic treatment with estrogen influences the OVX-triggered local periosteal microcirculatory reactions.

Secondly, we hypothesized that the periosteal microcirculation of osteoporotic rats would be more sensitive to the detrimental consequences of transient limb ischemia than that of estrogen-treated, age-matching controls. The results suggest that, even though osteoporosis itself does not amplify the IR-induced inflammatory responses, the periosteal granulocyte recruitment can be significantly reversed by chronic estrogen supplementation.

Materials and methods

Animals and experimental design

The experiments were carried out on female Sprague–Dawley rats housed in an environmentally controlled room with a 12-h light–dark cycle, and kept on commercial rat chow (Charles River, Wilmington, MA, USA) and tap water *ad libitum*. The experimental protocol was approved by the local animal rights protection authorities and followed the EC Directive 86/609/EEC and the National Institutes of Health guidelines for the care and use of laboratory animals.

In the first experimental series, 3-month-old animals (weighing 200–210 g) were ovariectomized (Group 1, OVX, n = 12), or sham-operation was performed (Group 2, Sham, n = 6). Five months later (at the age of 8 months), chronic estrogen therapy (Sims et al., 1996) was initiated 5 days/week with 20 μg kg^{-1} sc 17β-estradiol (E2, Sigma, St. Louis, MO, USA) in 6 animals of the OVX group (Group 1a). The E2 substitution was continued weekly until the end of the experiments (see time scheme in Fig. 1). The remaining OVX (Group 1b) and Sham animals received the vehicle for E2 (100% ethanol diluted in corn oil) in the same volume. Body weight changes and the development of osteoporosis were continuously followed in the proximal tibiae by means of ultrasonic densitometry (see later).

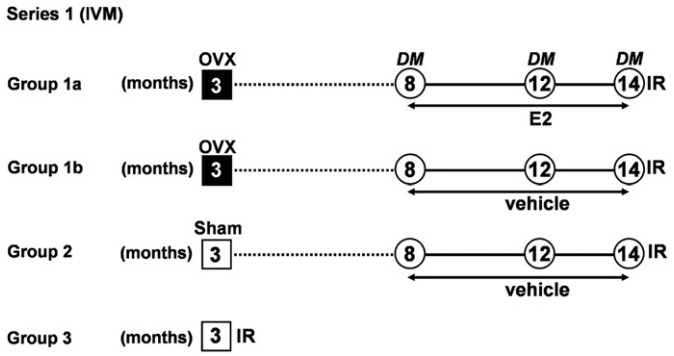

Fig. 1. The time sequence of surgical interventions, treatments and measurements in Series 1. OVX = ovariectomy; Sham = sham operation; DM = densitometry; IR = ischemia–reperfusion; IVM = intravital microscopy, E2 = 17β-estradiol treatment. In Series 2 identical protocols were applied to detect adhesion molecule changes.

Eleven months after OVX, the animals were subjected to a 60-min complete hindlimb ischemia followed by a 180-min reperfusion period (IR). Limb ischemia was induced by applying a tourniquet around the thigh and placing a miniclip on the femoral artery. Control hindlimb IR was conducted on another group of 3-month-old female rats (Group 3, n = 6). In this group the microcirculatory consequences of limb IR alone were investigated using intravital video microscopy (IVM).

A second series of experiments (Groups 4a, 4b, 5, and 6), with identical protocols (OVX or Sham combined with IR, n = 7–10), was performed to detect changes in the pro-inflammatory cytokine TNF-α concentrations in the plasma and in the expressions of adhesion molecules known to play a role in the process of PMN leukocyte adhesion to the endothelium. This was necessary because the fluorescent dyes used for IVM interfere with the flow-cytometry technique, and blood samples for the assessment of adhesion molecule expressions were therefore obtained separately. Tissue biopsies were also taken for detection of the tissue ICAM-1 changes by immunohistochemistry (see later).

Surgical procedure of ovariectomy

Animals were anesthetized with a combination of ketamine and xylazine (25 mg kg^{-1} and 75 mg kg^{-1} ip, respectively) and a median laparotomy was performed under sterile conditions. The connection of the Fallopian tubes was cut between hemostats, the ovaries were removed, and the stumps were then ligated. Thereafter, the abdomen was filled up with warm sterile physiological saline and the abdominal wall was closed in two layers. Sham-operated animals underwent identical procedures except that the Fallopian tubes and ovaries remained intact.

Quantitative ultrasound bone densitometry (QUS)

Under ketamine–xylazine anesthesia (as discussed earlier), bone density measurements were performed at the tibia and the tail with a DBM-Sonic 1200 (IGEA, Carpi, Italy) ultrasonic bone densitometry device; the changes in the average of the amplitude-dependent speed of sound (AD-SoS) were determined (Joly et al., 1999). After calibration, the AD-SoS values of the soft tissues (muscle and skin above the thigh) were determined, and the system deducted this value from the bone density. The AD-SoS values were calculated via a computer program and the average of 5 measurements was used at each time points. Twenty-one weeks after OVX, statistically significant density alterations were observed in the proximal tibia, and this location was therefore used for further QUS measurements at the ages of 8, 9, 12 and 14 months.

Surgical procedure for IVM

Intravital analysis of the periosteal microcirculation was performed at the end of the experimental protocol on 14-month-old animals. Anesthesia was induced with sodium pentobarbital (45 mg kg^{-1} ip) and sustained with small supplementary iv doses when necessary. The right carotid artery and the jugular vein were cannulated for the measurement of mean arterial pressure (MAP) and the administration of drugs and fluids, respectively. The animals were placed in a supine position on a heating pad to maintain the body temperature between 36 and 37 °C. Ringer's lactate was infused at a rate of 10 ml kg^{-1} h^{-1} during the experiments. 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 6× magnification operating microscope, using an atraumatic surgical technique (Varga et al., 2008).

Experimental protocols

In the first series, after a 30-min stabilization period, the baseline cardiovascular and microhemodynamic parameters were determined (baseline; −60 min). The periosteal microcirculation was observed every 60 min during the 180-min reperfusion period.

In the second experimental series, blood samples from the carotid artery were taken at baseline and during the reperfusion period for the detection of changes in the plasma concentrations of TNF-α and in the expression of the adhesion molecule CD11b. TNF-α levels were determined from plasma samples taken at the 60th min of reperfusion. In a pilot study, the dynamics of the CD11b expression changes in response to limb IR were characterized; significant elevations were not found earlier than 120 or 180 min in the reperfusion period. Accordingly, this time frame was selected for flow cytometric evaluations of blood samples (see later).

Intravital video microscopy

The right hindlimb with the exposed tibial periosteum was positioned horizontally on an adjustable stage, and the microcirculation of the proximal tibia was visualized by IVM (Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury lamp, Acroplan 20× water immersion objective, Carl Zeiss GmbH, Jena, Germany), using fluorescein isothiocyanate (Sigma, St. Louis, MO, USA)-labeled erythrocytes (0.2 ml iv) for red blood cell staining, and rhodamine 6G (Sigma, St. Louis, MO, USA, 0.2%, 0.1 ml iv) for leukocyte staining. The microscopic images were recorded with a charge-coupled device video camera (AVT HORN-BC 12, Aalen, Germany) attached to an S-VHS video recorder (Panasonic AG-MD 830, Matsushita Electric Industrial Co., Tokyo, Japan) and a personal computer.

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). Leukocyte–endothelial cell interactions were analyzed within 5 postcapillary venules (diameter between 11 and 20 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 reported 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 given as the number of cells/vessel circumference in mm.

In the examined anatomical fields of the tibia, the majority of the vessels were not capillaries, but venules (see later); hence the total vascular density was calculated from the length of recognized vessels divided by the corresponding area (an average of 3 measurements per observation field was used).

Immune labeling and flow cytometric analysis of CD11b expression

The surface expression of CD11b on the peripheral blood granulocytes was determined through flow-cytometric analysis of whole blood in duplicate (Varga et al., 2008). One-hundred μl of whole blood was incubated with 20 μl of (50 μg ml^{-1}) mouse anti-rat monoclonal antibody (BD Pharmingen, San Jose, CA, USA) for 20 min. Negative controls were obtained by omitting the monoclonal antibody. The cells were then washed twice in Hanks buffer and centrifuged at 13,000 rpm for 5 min and the resuspended pellet was incubated with fluorescein isothiocyanate-conjugated polyclonal rabbit anti-mouse immunoglobulin (10 μg ml^{-1}; DAKO Cytomation, Glostrup, Denmark; 20-μl aliquots of reagents to 180-μl aliquots of blood cells). The cells were again washed twice, and the erythrocytes were lysed with a Lysing kit (Biodesign, Saco, ME, USA), after which the cells were washed twice again (6000 rpm, 5 min) and resuspended in 200 μl Hanks buffer. Computer-assisted FACStar Plus Becton-Dickinson equipment was used for cytometry; the granulocytes were gated on the basis of their characteristic forward and side-scatter features. Generally, 10,000 events per sample were collected and recorded; the percentage of labeled (activated) granulocytes (relative to the overall marker-bearing cells) and the mean fluorescence intensity (average marker density) were calculated.

TNF-α measurements

TNF-α levels were measured in duplicate, using a commercially available anti-rat TNF-α ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Detection of tissue ICAM-1 by immunohistochemistry

At the end of the experiments, both the limb subjected to IR and the contralateral one were removed and the tibias and the surrounding muscles were fixed in 4% phosphate-buffered formalin for 2–3 days. The tissues were then decalcified with an electrophoretic apparatus (for 2×4 h) using a special decalcifying solution (Sakura TDE30; Sakura Finetek Corp. Torrance, CA, USA). The samples were embedded in paraffin, and the 4-μm sections were placed on silanized slides. After conventional methods of dewaxing and rehydration (initiated in xylene, followed by decreasing concentrations of ethanol and methanol), tissue endogenous peroxidase was blocked with a mixture of methanol and 1% H_2O_2 for 5 min, and the nonspecific tissue antigens with conventional 2.8% cow milk. For ICAM-1 immunohistochemistry, 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, Glostrup, 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 200× magnification. During the semiquantitative analysis, periosteal and intramuscular vessels were evaluated separately and the percentage of ICAM-1-positive vessels was calculated. The samples were allotted to one or another of the following semiquantitative categories (scores 1–8) (Table 1).

