Estrogen-Dependent Efficacy of Limb Ischemic Preconditioning in Female Rats

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ABSTRACT: Our aim was to examine the effects of ischemic preconditioning (IPC) on the local periosteal and systemic inflammatory consequences of hindlimb ischemia-reperfusion (IR) in Sprague–Dawley rats with chronic estrogen deficiency (13 weeks after ovariectomy, OVX) in the presence and absence of chronic 17beta-estradiol supplementation (E2, 20 $\mu g\,kg^{-1}$, 5 days/week for 5 weeks); sham-operated (non-OVX) animals served as controls. As assessed by intravital fluorescence microscopy, rolling and the firm adhesion of polymorphonuclear neutrophil leukocytes (PMNs) gave similar results in the Sham + IR and OVX + IR groups in the tibial periosteal microcirculation during the 3-h reperfusion period after a 60-min tourniquet ischemia. Postischemic increases in periosteal PMN adhesion and PMN-derived adhesion molecule CD11b expressions, however, were significantly reduced by IPC (two cycles of 10′/10′) in Sham animals, but not in OVX animals; neither plasma free radical levels (as measured by chemiluminescence), nor TNF-alpha release was affected by IPC. E2 supplementation in OVX animals restored the IPC-related microcirculatory integrity and PMN-derived CD11b levels, and TNF-alpha and free radical levels were reduced by IPC only with E2. An enhanced estrogen receptor beta expression could also be demonstrated after E2 in the periosteum. Overall, the beneficial periosteal microcirculatory effects of limb IPC are lost in chronic estrogen deficiency, but they can be restored by E2 supplementation. This suggests that the presence of endogenous estrogen is a necessary facilitating factor of the anti-inflammatory protection provided by limb IPC in females. The IPC-independent effects of E2 on inflammatory reactions should also be taken into account in this model. © 2017 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 36:97–105, 2018.

Keywords: osteoporosis; ischemia-reperfusion; limb; ischemic preconditioning; estrogen; microcirculation

During elective orthopedic interventions or traumas, tourniquet application may lead to iatrogenic ischemia-reperfusion (IR) injury of the affected extremities, which may influence the healing of the bone and the surrounding soft tissues[1] including the periosteum.[2] The local IR injury may lead to systemic inflammatory activation as well,[3] and the injury in distant organs (e.g., the liver and lungs) is mediated by many factors, among others by circulating pro-inflammatory cytokines and activated polymorphonuclear leukocytes (PMNs).[1,4] Nevertheless, it has been shown that short, repeated local IR periods termed ischemic preconditioning (IPC) confer anti-inflammatory protection both in the periosteum,[5] and in remote organs through humoral and neurogenic signals.[6–9]

The amelioration of IR-induced inflammatory complications by limb IPC should offer a therapeutic benefit in elderly patients when the prevalence of skeletal injuries increases and osteoporotic bones are more prone to accidental fractures. However, the influence of osteoporosis on the efficacy of IPC against IR-induced injury remains unexplored and the results obtained concerning the estrogen status in this condition seem contradictory. Earlier it was demonstrated that endogenous estrogen does not play a role in the protective effect of IPC.[10] Other studies have shown that the positive cardiac effects of IPC are lost when the endogenous estrogen levels are reduced by ovariectomy (OVX), but cardioprotection could be re-established by estrogen supplementation.[11–13] So far, available data on the effects of estrogen replacement during IPC appear to conflict.[11–14] We showed earlier that OVX per se did not predispose female rats to more severe inflammatory reactions, but estrogen supplementation reduced the harmful consequences of limb IR.[15] Therefore the present study was designed to ascertain whether IPC exerts its potentially positive anti-inflammatory effects on limb IR injury with chronic estrogen deficiency. In our study, we also sought to examine whether the periosteal microcirculatory reactions are modulated by exogenous estrogen supplementation. With the above in mind, we decided to characterize the effects of IPC with or without estrogen supplementation on local periosteal and systemic inflammatory changes in a rodent model of hindlimb IR injury with chronic estrogen deficiency.

MATERIALS AND METHODS

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) and tap water ad libitum.

Levente Pócs and Ágnes Janovszky contributed equally to this study.

Conflicts of interest: The authors declare that there were no conflicts of interest, financial or otherwise.

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The project was approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority), with the license number: V./144/2013. The study was performed in adherence with the EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and the National Institute of Health guidelines for the use of experimental animals. Animal welfare-related assessments and interventions were carried out prior to and during the experiments.

Experimental Protocol

Ovariectomy

Twelve-week-old female rats (weighing 180–200 g) were randomly allocated to ovariectomized ($N = 48$), or sham-operated ($N = 33$) groups. The animals were anesthetized with an intraperitoneal combination of ketamine and xylazine (25 mg kg^{-1} and 75 mg kg^{-1}, 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 with a 3–0 non-absorbable thread (Ethibond Excel®, Ethicon, Somerville, NJ). Thereafter, the abdomen was filled with warm sterile physiological saline and the abdominal wall was closed with a 4–0 absorbable suture and a 4–0 non-absorbable suture (Vicryl® and Prolene®, Ethicon, Somerville, NJ) in two layers. Sham-operated animals underwent identical procedures, except of course that the Fallopian tubes and ovaries were not touched.

