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

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## INTRODUCTION

Transient limb ischemia-reperfusion (IR) achieved with tourniquet (wrapping a band around the extremity) is primarily used for emergency or surgical indications. During elective orthopedic interventions, tourniquet is applied when the procedures (e.g. autotransplantion or free flap transfer*)* require a bloodless operative field. Along with the targeted bone and surrounding soft tissues, the periosteum is also affected. Since periosteum plays important roles in the bone nutrition and metabolism (via its osteoprogenitor cell content) as well as in normal bone healing, limb IR may critically impair periosteal and bone integrity.

IR injury is characterized by various detrimental biochemical and microcirculatory effects. A local reaction is closely linked to the nearly immediate production of reactive oxygen species (mostly produced by xanthine-oxidase and NADPH oxidases). The accompanying microcirculatory damage is hallmarked by impaired endothelium-dependent vasodilation and increased microvascular permeability, edema formation and an increased expression of adhesion molecules responsible for the interactions between endothelial cells and polymorphonuclear neutrophil leukocytes (PMNs). These microcirculatory manifestations of injury can be examined and quantified by intravital microscopy. Apart from its local inflammatory effects, the release of inflammatory mediators, and activation of PMNs and neurogenic pathways may induce remote effects, bringing about symptoms of the systemic inflammatory response syndrome.

The incidence of fractures and surgical procedures performed using tourniquet ischemia is particularly high in the elderly population suffering also from osteoporosis. The microcirculatory consequences of chronic ostrogen deficit and replacement, however, are not yet characterized in the periosteum.

One of the treatment modalities against IR injury is the ischemic preconditioning (IPC), a process where transient, brief periods of ischemia are followed by short intervals of reperfusion, affording protection against subsequent prolonged and deleterious ischemia. 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, termed as remote IPC (rIPC). 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. The effect of limb rIPC on the IR-induced microcirculatory reactions in liver, however, are

not known. Similarly, the efficacy of IPC on the postischemic microcirculatory injury of the periosteum in chronic estrogen deprivation is also to be characterized.

## 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 ovariectomy (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.

## MATERIALS AND METHODS

In Study 1, OVX was performed on 3-month-old female animals. Five months later (at the age of 8 months), chronic estrogen therapy was initiated 5 days/week with 20 μg $kg^{-1}$ subcutaneous 17β-estradiol (E2) and was continued weekly until the end of the experiments. Development of osteoporosis was continuously followed in the proximal tibiae by means of ultrasonic densitometry. Eleven months after OVX, the animals were subjected to a 60-min complete hindlimb ischemia followed by a 180-min reperfusion period. Microcirculatory consequences of limb IR were investigated by IVM at baseline and every 60 min during the 180-min reperfusion.

In the second study, the effects of limb IPC on the local tibial periosteal microcirculatory consequences were examined in response to 60 min of tourniqet ischemia followed by 180 min of reperfusion in OVX rats 2 months after OVX.

In the third study, microcirculatory and biochemical effects of rIPC (evoked by limb ischemia) on the postischemic inflammatory reactions of liver ischemia were assessed. The hepatic microcirculatory responses to 60-min complete ischemia followed by a 180-min reperfusion period were examined by using the noninvasive modified spectrometric O2C device (LEA Medizintechnik, Gieβen, Germany) and IVM (Zeiss Axiotech Vario 100HD microscope). rIPC was elicited by two cycles of 10-min complete hindlimb ischemia and 10-min reperfusion before the induction of liver ischemia.

For microcirculatory measurements, the medial/anterior surface of the left tibia and the left liver lobe were used. For IVM measurements, FITC-labeled erythrocytes, and rhodamine-6G-stained leukocytes were used. Determinations: expression of CD11b adhesion molecule on neutrophil leukocytes: flow cytometry; plasma TNF-α and high-mobility group protein B1 (HMGB1) levels: 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); liver transaminase release in plasma samples: standard photometric procedures (Vitros 250 analyzer, Ortho-Clinical Diagnostics, Raritan, NJ, USA); liver XOR activity: fluorometric kinetic assay; liver myeloperoxidase (MPO) activity: spectrophotometery (Shimadzu, Japan), liver NOX2 and NOX4 protein expression: western blot analysis; tissue ICAM-1expression: immunohistochemistry.

For statistical analysis: 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.

## RESULTS

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. The OVX-induced osteopenia was completely restored by E2 therapy. The IR-induced increase in the number of firmly adherent (sticking) leukocytes in the postcapillary venules was nearly completely prevented by chronic E2 supplementation in the OVX animals. An increased surface expression of adhesion molecule CD11b was not influenced by chronic E2 administration. Tissue ICAM-1 density in the vessels of the periosteum was significantly higher in the limbs subjected to IR in all examined groups.

In Study 2, primary and secondary PMN–endothelial interactions as well as CD11b expression were significantly reduced by IPC only the sham-operated group whereas these beneficial effects were lost after OVX.

In Study 3, microcirculatory perfusion parameters (flow, red blood cell velocity, oxygen saturation, hemoglobin content) were ameliorated in response to rIPC and cellular inflammatory reactions (rolling, sticking) were also decreased. Similarly, increased HMGB1 and TNF-α levels, PMN deposition (MPO activity) and XOR activity as well as liver necroenzyme levels were reduced by rIPC. 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, but did not affect the level of NOX4 expression.

**CONCLUSIONS**

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.