Statistical analysis

All data are expressed as means ± standard error of the mean (SEM). Data analysis was performed with the SigmaStat statistical software (Jandel Corporation, San Rafael, CA, USA). Changes in

Table 1
Scoring system for the analysis of ICAM-1 positivity in vessels from periosteal samples processed for immunohistochemistry.

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

variables within and between groups were analyzed by one-way ANOVA followed by the Bonferroni test. P values<0.05 were considered statistically significant.

Results

During the course of the experiments, the body weight of the animals increased in all groups (Fig. 2), but OVX was followed by a significantly higher weight gain as compared with that in the Sham group. After the initiation of the E2 therapy (at the age of 8 months), the weight gain decreased to the level for the non-OVX animals.

As shown by bone densitometry on the proximal tibia, osteoporosis developed 21 weeks after bilateral ovariectomy. The AD-SoS was significantly lower than that for the sham-operated animals (1674 ± 33 m s^{-1} *vs* 1850 ± 101 m s^{-1}, respectively). The OVX-induced osteopenia was completely restored by E2 therapy (Fig. 3).

At the beginning of IVM, the baseline values of the macrohemodynamic variables (including heart rate and MAP) in the various groups did not differ significantly and there were no significant hemodynamic changes relative to the baseline values during the experimental period. In all groups subjected to IR, a moderate decrease in MAP was observed in the first few minutes of reperfusion, but MAP thereafter stabilized at the control level (data not shown).

The number of firmly adherent (sticking) leukocytes was significantly increased in the postcapillary venules of the tibial periosteum in the 3-month-old controls, the Sham rats and the OVX animals at the end of the reperfusion phase (120–180 min). This phenomenon was nearly completely prevented by chronic E2 supplementation in the OVX animals (Fig. 4).

In the examined anatomical fields of the tibia, the majority of the vessels were not capillaries, but venules (Fig. 5). No significant differences were found in total vascular density between the 3-month-old controls (268.1 ± 10.1 cm^{-1}), the Sham (262.6 ± 7.1 cm^{-1}), the OVX (261.9 ± 7.6 cm^{-1}) or the OVX + E2 (265.1 ± 1.1 cm^{-1}) groups.

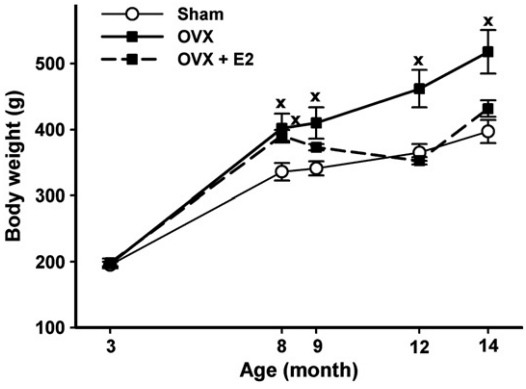

Fig. 2. Body weight changes in sham-operated (Sham) or ovariectomized animals treated with 17β-estradiol (OVX + E2) or vehicle (OVX). Data are presented as means ± SEM. ANOVA was followed by the Bonferroni test. $^{X}P<0.05$ *vs* Sham group; differences *vs* baseline (3 months of age) are not shown.

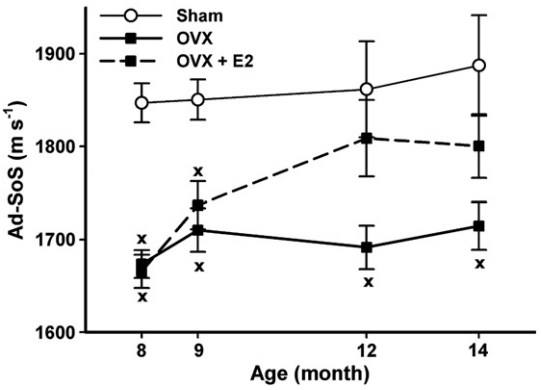

Fig. 3. Bone density changes in the proximal part of the tibia in sham-operated (Sham) or ovariectomized animals treated with 17β-estradiol (OVX + E2) or vehicle (OVX). Data are expressed as Ad-SoS and presented as means ± SEM. ANOVA was followed by the Bonferroni test. $^{X}P<0.05$ *vs* Sham group.

An increased surface expression of adhesion molecule CD11b was observed after 120 and 180 min of reperfusion; no major differences could be detected between the findings for the OVX and the Sham or the 3-month-old rats (Fig. 6). Chronic E2 administration did not influence this parameter.

The plasma TNF-α reached similar concentrations (young animals: 21.24 ± 3.30 pg ml^{-1}; Sham: 22.16 ± 5.79 pg ml^{-1}; OVX: 19.84 ± 1.35 pg ml^{-1}) by the 60th min of reperfusion. E2 treatment, however, resulted in significantly lower TNF-α values (OVX + E2: 11.14 ± 1.19 pg ml^{-1}) as compared with that for the OVX-challenged rats ($P<0.05$). No TNF-α was detected in control samples taken before the ischemic insult.

As assessed by immunohistochemical analysis (Fig. 7), the tissue ICAM-1 density in the vessels of the periosteum was significantly higher in the limbs subjected to IR (young animals: 1.67 ± 0.67; Sham: 1.83 ± 0.60; OVX: 2.17 ± 0.48; OVX + E2: 2.0 ± 0.52) than in the intact contralateral limbs in all experimental groups (young animals: 0.33 ± 0.21; Sham: 0.50 ± 0.22; OVX: 0.67 ± 0.21, OVX + E2: 0.50 ± 0.22). The intensity of the IR-induced ICAM-1-positive reaction was only moderate in the muscle tissue, and the data for the 3-month-old controls, the Sham, OVX and the OVX animals treated with E2 were also similar.

Discussion

Females are more resistant to circulatory shock and inflammation than males, but this gender-related anti-inflammatory protection is

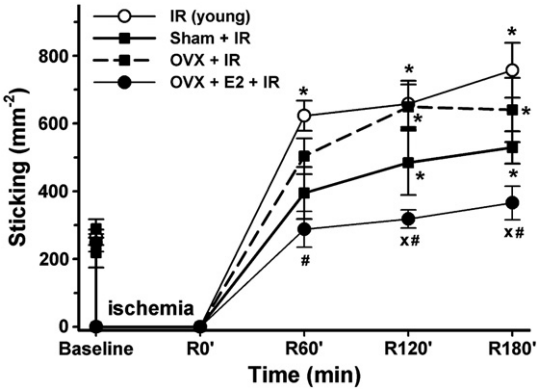

Fig. 4. Secondary leukocyte–endothelial cell interactions (sticking) in the postcapillary venules of the tibial periosteum in sham-operated (Sham + IR) or ovariectomized animals treated with 17β-estradiol (OVX + E2 + IR) or vehicle (OVX + IR) in comparison with 3-month-old (young) female rats (IR). Data are presented as means ± SEM. ANOVA was followed by the Bonferroni test. $^{*}P<0.05$ *vs* baseline, $^{X}P<0.05$ *vs* OVX + IR group, $^{\#}P<0.05$ *vs* IR group.

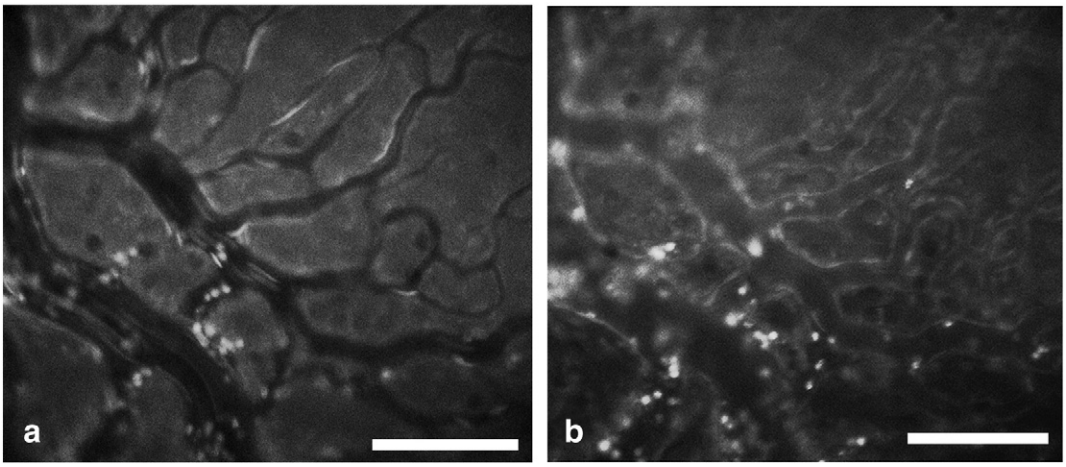

Fig. 5. Representative micrographs of the venular network of the tibial periosteum. Panel a: FITC-labeled erythrocytes, panel b: same field with rhodamine 6G-labeled leukocytes. Bar represents 100 μm.

lost during menopause or after OVX (Wichmann et al., 2000; Angele et al., 2000). As the incidence of traumas and osteoporotic fractures steadily rises in the elderly population (Gullberg et al., 1997), the question arises of whether osteoporotic women are more susceptible to postoperative or post-traumatic inflammatory bone complications than postmenopausal women receiving estrogen replacement therapy. We set out to answer this question by employing a chronic rat model of osteoporosis where the consequences of a long-term female hormone deficiency can be adequately estimated (Tan et al., 2003). The results showed that osteoporosis was reproducibly evolved after OVX, and the reversing efficacy of E2 therapy on osteopenia, together with the periosteal microcirculatory status of the tibia, could subsequently be evaluated in the long term. The data also showed, however, that the postischemic periosteal microcirculatory PMN recruitment and the expressions of the adhesion molecules CD11b and ICAM-1 (which play a role in the process of adhesion of the PMNs to the venular endothelium) undergo similar changes in this condition, irrespective of the age or the endogenous estrogen status of the animals. Thus, the primary message of our study is that a chronic estrogen deficit does not change the magnitude of the PMN-associated microcirculatory menace in the event of transient ischemia of the osteoporotic limb.