Chronic EstrogenTreatment

Eight weeks after OVX (i.e., at 20 weeks of age) (see Fig. 1), a chronic estrogen therapy was initiated in some of the OVX animals for 5 days/week with 20 μg kg^{-1} subcutaneous 17beta-estradiol[16] (E2, Sigma, St. Louis, MO) and it was continued for 5 weeks (i.e., until the end of the experimental protocol in week 25). The remaining OVX and Sham animals received the vehicle for E2 (100% ethanol diluted in corn oil) in the same volume.

Experimental Series

The later in vivo experiments were performed in two major series 13 weeks after the OVX and sham operations (in week 25) (Fig. 1). In the first series, the tibial periosteal microcirculatory consequences of a 60-min complete hindlimb ischemia followed by a 180-min reperfusion (with or without limb IPC) were investigated with intravital videomicroscopy (IVM). In a second series of experiments, identical protocols in the same groups were performed in order to detect changes in various systemic inflammatory reactions (see later on).

Series 1: Measurement of Local Inflammatory Reactions Using IVM

The experiments were performed under sodium pentobarbital (45 mg kg^{-1} ip) anesthesia and sustained with small supplementary intravenous doses when necessary. 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 their body temperature between 36 and 37°C. Here, 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 isolated, 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.[2]

In the final stage, the animals were randomly allotted to one of the following five groups. These are shown in Figure 1, week 25. Among vehicle-treated animals, a 60-min complete hind limb ischemia was induced by applying a tourniquet around the proximal femur and a miniclip on the femoral artery, which was followed by a 180-min reperfusion period in nine sham-operated animals (Sham + IR group) and 11 of the OVX animals (OVX + IR groups). Two other vehicle-treated groups were also subjected to two cycles of 10 min of limb IPC and 10 min of reperfusion (Sham + IPC + IR group, $N = 9$; OVX + IPC + IR group, $N = 9$). This IPC protocol has been shown to ameliorate local microcirculatory and systemic inflammatory complications caused by limb IR in male rats.[3] In all of the E2-treated animals, limb IR was combined with IPC (OVX + E2 + IPC + IR group, $N = 6$) and the experiments were started 18–24 h after the last E2 injection. In this series, the periosteal microcirculation was observed with IVM at baseline and every 60 min during the 180-min reperfusion period.

Microcirculatory Measurements

The right hindlimb with the exposed tibial periosteum 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)-labeled erythrocytes (0.2 ml iv), while PMNs were labeled with an iv injection of rhodamine 6G (Sigma, St. Louis, MO, 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

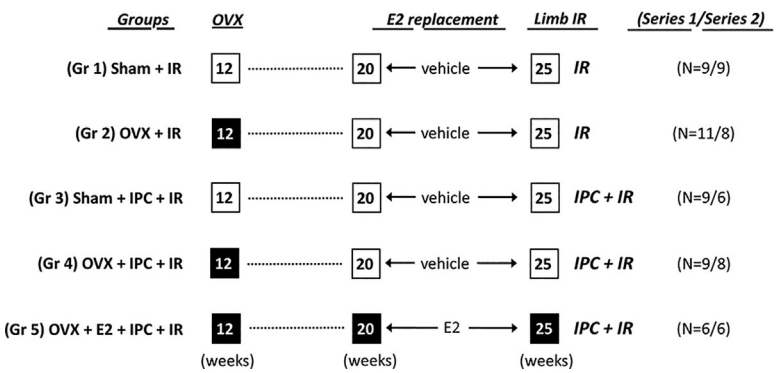

Figure 1. Groups and time sequence of surgical interventions, treatments and measurements: Ovariectomy (OVX) or a sham operation (Sham) was performed at 12 weeks of age; 17beta-estradiol treatment (E2) was performed for 5 weeks (5 days/week in a dose of 20 μg kg^{-1}); tourniquet-ischemia of a hindlimb followed by reperfusion (IR; 60′/180′) with or without ischemic preconditioning of the hindlimb (IPC; 2 × 10′/10′) was performed at the end of the protocol. In Series 1, the assessment of local inflammatory reactions in the tibial periosteum using intravital microscopy was carried out, while in Series 2, the detection of various systemic inflammatory parameters was performed. The number of animals used per group in each series is indicated in brackets.

(Panasonic AG-MD 830, Matsushita Electric Industrial Co., Tokyo, Japan) and a personal computer.

IVM—Video Analysis

A quantitative assessment of the microcirculatory parameters was performed off-line by a frame-to-frame analysis of the videotaped images, using image analysis software (IVM, Pictron Ltd., Budapest, Hungary) (Fig. 2). As for the periosteum, leukocyte–endothelial cell interactions were analyzed within five postcapillary venules (with diameters between 11 and 20 μm) per animal. IVM in the periosteum allows the observation of the primary and secondary PMN-endothelial interactions (rolling and adhesion, respectively). Rolling is a transient and reversible process, whereas adhesion represents a higher level of activation of leukocytes (when endothelial contact-dependent signals trigger the formation of the activation-dependent adhesion molecule expression of PMNs with accompanying NADPH oxidase activation and degranulation.[17] Based on their movements and contact with the endothelium of the postcapillary venules, 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 expressed here 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 expressed as the number of cells/vessel circumference in millimeters.