IVM allows direct observation and quantitative analysis of the microcirculation of the exposed tissues. With this technique, the consequences of an estrogen deficit and estrogen replacement have already been demonstrated in certain organs (Santizo et al., 2000; Watanabe et al., 2001), but never in the periosteum, though the protecting and feeding functions of the periosteum are hallmarked by the fact that restoration of periosteal microcirculation guarantees bone survival even in an environment with limited blood supply (Berggren et al., 1982). Additionally, osteoporosis has been shown to develop on the basis of an imbalance between bone resorption and formation, the effector cells being derived from mesenchymal osteoprogenitor cells found in the periosteum and endosteum, the former displaying predominance in this respect (Brighton et al., 1992). Furthermore, the experimental and clinical evidence suggests an important role of the periosteum not only in the pathogenesis of osteoporosis itself, but also in the effects of different anti-osteoporotic approaches (Allen et al., 2004). The periosteal region of the cortical bones exhibits a better anti-fracture efficacy than that of the endosteal region and it is proposed that the major target of different bone-reinforcing therapies is the periosteal site (Ferretti et al., 1995). Together with the nutritive functions of the periosteum, these observations underline the importance of the periosteum in the pathogenesis of different disorders also affecting the bone metabolism.

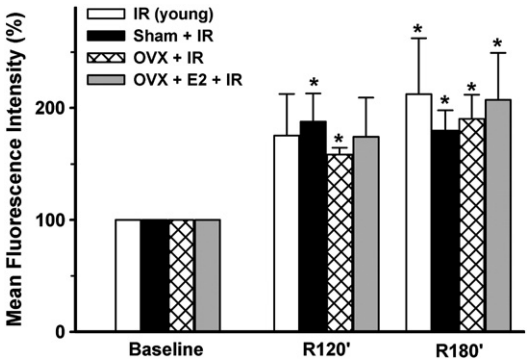

Fig. 6. Changes in expression of the CD11b adhesion molecule on the surface appearance of PMN leukocytes in response to 60 min of ischemia followed by 180 min of reperfusion in sham-operated (Sham + IR) or ovariectomized animals treated with 17β-estradiol (OVX + E2 + IR) or vehicle (OVX + IR) in comparison with 3-month-old (young) female rats (IR). Data are presented as means ± SEM. *$P<0.05$ *vs* baseline.

In our study, IR induced considerable increases in the periosteal leukocyte–endothelial interactions during the reperfusion phase, but these changes were similar in the aged OVX, the sham-OVX groups and the young animals. This observation adds a new aspect to the previous finding of enhanced PMN adhesion in the venules of the femur in the short run, i.e. 2 weeks after OVX in unstressed animals (Kasiyaphat et al., 2008). Similarly, an early enhancement of PMN infiltration has been demonstrated in the muscle of OVX rats after IR (Stupka and Tiidus, 2001). In fact, there is currently no consensus on the magnitude or pathologic role of leukocyte adhesion in non-skeletal tissues after OVX. OVX has been reported to enhance PMN accumulation in the brain of resting animals (Santizo et al., 2000) but other data indicated that OVX does not influence the PMN adherence in the brain tissue after transient ischemia (Xu et al., 2004). We suggest that these differences in baseline leukocyte adhesion might be explained by the different timelines. Use of a standardized experimental setup with matching sex, age and bone density conditions would solve this question.

The microcirculatory patency is influenced by functional and morphological changes in the microvasculature. As concerns the structural aspects, it has been shown that OVX leads to reduced blood flow in the bone (Kapitola et al., 1995) and a lower total capillary density in the heart (Jesmin et al., 2002) and brain (Jesmin et al., 2003), a phenomenon probably related to the well-known effect of estrogen on angiogenesis. In our chronic OXV rat model, the periosteal vessel density was not affected by OVX or E2 replacement therapy. It

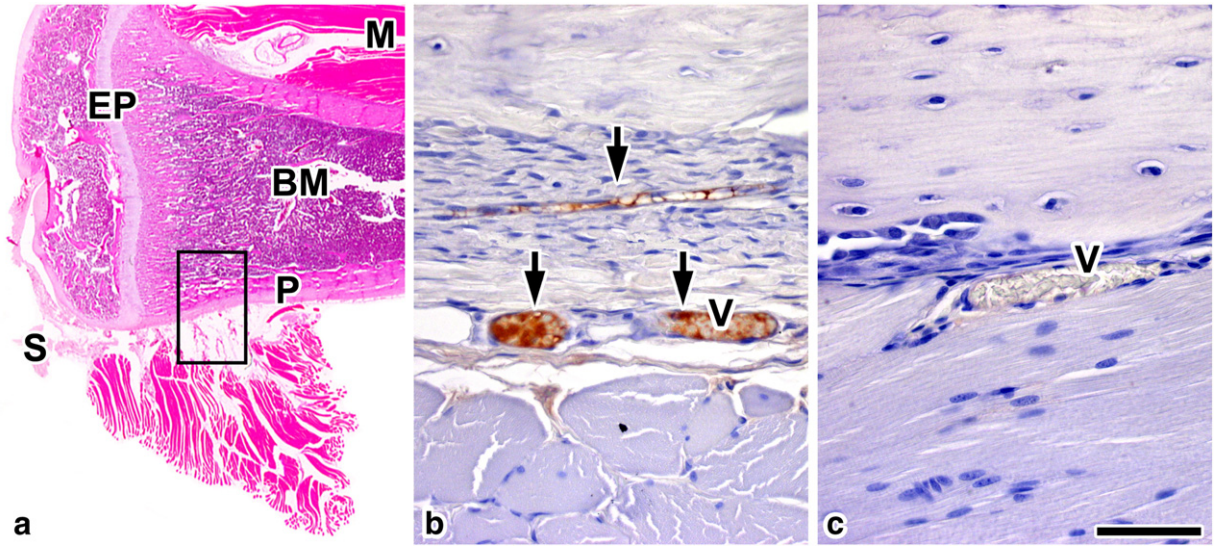

Fig. 7. Representative longitudinal section of the rat tibia surrounded by soft tissues (stained with ICAM-1 antibody plus hematoxylin). Left panel (a): tibial epiphysis (EP), bone marrow (BM), knee-joint synovia (S), muscle (M) and tibial periosteum (P) are indicated. Middle panel: positive staining for ICAM-1 (arrows) was found in the periosteal venules (V) after IR (b), but not in the contralateral (non-IR) limbs (c). Bar represents 50 μm.

is important to note that these changes were followed in the proximal tibial periosteum, where venules are the predominant vessel type. The postcapillary venules are predilectory sites for PMN–endothelial interactions, and thus a higher density may predispose to local inflammatory complications. The particularly high venular density of the periosteal microarchitecture may account, at least in part, for a tissue-specific response.

Although relatively little information is available on the effects of chronic OVX itself, there are numerous experimental studies on the microcirculatory changes of hormone substitution (e.g. E2). Since estradiol is protective in many forms of injury, it is possible that E2 is protective independently of the presence of OVX. Nonetheless, E2 has been shown to reduce leukocyte accumulation, infarct size (Jeanes et al., 2006) and oxygen free radical production (Florian et al., 2004) in OVX rats; PMN adherence and infiltration for instance can be effectively prevented by estrogen supplementation after OVX (Stupka and Tiidus, 2001).

The mechanism of action by which E2 influences PMN–endothelial interactions is not known in detail. It has been suggested that E2 reduces the expression of endothelium-derived adhesion factors such as P-selectin, vascular cell adhesion molecule-1, and ICAM-1 (Miller et al., 2004). Interestingly, some of these molecules also have a role in bone development, since LFA and ICAM-1 have been shown to influence the osteoclast function (Kurachi et al., 1993). It is hypothesized that an estrogen deficiency results in increased ICAM-1 expression on osteoclast precursors, which may contribute to the bone loss following menopause or OVX (Gao et al., 2000). The smaller diameter of the medullary resistance arterioles in OVX rats also suggests that endogenous estrogen exerts a significant dilator influence within the bone circulation (Soukhova-O'Hare et al., 2005). We investigated the changes in expression of CD11b, a key determinant of PMN-mediated injuries (Hentzen et al., 2000). Upon activation, CD11b (the αM part of the CD11b–CD18 complex) is transferred to the cell surface from preformed intracellular pools (Jones et al., 1988) and, as opposed to the constitutive CD11a, its expression is increased several-folds in response to IR stimuli. The considerably increased CD11b expression implies that complete limb ischemia leads to severe systemic inflammatory consequences in this model. The local expression of ICAM-1, the endothelial counterpart of CD11b, was also enhanced in the postischemic periosteum, where the PMN–endothelial interactions were simultaneously and visibly increased. These reactions were not influenced by E2 repletion, which suggests that E2 exerts its anti-adhesive effect independently of CD11b or ICAM-1. The mechanism of how estrogen can modulate *in vivo* PMN reactions demands further attention, but our results are in agreement with those of *in vitro* studies, where OVX did not influence the CD11b expression of the PMNs (Deitch et al., 2006). There is evidence of a cellular sexual dimorphism in the activation of PMNs, implying that PMNs from females respond to trauma and humoral stimuli to a lower extent than do those from males (Deitch et al., 2006). Further, estrogen directly modulates the expression of the neuronal isoform of nitric oxide synthase in PMNs (García-Durán et al., 1999). In our study, E2 treatment clearly reduced the IR-induced secondary PMN–endothelial interactions (i.e. sticking), but this potentially protective effect was not mediated by a mechanism involving tissue ICAM-1 expression changes. Although an increased PMN-derived NO production might reduce PMN adhesion, this reaction is ICAM-1 dependent (Dal Secco et al., 2006). Overall, the earlier findings may implicate that chronic estrogen supplementation modulates PMN activation directly, through a novel, specific, estrogen-sensitive pathway.

In our rat model, OVX was followed by definite diagnostic signs of osteoporosis 21 weeks later. In this condition, the postischemic periosteal microcirculatory complications were not aggravated further relative to the non-OVX age-matched controls. Similar changes were observed in TNF-α release, providing further evidence that OVX itself does not modify the inflammatory complications, but E2 supplementation greatly reduces this reaction. It has also been demonstrated by others that E2 inhibits TNF-α gene transcription via the beta estrogen receptors (Srivastava et al., 1999) and via the TNF-α-mediated increases in the expressions of adhesion molecules and chemoattractants (Xing et al., 2007). A downregulation of nitric oxide synthesis by TNF-α may also contribute to the mechanisms of these microcirculatory reactions (Yoshizumi et al., 1993). Our present data indicating the positive effect of prolonged E2 substitution on the PMN reactions and TNF-α release, however, points to another clinical implication of our findings; ovarial hormone deprivation supplemented with estrogen therapy (apart from the well-known positive effect in reducing the risk of osteoporotic fractures) also affords marked protection against inflammatory mediator release.

Conclusion

The periosteal microcirculatory consequences of tourniquet-caused ischemia were first quantified by IVM in an adequately long-term follow-up of osteoporosis. The efficacy of E2 therapy was

objectively judged in this condition. The overall message is that chronic estrogen supplementation not only ameliorates bone demineralization, but also decreases systemic and PMN-driven local periosteal inflammatory reactions.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Remote ischemic preconditioning differentially affects NADPH oxidase isoforms during hepatic ischemia–reperfusion

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ABSTRACT

Aims: We investigated the function of major superoxide-generating enzymes after remote ischemic preconditioning (IPC) and hepatic ischemia–reperfusion (IR), with the specific aim of assessing the importance of the most relevant NADPH oxidase (NOX) isoforms, NOX2 and NOX4, in the mechanism of action.
Main methods: 60-min partial liver ischemia was induced in Sprague–Dawley rats in the presence or absence of remote IPC (2 × 10-min limb IR), and hepatic microcirculatory variables were determined through intravital video microscopy and lightguide spectrophotometry during reperfusion. Inflammatory enzyme activities (myeloperoxidase (MPO) and xanthine oxidoreductase (XOR)), cytokine production (TNF-α and HMGB-1), liver necroenzyme levels (AST, ALT and LDH) and NOX2 and NOX4 protein expression changes (Western blot analysis) were assayed biochemically.
Key findings: In this setting, remote IPC significantly decreased the IR-induced hepatic NOX2 expression, but the NOX4 expression remained unchanged. The remote IPC provided significant, but incomplete protection against the leukocyte–endothelial cell interactions and flow deterioration. Hepatocellular damage (AST, ALT and LDH release), cytokine levels, and XOR and MPO activities also diminished.
Significance: Remote IPC limited the IR-induced microcirculatory dysfunction, but the protective effect did not affect all NOX homologs (at least not NOX4). The residual damage and inflammatory activation could well be linked to the unchanging NOX4 activity.

Introduction

The application of repetitive short periods of ischemia, referred to as ischemic preconditioning (IPC), is a well-established approach to the achievement of increased ischemic tolerance in various tissues. Interestingly, remote, inter-organ IPC also confers protection against subsequent ischemia–reperfusion (IR) injury (Koti et al., 2002). The elements and the sequence of events through which IPC exerts distant beneficial effects have not been fully explored, but adaptation of the gene expression of the cellular redox homeostasis is one of the key protective mechanisms through which a lower degree of radical formation can be attained in the endothelial compartment (Koti et al., 2002; Mallick et al., 2005). Signaling processes catalyzed by oxygen and nitrogen radical-producing enzymes, including neuronal nitric oxide synthase (nNOS), endothelial NOS (eNOS) and xanthine oxidase (XO) (Abu-Amara et al., 2011; Yuan et al., 2012), have been demonstrated in the course of defensive action of IPC, and a number of additional data have also established the decisive roles of NADPH oxidases (NOXs) (Wang et al., 2007; Tejima et al., 2007). With variation in the catalytic subunits, the NOXs comprise 7 family members (NOX1 to NOX5 and DUOX1 and DUOX2), which exhibit tissue-specific differences in their baseline expression (Bedard and Krause, 2007). In the case of liver parenchyma, NOX2 and NOX4 proteins have been found in hepatocytes, NOX2 predominates in the Kupffer cells, while NOX4 is more abundant in the microvessels (Bengtsson et al., 2003; Ellmark et al., 2005). Furthermore, the expression of NOX4 is at least 20-fold greater than that of NOX2 in the endothelial cells (Sorescu and Griendling, 2002), while the expression of NOX2 cannot be detected in the vascular smooth muscle cells (Görlach et al., 2000; Lassègue et al., 2001).

NOXs are specifically activated by many stimuli that are known to cause an endothelial dysfunction (Anilkumar et al., 2009), and previous studies have provided evidence of elevated mRNA levels of both NOX2 and NOX4 in response to a liver IR injury (Marden et al., 2008). Moreover, the mortality rate due to hepatic ischemia was reduced in NOX2-deficient mice (Harada et al., 2004) and the role of the phagocytic form of NOX in Kupffer cells has been demonstrated after preconditioning with a chemical agent that induces hypoxia (Tejima et al., 2007). Collectively, these data suggest that influencing NOX4 (derived from hepatocytes and/or

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vascular cells) and NOX2 (produced by phagocytic PMN leukocytes and/or Kupffer cells) may contribute to the protective mechanism of remote IPC. We therefore hypothesized that the effects of remote IPC can be linked to an alleviated inflammatory reaction in the postischemic hepatic microcirculation associated with NOX2 and NOX4 activation. To address this issue, we set out to investigate the consequences of limb IPC on major intracellular superoxide-generating enzyme systems in a rat model of hepatic IR injury, with special emphasis on changes in expression of NOX2 and NOX4 proteins.

Materials and methods

The experiments were carried out on male Sprague–Dawley rats (Charles River, Sulzfeld, Germany; average weight 300 ± 20 g) housed in an environmentally controlled room with a 12-h light–dark cycle, and kept on commercial rat chow (Charles River, Wilmington, MA, USA) and tap water *ad libitum*. The experimental protocol was in accordance with EU directive 2010/63 for the protection of animals used for scientific purposes and was approved by the Animal Welfare Committee of the University of Szeged. This study also complied with the criteria of the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Surgical procedures

Anesthesia was induced with a combination of 25 mg ml^{-1} (S)-ketamine (Ketanest; Parke Davis, Berlin, Germany) and 20 mg ml^{-1} xylazine (Rompun; Bayer, Leverkusen, Germany) in a ratio of 8:1, injected i.p. and sustained with small supplementary i.v. doses every 30 min. The trachea was intubated 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, respectively. The animals were placed in a supine position on a heating pad to maintain the body temperature between 36 and 37 °C, and lactated Ringer's solution was infused at a rate of 10 ml kg^{-1} h^{-1} during the experiment.

Before surgery, the fur over the abdomen was shaved, and the skin was disinfected with povidone iodide. After midline laparotomy and bilateral subcostal incisions, the liver was carefully freed from all ligamentous attachments and the liver was exposed and the left branches of the portal vein and the hepatic artery were mobilized. Complete ischemia of the median and left hepatic lobes was achieved by clamping the left lateral branches of the hepatic artery and the portal vein with a microsurgical clip for 60 min. After the ischemic period, the clips were removed and the wound was temporarily covered with water-impermeable foil during the 180-min reperfusion period (Taniguchi et al., 2007).

Experimental protocols

The experiments were performed in two major series, with the animals randomly assigned to one or another of the following experimental groups. In the first series, we evaluated the microcirculatory consequences of partial hepatic ischemia by using the noninvasive modified spectrometric O2C method (O2C system, see later). In one group, the hepatic microcirculatory responses to 60-min complete ischemia followed by a 180-min reperfusion period were examined (IR group, n = 6). After recording of the baseline microcirculatory variables (t = −100 min), ischemia was induced in the median and left hepatic lobes. The occlusions were then released (t = 0 min), and the microcirculation in the affected lobes was observed *via* O2C at t = 60, 120 and 180 min in the reperfusion phase. In another group, 2 cycles of a 10-min complete hindlimb ischemia and 10-min reperfusion was used as a preconditioning trigger before the induction of liver ischemia (remote IPC + IR group, n = 6). Limb ischemia was achieved by placing a tourniquet around the proximal femur, with simultaneous occlusion of the femoral artery with a miniclip (Szabó et al., 2009). The animals in a third group were subjected to the same surgical procedures, except for the induction of liver or limb ischemia (Sham group, n = 6). Blood samples for biochemical determinations were taken at t = 0, 60, 120 and 180 min of the experiments. Tissue biopsies for enzyme activity and Western-blot analyses were taken at the end of the experiments. Tissue biopsies were stored at −80 °C, and plasma samples at −20 °C before later analysis.