Series 2: Detection of Systemic Inflammatory Reactions

In a second series of experiments, identical protocols for the same groups were applied to detect changes in the proinflammatory cytokine TNF-alpha concentrations in the plasma and in whole blood free radical productions, as well as in the expressions of a circulating PMN-derived adhesion molecule (see the groups above, $N = 6$–9). The separation of the two series was necessary in order to avoid any interference between the fluorescent dyes used for IVM and acquisition techniques used with flow cytometry and luminometry. In this series of experiments, measurements were made from blood samples taken at baseline and at every 60 min of the reperfusion phase. And at the end of the protocol, periosteal specimens were harvested under RNase- and DNase-free circumstances to detect periosteal estrogen receptor (ER) expressions, then the samples were stored at −80°C until assay.

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 via a flow-cytometric analysis of whole blood in duplicate.[2] 100 μl of whole blood was incubated with 20 μl of (50 $\mu g\,ml^{-1}$) fluorescein isothiocyanate-conjugated mouse anti-rat monoclonal antibody (clone OX-42, AbD Serotec, Kidlington, UK) for 20 min. Negative controls were obtained by omitting the monoclonal antibody. The cells were then washed twice in Hanks buffer and centrifuged (Heraeus Biofuge primoR, Thermo Scientific, Waltham, MA, rotor diameter: 65 mm) at 12,281 g for 5 min. The cells were again washed twice, and the erythrocytes were lysed with a lysis puffer (Erythrolyse Red Blood Cell Lysing Buffer (10x) Reagent, GenWay, San Diego, CA) for 8 min, after which the cells were washed twice again (2,616 g, 5 min) and resuspended in 750 μl of Hanks buffer. CyFlow ML (Partec GmbH, Münster, Germany) equipment was used for cytometry; the granulocytes were gated on the basis of their characteristic forward and sidescatter features. Here, 10,000 events per sample were collected and recorded, then the percentages of labeled (activated) granulocytes (relative to the overall marker-bearing cells) and the mean fluorescence intensity (average marker density) were calculated.

Determination of Plasma TNF-Alpha Levels

Blood samples (0.5 ml) were taken from the carotid artery and placed into precooled EDTA-containing polypropylene tubes, centrifuged at 13,500 rpm for 5 min at 4°C, and then stored at −70°C until assay. Proinflammatory cytokine TNF-alpha concentrations were determined in plasma samples by means of commercially available enzyme-linked immunosorbent assays (Quantikine Ultrasensitive ELISA kit for rat TNF-alpha; R&D systems, Minneapolis).

Free Radical-Producing Capacity of the Blood

10 μl of blood dissolved in Hanks buffer was incubated for 20 min at 37°C in lucigenin (5 mM; dissolved in Hanks buffer) solution in the presence or absence of zymozan (190 μM, dissolved in Hanks buffer). Superoxide production was

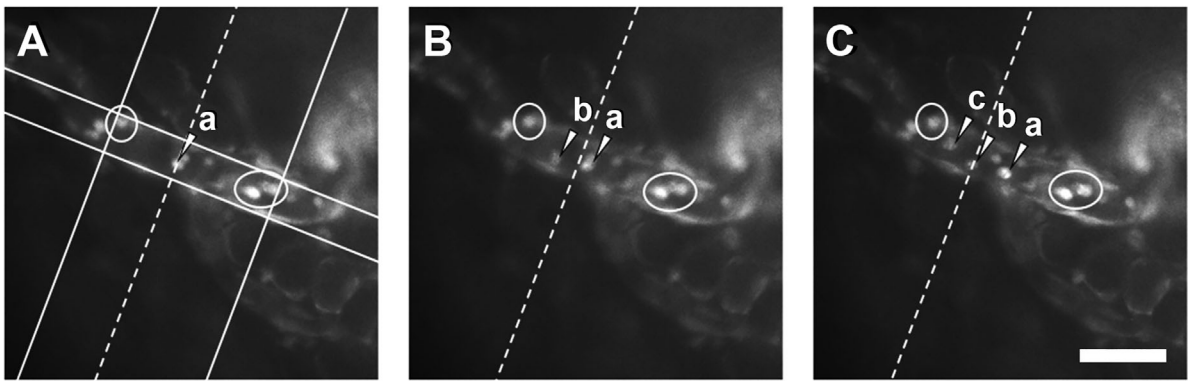

Figure 2. Representative micrographs showing the sequence of PMN–endothelial interactions on three consecutive images (Panels A–C) recorded by using intravital microscopy (recording rate: 20 frames/s). The segment of the examined tibial postcapillary vein is surrounded by lines in Panel A. Movement of rhodamine 6G-labeled PMN (marked by a–c) is demonstrated frame-by-frame referring to a dashed line. Stationary (adhesive) leukocytes are marked by ellipses. The bar in Panel C denotes 50 μm scale and this applies to all photomicrographs.

estimated via the rate of zymozan-induced increase in chemiluminescence (measured with an FB12 Single Tube Luminometer (Berthold Detection Systems GmbH, Bad Wildbad, Germany) and normalized for leukocyte counts in the peripheral blood.