In the second series of experiments, the groups (n = 6 each) and the protocols were identical with those in the first series, with the exception that the microcirculation in the affected liver lobes was investigated by means of intravital video microscopy (IVM, see later) at t = 60 min in the reperfusion phase.

Modified lightguide spectrophotometry (O2C) device

We used the O2C system (LEA Medizintechnik, Gießen, Germany) for noninvasive and online examination of the microcirculation, which allows the simultaneous recording of tissue oxygen saturation (S_{O_2} percentage, absolute value), tissue hemoglobin (rHb, AU), capillary blood flow (AU) and capillary blood flow velocity (RBCV, AU). The O2C device combines white light spectroscopy with laser-Doppler measurement in one flat probe. To prevent the influence of regional heterogeneity and temporal blood flow variations, measurements were performed at three predetermined locations on the liver surface for 30 s each (Schreinemachers et al., 2009) with an ambient light correction before measurement.

IVM

Polymorphonuclear (PMN) leukocytes of individual vessels were examined by means of conventional fluorescence IVM (Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury lamp, Acroplan 20× water immersion objective), using *in vivo* fluorescence labeling. The posterior surface of the left liver lobe was exteriorized and placed on a specially designed pedestal, providing a suitable horizontal plane (Ábrahám et al., 2008). PMNs were stained *in vivo* by means of rhodamine-6G (Sigma, St. Louis, MO; 0.2%, 0.1 ml, i.v.). The microscopic images were recorded with a charge-coupled device video camera (AVT HORN-BC 12) attached to a personal computer. The microcirculatory parameters were assessed off-line by frame-to-frame analysis of the recorded images, using image analysis software (IVM, Pictron Ltd., \Budapest, Hungary). The microcirculatory inflammatory reaction was assessed by calculating the number of rolling and sticking PMN leukocytes within 5 central acinar venules (diameter between 20 and 40 μm) per animal (Ábrahám et al., 2008). Rolling leukocytes were defined as cells moving at a velocity less than 40% of that of the erythrocytes in the centerline of the microvessel passing through the observed vessel segment within 30 s, and their number was given as the number of non-adherent leukocytes per second per vessel circumference. 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.

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 dithiothreitol, 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 24,000 *g* 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 (Beckman et al., 1989).

Myeloperoxidase (MPO) activity

Tissue MPO activity was measured in liver biopsies by the method of Kuebler et al. (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 24,000 *g*. 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.

TNF-α and HMGB1 levels

Blood samples (0.5 ml) were taken from the carotid artery into precooled DTA-containing polypropylene tubes. Samples were centrifuged at 1000 *g* for 30 min at 4 °C, and then stored at −70 °C until assay. Plasma TNF-α and HMGB1 concentrations were determined with commercially available enzyme-linked immunosorbent assays (Quantikine Ultrasensitive ELISA kit for rat TNF-α; Biomedica Hungaria Kft, Hungary and Shino-Test Corporation ELISA kit for HMGB1; Kanagawa, Japan).

Western blot analysis of NOX2 and NOX4

Liver samples were homogenized and then lysed with RIPA buffer (Santa Cruz Biotech). Protein extracts (20 μg of total protein) were heated at 95 °C for 10 min, then placed in ice to cool, electrophoresed in 4–15% gradient sodium dodecyl sulfate–polyacrylamide gels, and transferred onto nitrocellulose membranes (Millipore). Membranes were blocked with Tris-buffered saline (TBS) and 5% skim milk at room temperature for 1 h prior to overnight incubation at 4 °C with primary antibodies against gp91phox (1:2000 dilution; Epitomics, Burlingame, CA, USA), and NOX4 (1:2000 dilution; Epitomics, Burlingame, CA, USA). After washing with TBS-T, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugate-corresponding secondary antibodies (anti-rabbit, 1:2500 dilution; Promega, Madison, WI, USA). The membranes were then developed with the SuperSignal West Pico horseradish peroxidase substrate kit (Pierce, Rockford, IL, USA) and intensities of protein bands were quantitated and photographed on a Lumi-Imager™ (Roche-Diagnostics, Boehringer Mannheim, Germany) image station. For the control of sample loading and protein transfer, the membranes were stripped and reprobed with β-actin antibody (1:1000 dilution; Sigma-Aldrich, St. Louis, MO, USA).

Liver transaminase (AST, ALT and LDH) release

Blood samples withdrawn from the carotid artery were analyzed for aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) and lactate-dehydrogenase (LDH) by standard photometric procedures (Vitros 250 analyzer, Ortho-Clinical Diagnostics, Raritan, NJ).

Statistical analysis

Data analysis was performed with the SigmaStat statistical software (Jandel Corporation, San Rafael, CA, USA). Changes in microcirculatory parameters and liver enzyme activities between groups and within groups were analyzed by two-way ANOVA, followed by the Bonferroni test. For the evaluation of biochemical assays and ELISA data, changes in variables between groups were analyzed by one-way ANOVA on ranks, followed by the Holm–Sidak test. Western blot data were analyzed with no normal distribution with the Mann–Whitney test. *P* values <0.05 were regarded as significant.

Results

Microcirculatory changes

Modified lightguide spectrophotometry

The microhemodynamic parameters, capillary blood flow, capillary blood flow velocity (RBCV), tissue oxygen saturation (S_{O_2}), and tissue hemoglobin content (rHb) were assessed simultaneously and on-line in the left liver lobes (Fig. 1). Reperfusion after 60-min ischemia was not associated with significant changes in intrahepatic blood flow (Fig. 1A) as compared with the Sham group. When IR was preceded by remote IPC, however, the postischemic hepatic blood flow was significantly higher than the pre-ischemic values throughout the examination period. The RBCV in the IR group was significantly lower during reperfusion in comparison with the pre-ischemic values, and no recovery was observed during the examination period (Fig. 1B). Remote IPC, however, reversed the RBCV changes to the level measured in the Sham group. Taken together, the flow and velocity changes caused by the 60-min partial ischemia were manifested in deteriorated levels of tissue S_{O_2} and rHb, which were restored by the remote IPC protocol (Fig. 1C, D).

Fluorescent IVM data

Due to the lack of selectin molecules in the post-sinusoidal endothelium, "classical rolling" cannot be observed, but the number of PMNs exhibiting the rolling phenomenon could nevertheless be determined in the central venules. Liver IR was accompanied by an approximately 4-fold increase in the number of rolling leukocytes relative to that of the sham-operated animals (Fig. 2A). In accordance with this, the number of sticking cells also displayed a significant increase in response to liver IR (Fig. 2B). Remote IPC resulted in significant improvements in both forms of cell-to-cell interactions.

Inflammatory enzyme (MPO and XOR) levels

The PMN deposition analyzed *via* the MPO activity was increased significantly 180 min after the ischemia, together with the XOR activity, which was elevated approximately 2-fold as compared with the sham-operated group. Remote IPC applied prior to the partial liver IR insult reduced the MPO and XOR activities almost to the sham-operated levels (Fig. 3A, B).

Plasma HMGB1 and TNF-α levels

Following 60-min partial liver ischemia, significantly increased HMGB1 and TNF-α levels were observed at 180 min of the reperfusion period (Fig. 4A, B). The IR-induced elevations of the plasma HMGB1 and TNF-α were effectively attenuated by remote IPC (Fig. 4A).

NOX2 and NOX4 protein expression

Western blot analysis of the NOX2 and NOX4 proteins revealed significant increases after partial liver IR in comparison with the sham-operated animals. The application of remote IPC before IR decreased the expression of NOX2 significantly (Fig. 5A), but did not affect the level of NOX4 expression (Fig. 5B).

Hepatic enzymes in the plasma

The animals in the sham-operated group displayed minimally increased necroenzyme levels throughout the experimental protocol as compared with the baseline values. In comparison, the IR group exhibited significantly higher AST, ALP and LDH activities during the reperfusion period, indicating significant functional damage. When the remote IPC protocol preceded liver IR (remote IPC group), the plasma levels of necroenzymes were significantly reduced in comparison with the IR group (Fig. 6).

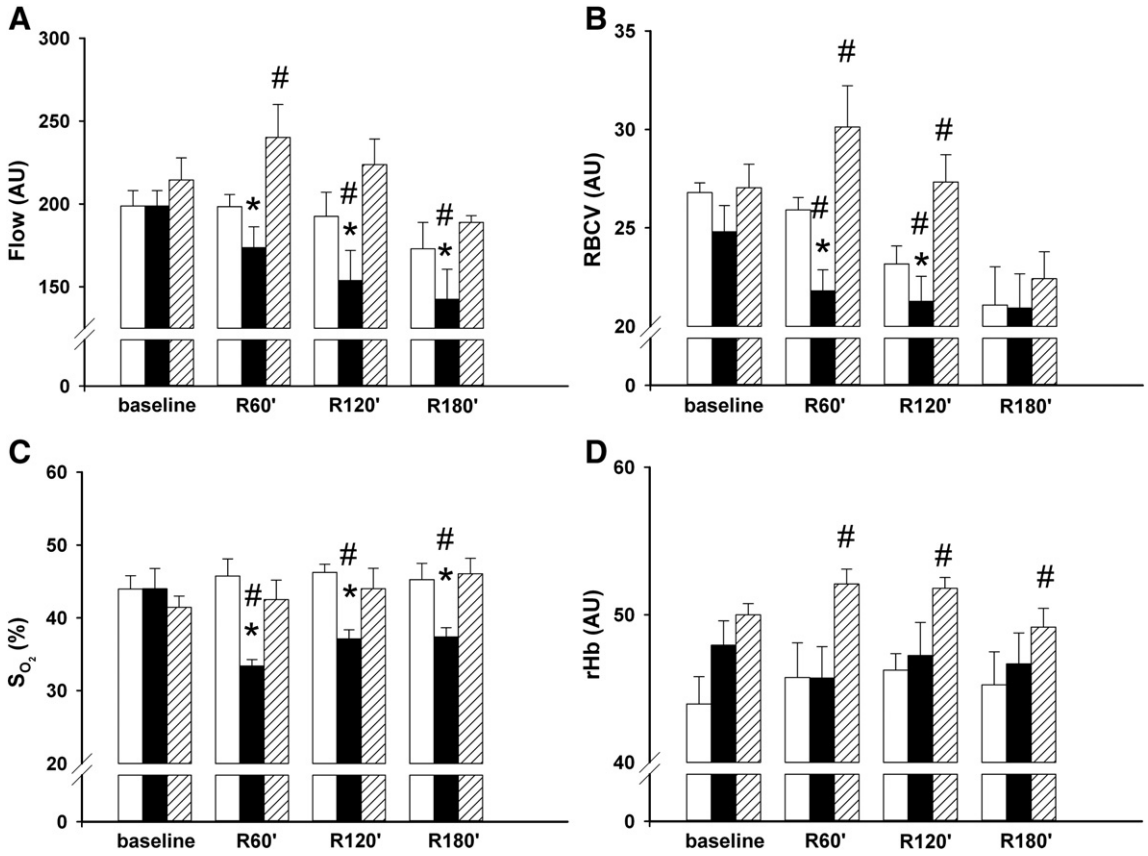

Fig. 1. Changes in hepatic microcirculatory variables. The animals were sham-operated (Sham group, white columns) or subjected to 60-min partial hepatic ischemia followed by 180 min of reperfusion (IR group, black columns), or 2 cycles of 10-min complete hindlimb ischemia followed by a 10-min reperfusion period was applied as a preconditioning trigger before liver ischemia (remote IPC + IR group, striated columns). A. Capillary blood flow (given in arbitrary units, AU), B. velocity in the capillaries (RBCV, given in AU), C. tissue oxygen saturation (S_{O_2}, given in %), D. tissue hemoglobin (rHb, given in AU). Data are presented as means ± SEM. *$P < 0.05$ *vs* baseline; #$P < 0.05$ *vs* Sham group (two-way ANOVA, Bonferroni test).

Discussion

The major aim of this study was to investigate the effects of remote IPC after partial liver IR on enzymes that generate intracellular reactive oxygen species (ROS). The scope of this project was narrowed down to the most likely ROS-producing sources, and we focused on the most relevant isoforms, NOX2 and NOX4, whose roles in the mechanisms of IR damage or IPC have already been implicated. A partial hepatic ischemia model was used to evaluate the effects solely in response to IR, excluding poorly tolerated mesenteric congestion with the concomitant mediator release and deterioration of the systemic hemodynamics (Vollmar et al., 1994). We previously characterized the microcirculatory effects of IPC achieved by using 2 cycles of complete hindlimb ischemia on IR-induced periosteal microcirculatory derangement and systemic inflammatory responses (Szabó et al., 2009). As beneficial local effects were observed, the identical protocol was utilized to investigate the consequences of limb IPC on IR-induced inflammatory responses in a distant organ, and our study clearly demonstrated the microcirculatory benefits of remote IPC. The IR-related increases in the levels of necroenzymes and inflammatory cytokines were ameliorated and the activities of potential sources of ROS production, *i.e.* XOR, MPO and NOX2, were significantly reduced. The most interesting finding, however, was that the enhanced NOX4 expression was not influenced by remote IPC.

There are several possible explanations of this result. Firstly, the present findings are consistent with those of other research, which revealed that IPC inhibits the effects of mediators involved in the microcirculatory dysfunction, including ROS (Cutrn et al., 2002; Jabs et al., 2010). The main cellular sources of ROS generation in the blood vessels are the NOXs in the smooth muscle cells (Griendling et al., 2000), endothelial cells (Bayraktutan et al., 2000) and adventitial fibroblasts (Pagano et al., 1998). Among the NOX homologs, NOX4 has been reported to be the main isoform in the vascular cells (Bengtsson et al., 2003; Ellmark et al., 2005), although the expression of NOX2 or even NOX1 has also been demonstrated. Enhanced NOX4 activity has been implicated in the development of various cardiovascular pathologies, because NOX-derived superoxide rapidly reduces the bioavailability of endothelium-derived NO, thereby promoting vasoconstriction and enhancing the vascular resistance (Dusting et al., 1998; Zicha et al., 2001). Nevertheless, NOX4 within the vessel wall generates a low level of ROS continuously, even in the absence of extrinsic stimulation, and unlike phagocytic NOXs, is involved in physiological vascular processes such as maintenance of the vascular tone, regulation of the endothelium-dependent vasodilator function, oxygen sensing and modulation of the redox-sensitive signaling pathways (Dworakowski et al., 2008; Cave et al., 2006). Moreover, endothelial NOX4 acts as a constitutive endothelial generator of H_2O_2, which positively affects the vascular function, linked to signaling events that promote vasodilatation and cell protection (Dikalov et al., 2008; Takac et al., 2011). The exact factors determining the action of NOX4 in producing superoxides or H_2O_2 are unclear, but in overexpression systems NOX4 releases predominantly H_2O_2 (Ray et al., 2011). Additionally, molecular mechanisms implicated in NOX4 vasoprotection include the activation of eNOS and the increased production of NO, and the increased expression of antioxidant systems such as Nrf-2 (Schröder et al., 2012). Indeed, a significant decrease in myocardial infarct size was recently observed in NOX2- and NOX1/NOX2-, but not in NOX4-deficient mice (Braunersreuther et al., 2013). Thus, data are accumulating concerning a Janus-faced mechanism by which NOX4 can protect or cause harm to the circulatory system (Schmidt et al., 2012) and, if a defensive action of

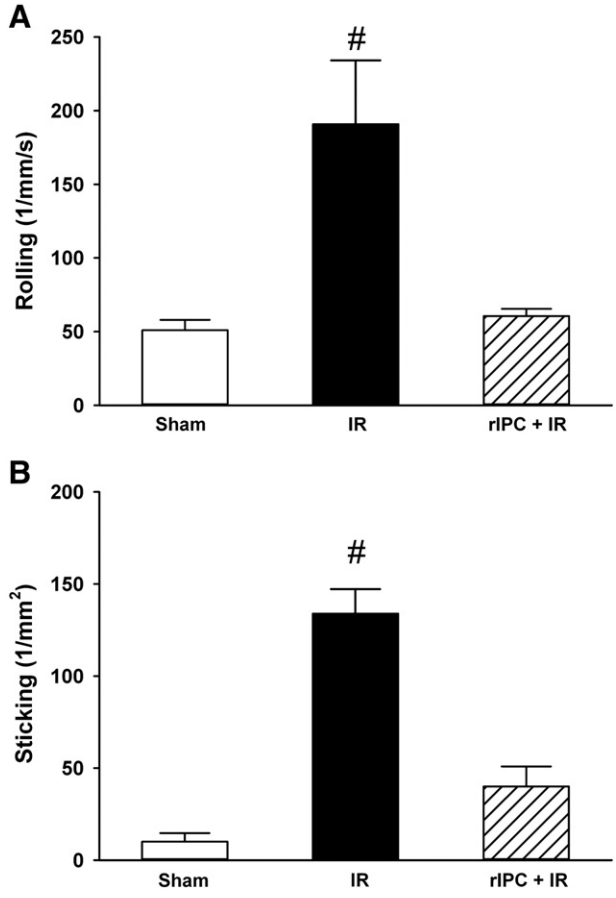

Fig. 2. Changes in PMN–endothelial interactions. A. The number of rolling PMNs in the central venules of the liver (rolling, given in mm^{-1} s^{-1}), B. number of sticking PMNs (sticking, given in mm^{-2}). Data are presented as means ± SEM. #$P < 0.05$ *vs* Sham group (one-way ANOVA, Holm–Sidak test).

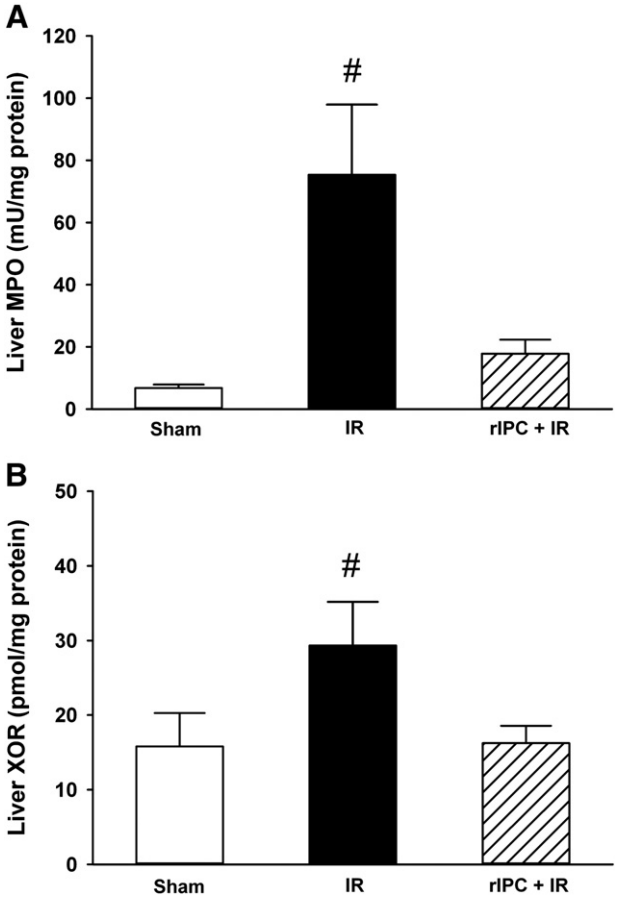

Fig. 3. Hepatic MPO and XOR activities. A. XOR activity, B. MPO activity. Data are presented as means ± SEM. #$P < 0.05$ *vs* Sham group (one-way ANOVA, Holm–Sidak test).