Determination of Plasma E2 Levels

Endogenous E2 levels were determined using the Elecsys Estradiol III kit (Roche Diagnostics GmbH, Mannheim, Germany) and the Roche Cobas e 601 immunology analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Determination of Periosteal Estrogen Receptor-Alpha (ER-Alpha) and Beta (ER-Beta) mRNA Expressions

Tissue Collection

Anteromedial tibial periosteal samples were harvested via sterile surgical exposure of the contralateral (non-ischemic) limbs under an operating microscope. The samples were washed in 0.3 ml of sterile DNase, RNase and protease-free water (Sigma, St. Louis, MO) and placed in RNA stabilization solution (0.2 ml/each sample; RNAlater, Ambion®, Thermo Fisher Scientific, Waltham, MA). After overnight storage at 4°C, the RNA stabilization solution was removed, and tissue samples were stored at −80°C until RNA purification. Here, uterus samples were used as internal controls.

RNA Purification

The total RNA taken from the tibial periosteum and the uterus of each animal was purified with the NucleoSpin® RNA XS kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the protocol provided by the manufacturer.

Real Time PCR for ER-Alpha and ER-Beta

100 ng of RNA template in a 10 μl reaction mix were measured, using a quantitative reverse transcriptase-mediated PCR kit (Verso 1-step RT-qPCR Mix, ROX kit; Thermo Fisher Scientific, Waltham, MA). The amplification conditions were 50°C for 15 min, 95°C for 15 min, 40 cycles of 95°C for 15 s, and 58°C for 15 s. RNA levels were calculated using the ΔΔCT method and were normalized to 18S mRNA. The Universal Probe Library (UPL) system (Roche, Basel, Switzerland) was used to design primers and probes for the experiments (see Table 1).

Statistical Analysis

The required number of animals (i.e., sample size) was assessed by using the PS Power and Sample Size Calculations software package (version 3.1.2) prior to the experiments. Data analysis was performed with the SigmaStat statistical software package (Jandel Corporation, San Rafael, CA). The normality of data sets was checked, and in case of normal distribution, changes in variables within and between groups were analyzed by the two-way repeated measures ANOVA test, followed by the Holm–Sidak test. Data are expressed as means ± standard error of the mean (SEM). Due to the non-Gaussian distribution, PCR data were analyzed by the Kruskal–Wallis test, followed by the Dunnett test; the box plot figure shows the mean, the median, and the 25th and 75th percentile values. p values <0.05 were considered statistically significant at all parameters.

RESULTS

Effects of Local IPC on the Postischemic Tibial Periosteal Microcirculatory Inflammatory Reactions With Estrogen Depletion

When compared with the baseline values, the values of primary PMN–endothelial interactions (termed rolling) in the postcapillary venules of the tibial periosteum increased to a similar extent in the Sham + IR and OVX + IR animals at all examined time-points of reperfusion after limb IR (see Fig. 3). When limb IR was combined with local IPC, moderately reduced rolling values were observed in non-ovariectomized rats (Sham + IPC + IR group) at later stages of reperfusion (120 and 180 min), but no reduction was seen in OVX rats (OVX + IPC + IR group). At 60 and 120 min of reperfusion, the lowest rolling values were detected in animals treated with chronic E2 (OVX + E2 + IPC + IR group), but these differences were not statistically significant.

Leukocyte adherence (sticking) revealed a similar pattern to that seen with PMN rolling; no ameliorating effect of IPC was seen in OVX animals (in the OVX + IPC + IR group), but some alleviating effect was observed after E2 treatment (in OVX + E2 + IPC + IR group) (see Fig. 4).

Systemic Inflammatory Reactions

An increased expression of the adhesion molecule CD11b on the PMN surface was observed after 120 and 180 min of reperfusion. After, no major differences could be seen between the values for the Sham + IR and OVX + IR groups, but a slight decrease was observed after IPC in sham-operated animals (Sham + IPC + IR) (see Fig. 5). This amelioration, however, was not seen after OVX (in the OVX + IPC + IR group). It seems that chronic E2 treatment effectively prevented the IR-induced increase in CD11b expression (OVX + IPC + IR + E2).