NOX4 activity is possible, it could be hypothesized that the potentially vasoprotective characteristics of NOX4 could, at least in part, explain the ineffectiveness of remote IPC in reducing the IR-related NOX4 expression.

Another possibility is that the influence of IPC is not universally effective, in consequence of the different sensitivities of the various target genes and enzymes. The remote IPC provided significant, but incomplete protection, and the residual damage and IR-induced inflammatory activation could therefore well be linked to the unchanging NOX4 activity. In this sense, the effect of IPC does not extend to all homologs (at least not to NOX4). Recent findings suggest that, unlike other ROS-generating NOXs, the tightly-assembled active conformation of NOX4 cannot be disrupted by conventional means, because the membrane-bound subunit does not require interaction with the cytosolic subunits (Csányi and Pagano, 2013).

Previous studies demonstrated that the beneficial effect of IPC extends to reducing the adherence of PMNs to the ischemic–reperfused sinusoidal endothelium, attenuating the PMN-mediated endothelial dysfunction, and limiting leukocyte accumulation in the postischemic liver tissue (Yuan et al., 2005). In accordance with these observations, the influx of activated PMNs into the postsinusoidal venules was observed in the present model, and as a result of remote IPC, the extent of rolling and sticking of the leukocytes was significantly reduced in the central venules. The mechanisms underlying PMN trafficking after IR and remote IPC can be explained in terms of several pathways leading to a modified adhesion molecule expression. The characteristic of the hepatic sinusoidal endothelium may contribute to leukocyte trapping: non-classical rolling and mainly vascular adhesion molecule-1 (VCAM1)-mediated strong adhesion occur in response to inflammation (Lee et al., 2004). NOXs have been shown to be particularly involved in regulating VCAM1 expression through TNF-α release (Cayatte et al., 2001). When applied *in vitro*, S17834, a flavonoid NOX inhibitor, directly repressed the vascular NOX activity, and it reduced TNF-α-stimulated VCAM, ICAM-1 and E-selectin expression *in vivo* (Cayatte et al., 2001). Further, the lower degree of ROS formation after remote IPC partially produced by NOXs can also be linked to reduced adhesion molecule expression. *Via* mineral corticoid receptor activation, aldosterone induced adhesion molecule expression also involves activation of the NOXs (Hashikabe et al., 2006). Moreover, LPS induces the expression of surface adhesion molecules through the activation of TLR4, which has been shown to be involved in the NOX-dependent activation of NF-κB (Park et al., 2006).

The activity of MPO, the commonly used index of PMN priming and activation (Yuan et al., 2005), further strengthened our IVM observations. As shown in the present study, the activity of MPO is significantly correlated with the number of visualized PMNs. This observation is in agreement with a previous report that accumulated PMNs were positively correlated with the development of hepatocellular damage after IR (Yuan et al., 2005). Upon activation, the prototypic isoform NOX2 has been proven to be responsible for the superoxide generation of PMNs (Babior, 1999). This isoform, also referred to as "respiratory burst oxidase", was first described in PMNs as the starting point of ROS production (Babior, 1999). The effects of NOX2-derived ROS and proteases released from activated PMNs in promoting cell death in the liver have been widely documented (Yuan et al., 2005; De Minicis et al., 2010). In the present study, remote IPC attenuated both arms of PMN-related injury: it lowered the PMN priming/MPO activity and reduced the expression of NOX2. This is in accordance with previous observations where preconditioning was induced *via* NOX2 in ischemic brain, myocardium and endothelial cells (Kawano et al., 2007; Milovanova et al., 2008; Thirunavukkarasu et al., 2012).

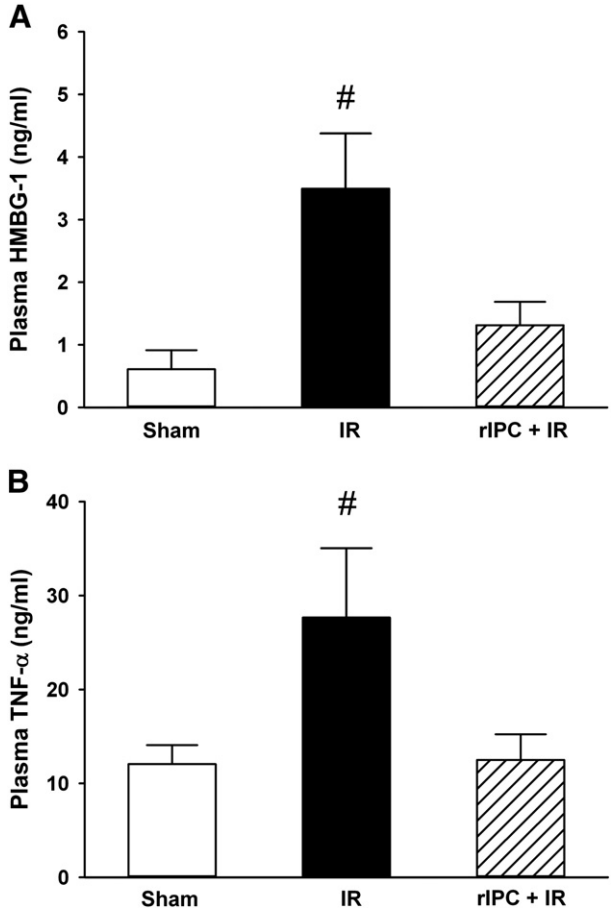

Fig. 4. Plasma cytokine levels. A. TNF-α level; B. HMGB1 level. Data are presented as means ± SEM. #$P < 0.05$ *vs* Sham group (one-way ANOVA, Holm–Sidak test).

Cellular ROS (superoxide and H_2O_2) are now well recognized as playing an integral role in several growth factors and cytokine signal transduction pathways (Lander, 1997) and serve as second messengers for cellular tyrosine kinase signaling (Goldstein et al., 2005). NOXs appear to be especially involved in redox signaling because they are the only enzymes (among the many intracellular sources of superoxide formation) whose primary function is to generate ROS (Lambeth, 2004). The tissue specificity and difference in catalytic subunits offer an opportunity for the development of NOX inhibitors that do not compromise the essential physiological signaling and phagocytic functions carried out by ROS.

In this study, remote IPC significantly attenuated systemic cytokine release. It has previously been shown that NOX-mediated ROS production has a major role in the ischemia-induced inflammatory responses involving activation of the NF-κB (Dworakowski et al., 2008). TNF-α and HMGB1 have a common NF-κB-dependent transcription; both are involved in inflammatory responses (Dworakowski et al., 2008; Kamo et al., 2013). Moreover, hepatocytes have been demonstrated to produce TNF-α in a NOX-dependent manner following hypoxia–reoxygenation and liver IR (Spencer et al., 2013). The relationship between TNF-α and HMGB1 expression and ROS production is bidirectional: TNF-α and HMGB1 are involved in regulating the expression of cytokines and other mediators that participate in acute inflammatory responses, many of which are associated with the increased generation of ROS (Kamo et al., 2013). On the other hand, oxidative stress has been reported to up-regulate the expression of cytokines that promote inflammatory responses during reperfusion. These antigen-independent responses interact and amplify each other, finally leading to impaired microhemodynamics, functional and structural cell damage, and remote or systemic inflammatory complications.

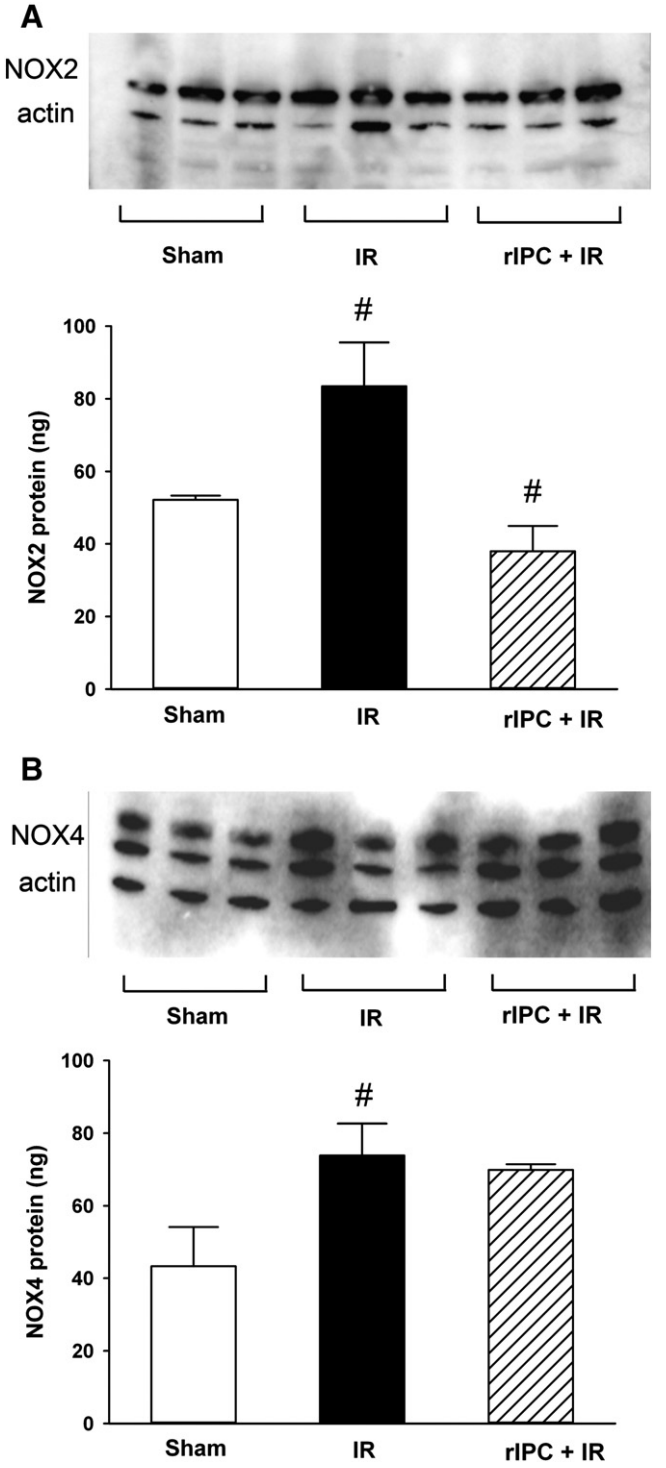

Fig. 5. Hepatic expressions of NOX2 and NOX4 proteins. A. NOX2 expression; B. NOX4 expression. Data are presented as medians ± SD. #$P < 0.05$ *vs* Sham group (Mann–Whitney test).