The free radical-derived chemiluminescence of the whole blood (as determined by the superoxide radical-dependent chemiluminescence measurements) gave the earliest increase (after 60 min of reperfusion) after IPC both in the sham-operated and OVX animals (Sham + IPC + IR and OVX + IPC + IR), but it rose only slightly in the E2-treated OVX + IPC + IR animals (OVX + E2 + IPC + IR) at this time point (see Fig. 6). Free radical production did not reveal any

Table 1. Primers and Probes for Quantitative RT-PCR Used in This Study

Target	Forward Primer	Reverse Primer	Probe
ER-alpha	TTCTTTAAGAGAAGCATTCAAGGAC	TCTTATCGATGGTGCATTGG	# 130; 04693663001
ER-beta	GGCTGGGCCAAGAAAATC	TCTAAGAGCCGGACTTGGTC	# 111; 04693442001
18S	CTCAACACGGGAAACCTCAC	CGCTCCACCAACTAAGAACG	# 77; 04689003001

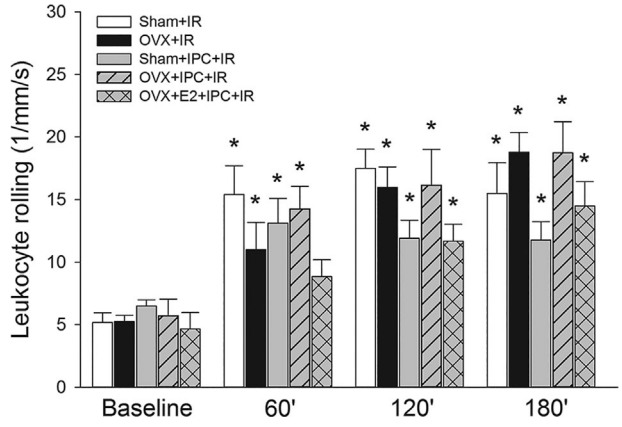

Figure 3. Changes in primary leukocyte–endothelial cell interactions (rolling) in the postcapillary venules of the tibial periosteum at baseline, 60, 120, and 180 min after a 60-min limb ischemia. Sham: Sham operation; OVX: ovariectomy; IR: tourniquet-ischemia of a hindlimb followed by reperfusion (60′/180′); IPC: hindlimb ischemic preconditioning ($2 \times 10'/10'$); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm-Sidak test. Here, data values are given as means ± SEM, and $^{*}p < 0.05$ versus baseline.

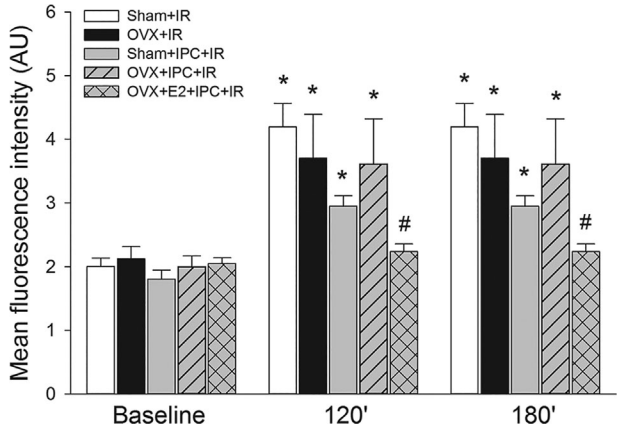

Figure 5. Changes in expression of the CD11b adhesion molecule on the surface of PMNs at baseline and in response to 60 min of limb ischemia followed by 120 and 180 min of reperfusion. Sham: Sham operation; OVX: ovariectomy; IR: tourniquet-ischemia of a hindlimb followed by reperfusion (60′/180′); IPC: hindlimb ischemic preconditioning ($2 \times 10'/10'$); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Here, data values are given as means ± SEM, and $^{*}p < 0.05$ versus baseline and $^{\#}p < 0.05$ versus Sham + IR.

more differences between the different experimental groups at later time points.

From the experiments, we found that IR brought about a significant increase in TNF-alpha levels in the plasma in all of the groups (see Fig. 7). Due to the high data dispersion, no statistically significant differences were seen between the groups at any time point, but the lowest increase was observed in the E2-treated animals.

The protocol was not synchronized with the estrous cycles of the animals and vaginal smear tests were not performed. The serum E2 concentrations ranged from 9.57 to 15.87 pg/ml in the Sham-operated animals, while these levels were significantly lower in the OVX animals ($p < 0.001$), not even attaining the detection limit of the assay (>5 pg/ml). However, plasma E2 was restored by chronic E2 supplementation in the OVX animals and the values were slightly higher than those in the Sham group (20.06 median value pg/ml, $p < 0.05$).

Periosteal Estrogen Receptor Expression

In the periosteum, a similar level of ER-beta transcription was observed in the sham-operated and in the OVX animals; and the highest transcription level was noted after chronic E2 supplementation (see Fig. 8). Periosteal ER-alpha mRNA levels, however, remained below the detector threshold. We excluded any methodological issues related to the detection of ER-alpha by simultaneously examining uterus samples taken from the same animals for an mRNA analysis of both receptors. Similar to Mohamed and Abdel-Rahman,[18]

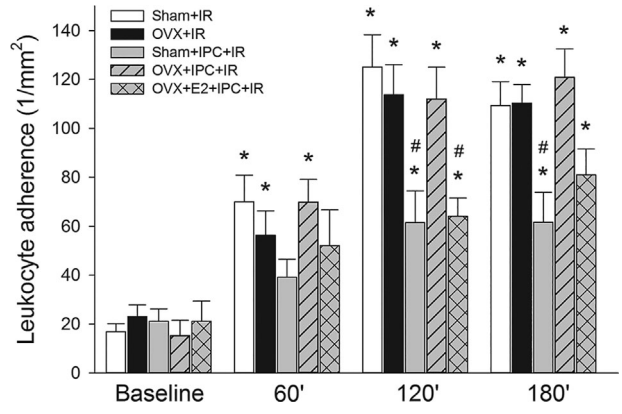

Figure 4. Changes in secondary leukocyte–endothelial cell interactions (adherence) in the postcapillary venules of the tibial periosteum at baseline, and 60, 120, and 180 min after a 60-min limb ischemia. Sham: Sham operation; OVX: ovariectomy; IR: tourniquet-ischemia of a hindlimb followed by reperfusion (60′/180′); IPC: hindlimb ischemic preconditioning ($2 \times 10'/10'$); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Here, data values are given as means ± SEM, and $^{*}p < 0.05$ versus baseline and $^{\#}p < 0.05$ versus Sham + IR.

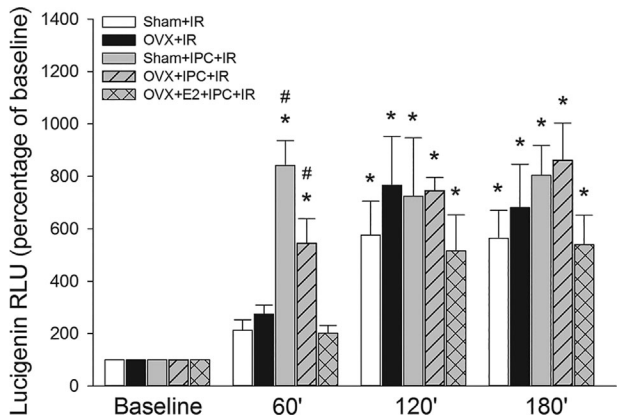

Figure 6. Whole blood superoxide production at baseline and in response to 60 min of limb ischemia followed by 60, 120, and 180 min of reperfusion. Sham: Sham operation; OVX: ovariectomy; IR: tourniquet-ischemia of a hindlimb followed by reperfusion (60′/180′); IPC: hindlimb ischemic preconditioning ($2 \times 10'/10'$); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Here, data values are given as means ± SEM, and $^{*}p < 0.05$ versus baseline, $^{\#}p < 0.05$ versus Sham + IR.

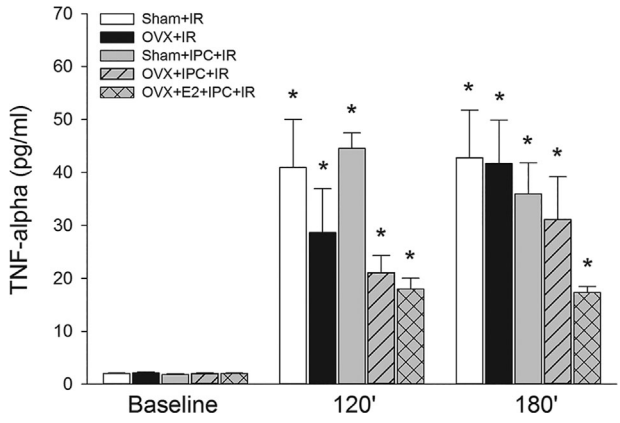

Figure 7. TNF-alpha levels in plasma samples at baseline and in response to 60 min of limb ischemia followed by 120, and 180 min of reperfusion. Sham: Sham operation; OVX: ovariectomy; IR: tourniquet-ischemia of a hindlimb followed by reperfusion (60′/180′); IPC: hindlimb ischemic preconditioning ($2 \times 10'/10'$); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Here, data values are given as means ± SEM, and $^{*}p < 0.05$ versus baseline.

we found higher mRNA levels (for both ER-alpha and beta) in the uterus in OVX group than in the Sham group (data not shown).

DISCUSSION

Previously we examined the periosteal microcirculatory consequences of tourniquet-induced ischemia in a clinically relevant, long-term follow-up study with osteoporotic rats.[15] We showed that OVX did not enhance IR-induced periosteal microcirculation dysfunction, but chronic estrogen supplementation ameliorated the local inflammatory complications. In the present protocol we employed a shorter term of OVX, which does not cause osteopenia, but it is sufficient to evoke a chronic estrogen deficit in rats.[19] It appears that IPC mostly influences the second stage of IR-induced periosteal PMN-endothelial interactions (sticking) both here in females and in males,[3] which might be explained by the effect of IPC on adhesion molecule expression responsible for leukocyte adhesion to the postischemic endothelium.[3] This protection, however, disappeared in the OVX animals in this study, as both PMN rolling and adhesion increased. Hence, it appears that the IPC-induced periosteal protection against postischemic inflammatory complications is lost after estrogen depletion and this observation has potential clinical implications. In a similar way, CD11b expression (a marker of activation of circulating PMNs[20] was lower in IPC animals only if OVX was not performed. It is therefore reasonable to suppose that endogenous estrogen in females plays a facilitating role in the anti-inflammatory mechanisms provided by IPC in the periosteum. This hypothesis is supported by the observation that E2 supplementation reverses the protection that was lost in OVX + IPC + IR animals. Similarly to our present results, the positive effects of IPC were shown to vanish in postischemic hearts harvested from OVX rats, and reversed by E2.[11] Prior to this, the microcirculatory benefits of E2 supplementation were examined after IR, without IPC. The postischemic periosteal microcirculatory complications of tourniquet ischemia could be reversed by E2 supplementation[15] and E2 has also been shown to have beneficial microcirculatory effects in numerous other models of IR.[21–22] Since the alleviating effects of E2 are present with or without IPC, it is difficult to differentiate between the beneficial effects of E2 treatment per se and its effect on IPC. Hence, one may suppose that the beneficial effects of E2 seen in this model might be independent of its effects on IPC.