Conclusions

Remote IPC exerted marked protection against the potentially detrimental microcirculatory consequences of hepatic IR. This accords with the lower levels of generation of inflammatory mediators, preserved liver blood flow and oxygenation. The signs of PMN activation and the induction of NOX2 were likewise weaker, but in contrast with expectations, the expression of NOX4, the main vascular NOX isoform, was not

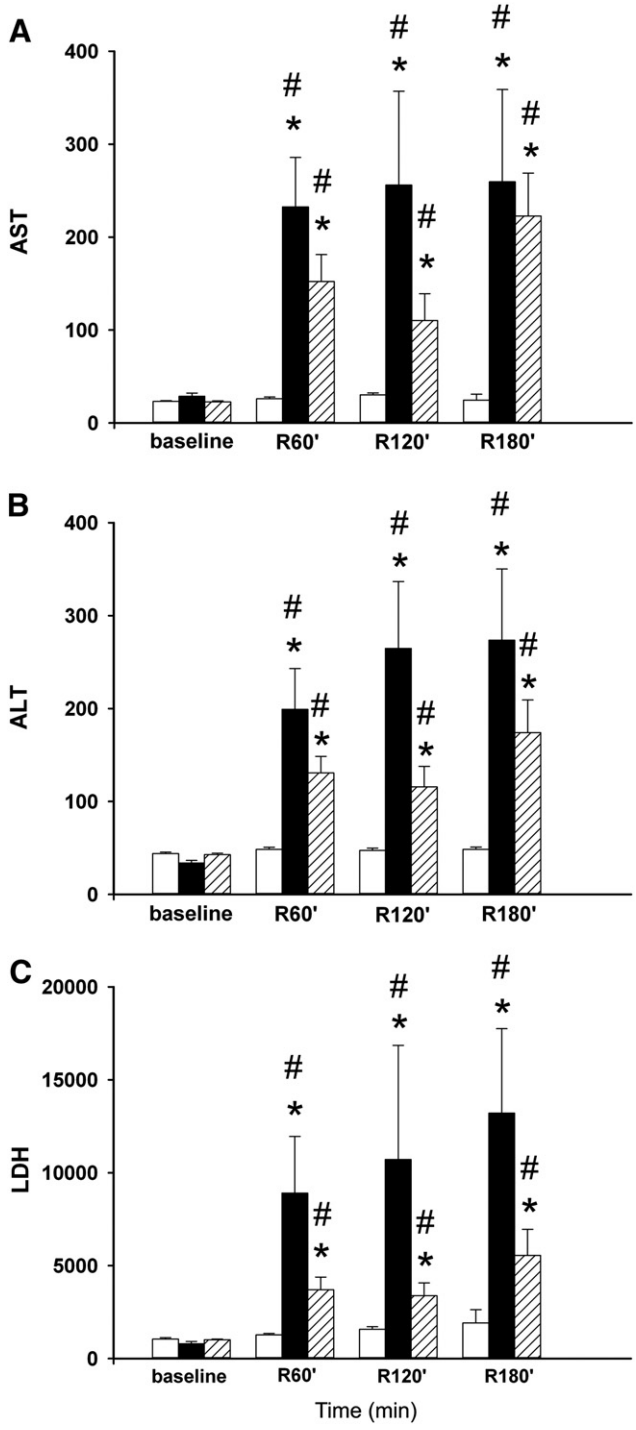

Fig. 6. Time courses of plasma AST, ALT and LDH changes. The animals were sham-operated (Sham group, white columns) or subjected to 60-min partial hepatic ischemia followed by 180 min of reperfusion (IR group, black columns) or 2 cycles of 10-min complete hindlimb ischemia followed by a 10-min reperfusion period was applied as a preconditioning trigger before liver ischemia (remote IPC + IR group, striated columns). A. AST levels (AST, given in U/L), B. ALT levels (ALT, given in U/L), C. LDH levels (LDH given in U/L). Data are presented as means ± SEM. *$P < 0.05$ *vs* baseline; #$P < 0.05$ *vs* Sham group (two-way ANOVA, Bonferroni test).

attenuated. Several questions remain unanswered, but further characterization of "ischemia-specific" enzymatic sources may lead to the preventive targeting of oxido-reductive stress, rather than the scavenging of ROS after they have been formed.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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IV.

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Result: For 50kPa indentation the percentage reduction of the submucosal depth increased with increasing time (15% for 5s, 28% for 30s and 45% for 60s). In the 160kPa indentation the submucosal depth also reduced by 45% over a 60s indentation even despite a sample with a thick submucosal layer. In 255kPa samples the longitudinal muscle and serosal depth were affected but not as markedly (20% reduction over 5 seconds and 27% reduction over 60 seconds). The 255kPa samples were unique as in the 30s and 60s group there were slides with completely obliterated longitudinal muscle, indicating that at this level the submucosal layer may be unable to withhold the applied stress.

Conclusion: Mechanical stresses, equivalent to those in laparoscopic grasping, result in changes to the depth of the colon wall, particularly in the submucosa and at longer durations of stress application. Further analysis will determine safe tissue handling thresholds.

OP 61

Effects of Limb Ischaemic Preconditioning in Ovariectomized Rats

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Background: During elective orthopedic surgery or fracture repair, tourniquet ischaemia of osteoporotic bones may carry the risk of postischaemic inflammatory complications. We previously demonstrated that exogenous estrogen and ischaemic preconditioning (IPC) ameliorates periosteal inflammatory reactions induced by limb ischaemia–reperfusion (IR). In the present experiments, the effect of IPC in these reactions was examined during experimental osteoporosis.

Material and Methods: Three–month old female Sprague–Dawley rats were bilaterally ovariectomized (OVX) or sham–operated (SH). 12 weeks later, the periosteal microcirculatory consequences (neutrophil leukocyte–endothelial interactions) of 60–min complete limb ischaemia and 180 min reperfusion were examined by fluorescence intravital microscopy in sham–operated (SH+IR) and OVX animals (OVX+IR). In further groups, limb IPC (2 × 10'/10" I–R) was elicited before I–R in sham–operated (SH+IPC+IR) and OVX rats (OVX+IPC+IR) (n=6–9). In another series, intravascular neutrophil leukocyte activation (CD11b adhesion molecule expression) changes were assessed by flow cytometry (n=7–9).

Result: Rolling and adhesion of leukocytes to the periosteal postcapillary venules and leukocyte CD11b expression were increased after limb IR; these reactions were attenuated by IPC. In OVX animals, the number of rolling leukocytes was moderately reduced, but adherent leukocyte numbers and CD11b expression were not affected by IPC.

Conclusion: Our data shows that the ameliorating effects of IPC against limb IR–induced inflammatory complications are partially lost in the presence of chronic estrogen withdrawal. These data suggest a limited efficacy of IPC to improve the postischaemic microcirculatory inflammatory consequences of tourniquet ischaemia in osteoporotic situations. (Supported by: OTKA K104656, TÁMOP 4.2.4. A/2–11–1–2012–0001, TÁMOP 4.2.2A–11/1/KONV–2012–0035, TÁMOP 4.2.2A–11/1/KONV–2012–0073).

OP 62

Postconditioning Is Able to Improve Small Intestinal Microcirculation after Lower Limb Ischemia

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Background: Major lower limb vascular surgeries may result in severe, remote injury of the gastrointestinal system, which has high mortality rates. Postconditioning is a technique with potential capability of reducing remote gastrointestinal complications. Our aim was to assess the remote macro- and micro-hemodynamic changes of the small intestine following an infrarenal aortic occlusion and to evaluate the effects of postconditioning on these alterations.

Material and Methods: Male Wistar rats underwent 3 hours of infrarenal aortic occlusion followed by 4 hours of reperfusion. In one group, postconditioning was applied. During the experiment blood pressure, superior mesenteric artery flow and mucosal microcirculation of duodenum, jejunum and ileum were assessed. At the end of the experiment, samples were taken from each intestinal segment for histological examinations.

Result: Superior mesenteric artery flow, as well as segmental small bowel microcirculation showed significant impairment in the IR group in contrast to the sham-operated group, (flow: p=0.012; microcirculation(duodenum): p=0.012; microcirculation(jejunum): p=0.008; microcirculation(ileum): p=0.002), while histological damage was significantly elevated. Strong negative correlation was found between microcirculatory values and histological damage (r= -0.911, p<0.001). Postconditioning was able to limit flow reduction in all small bowel segments and in the superior mesenteric artery (flow: p=0.009; microcirculation(duodenum): p=0.009;