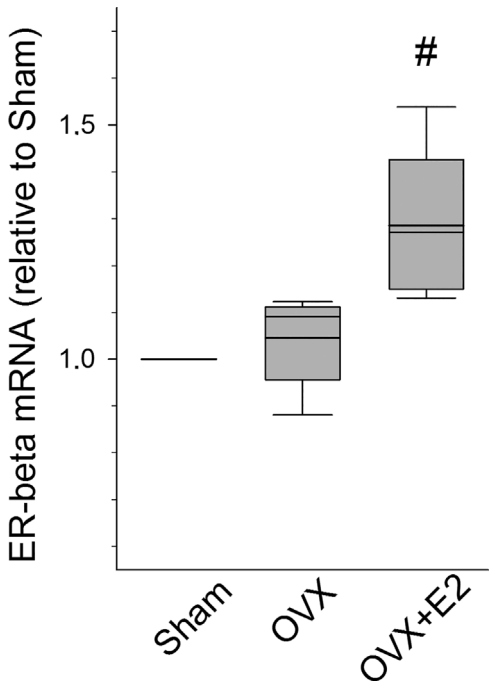

Figure 8. ER-beta mRNA expression levels in the tibial periosteum taken from sham-operated (Sham), ovariectomized (OVX) and OVX animals that were treated with 17beta-estradiol (OVX + E2). Kruskal-Wallis test was followed by the Dunnett test. Here, data values are given as mean, median, 25th and 75th percentiles, and $^{\#}p < 0.05$ versus Sham.

In our study, the microcirculatory manifestations of reduced efficacy of IPC after OVX were demonstrated for the first time, but similar reactions were observed with other manifestations of postischemic tissue injury in other organs by others (i.e., cardiac dysfunction).[11–14] The consequences of E2 supplementation in these scenarios, however, are not at all clear. As such, the OVX-related loss of IPC-induced protection in cardiac functions could be restored by E2 in certain studies with rats.[11–12] The results are somewhat controversial, as the protective effects of IPC were present in OVX rabbits.[10] Also, E2 did not exert any alleviating effects in other studies where IPC was combined with OVX.[13–14] Furthermore, long- and short-term estrogen administration produced different effects,[12,23] and inter-species and inter-organ differences and dissimilarities cannot be ruled out either.[10,12–13,24] The reason for the differences

between endogenous and exogenous estrogen effects in different experimental models is not well understood.

Some of the above differences might be due to the number and function of estrogen receptors within the affected tissue and also due to the effect of OVX and E2 on these receptor expressions. E2 is known to act as a transcription factor, as the binding of E2 to its ER-alpha or ER-beta receptors within the nucleus causes well-known genomic effects by inducing expression changes of different genes (e.g., nitric oxide synthase).[25] In addition, the action of binding E2 to its (plasma and mitochondrial) membrane-associated receptors also mediates non-genomic events[26–27] including the prevention of injury/stress-induced apoptosis[26] and cytochrome c release from myocardial mitochondria.[28] In our investigations, the ER-beta expression in the periosteum did not vary in response to OVX, but displayed an elevation in response to chronic E2 treatment (whereas the ER-alpha expression remained below the detector threshold). The up-regulation of the ER-beta receptor expression by E2 in the mitochondria and inhibition of apoptotic processes seems to be linked to the protective effect of E2 in trauma-hemorrhage.[29] Moreover, cardioprotective effects of E2 were attributable to the ER-beta receptor-related changes in the transcription on metabolic genes in another study.[30] In all likelihood, ER-beta is involved in regulating the estrogen-related increase in nitric oxide synthase activation[25] and others demonstrated the impact of ER-alpha as well.[31] PMN-related inflammatory processes were enhanced in OVX rats after trauma-induced hemorrhagic shock, which was prevented by the acute administration of E2 and an ER-beta agonist.[24] In vivo gene delivery of ER-beta to the endothelium greatly reduced the IR-induced formation of reactive oxygen species, increased nitric oxide formation and restored mitochondrial function in the adjacent cardiomyocytes.[32] In our study, some of the inflammatory processes (the CD11b expression of PMNs and free radical content in the blood) could be ameliorated by chronic E2; and the possible role of the up-regulation of ER-beta in these reactions cannot be ruled out. It should be noted, however, that estrogens also have a direct free radical scavenging effect via their phenolic A-ring,[33] a glutathione increasing effect,[34] and a direct modulatory action on NADPH activity.[35] Antioxidant effects of E2 may also be related to its influence on NFκB signaling[36] and the up-regulating of Nrf2.[37] As for the systemic effects, the involvement of ER-alpha-related actions of E2 also plays a role (in heart IR without IPC[38]), but discussion of these reactions as well as those evoked by selective estrogen modulators lies outside the scope of the present study. As was suggested by Murphy and Steenbergen, the shorter-term effects of E2 may be caused by ER-alpha, whereas longer-term effects may be mediated mainly through ER-beta.[39] Moreover, ER-independent effects of E2 in this study should not be ruled out either. It should be mentioned that the periosteal expression of ERs has not yet been examined in humans, but in the cortical and trabecular bone tissue, both ER proteins can be detected (via immunohistochemistry) with a different density during bone development.[40] It appears that only the ER-beta mRNA expression was examined in the tibial periosteum in the rat[41] and here we were unable to detect any ER-alpha mRNA expression of in the periosteum. This might mean that ER-alpha mRNA expression is not detectable in the periosteum. However, the translation of our present findings (the absence of periosteal ER-alpha mRNA expression) to the human situation requires further in-depth investigation.

Systemic inflammatory parameters also displayed characteristic changes. That is, the IR-induced increase in CD11b expression of circulating PMNs (a marker of their activation) was reduced by IPC only in sham-operated animals, but not in those with OVX. This reaction was also reversed by E2. The PMN-derived CD11b expression was likewise reduced by E2 in vitro[42] and in trauma-hemorrhagic shock[43] as well as in levels of some of other adhesion molecules such as the E-selectin.[44] We have not come across any studies that investigated the effect of IPC in OVX animals from the viewpoint of adhesion molecule expressions. In the present study, whole blood free radical content was significantly increased in all groups. In the Sham + IR and OVX + IR groups, local (periosteal) and systemic inflammatory reactions had a slightly different timeframe, since IVM data revealed increased PMN rolling and adhesion after 60 min of reperfusion (indicating an early activation of the affected endothelium and a simultaneous availability of primed leukocytes), but the superoxide levels displayed later changes (occurring after 120 min). The background of this phenomenon is not yet understood, but since increased CD11b expression in peripheral leukocytes also occurred at later stages of reperfusion (after 120 min), the contribution of other elements (e.g., activated macrophages) to the increased superoxide production may be assumed. Interestingly, IPC failed to induce any amelioration in whole blood free radical production, and furthermore, it induced an earlier increase in this parameter in both sham-operated and OVX groups. It should also be mentioned that this increase was not present in the E2-treated group. Actually, free radicals are known to play a role in the pathomechanism of IPC because their accumulation could be detected in vivo and superoxide scavengers reversed the tissue protective effects of IPC.[45–46] ER-beta has been shown elsewhere to be involved in reducing neutrophil activation[24] and the free radical reducing effect of E2 was also highlighted.[47] Interestingly, levels of one of the central regulators of inflammation TNF-alpha were not influenced by IPC. Quite surprisingly, the phenomenon observed in humans[48] indicating increased serum TNF-alpha levels after OVX could

not be confirmed in the present study (i.e., the baseline TNF-alpha values were not dissimilar after OVX), and even slightly lower values were found in all of the OVX animals (after 120 min of reperfusion). These differences might be the result of interspecies differences or changes in the immunological responses seen after OVX (which are outside the scope of the present study). TNF-alpha release has been shown to be reduced by E2 in numerous studies (with or without OVX),[23,49] even in male patients.[50] In this respect, the changes induced by reperfusion or IPC + IR have yet to be compared in OVX studies elsewhere. Here, the lowest postischemic values were found after applying E2 (although not attaining any statistical significance due to the relatively high data dispersion). This parameter together with reduced CD11b expression and the slower postischemic increase in superoxide production represent manifestations of the alleviated systemic inflammatory reactions after E2 supplementation.

CONCLUSIONS

In our study, we found that the beneficial periosteal microcirculatory effects of local limb IPC vanished after OVX in rats. These observations suggest that in postmenopausal females during orthopedic-trauma interventions, the efficacy of limb IPC in preventing the inflammatory complications of tourniquet ischemia might be limited. This conclusion is strengthened by our findings which show that E2 supplementation reversed these changes by alleviating the local and systemic inflammatory reactions. Based on our previous and present findings in rats, some of the alleviating effects of E2 seen here might be independent of its effects on IPC and may be linked to those seen with periosteal ER-beta expression. The clinical significance of this finding, however, remains to be elucidated.

AUTHORS' CONTRIBUTIONS

The experiments, the analysis of microcirculation data, the interpretation of data and drafting were carried out by LP, AJ, DG, and JK. The PCR analysis was supervised by GT and the FACS analysis was supervised by IO. The research design, drafting, and critical revision of the manuscript were performed by MB, JP, and AS. All the authors have read and approved the final submitted manuscript.

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