Examination of the periosteal microcirculation of long bones. Consequences of estrogen deficiency

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1. INTRODUCTION

Apart from its nutritive functions, the periosteum affects bone regeneration via its stem/osteoprogenitor cell content. Normal healing after bone fractures, trauma-orthopedic interventions and invasive dental procedures is critically linked to the reestablishment of the periosteal microcirculation. Besides the initiation of cell differentiation during bone repair and remodeling processes, the periosteum together with the endosteum plays significant roles in the pathogenesis of both hormone-related and trauma-induced osteoporotic alterations in the bone metabolism. Nevertheless, the axial bones, and in particular the jawbones, and the appendicular bones display differences not only in their blood supply and fracture healing characteristics, but also in respect of the development of osteoporosis and their reactions to treatment modalities (such as bisphosphonates). These reactions may also be linked to the differences in periosteal microcirculatory reactions. Due to methodological limitations, however, the periosteum is a relatively infrequent site for microcirculatory studies.

In emergency or elective surgical indications, transient limb ischemia is often achieved with the application of a tourniquet (i.e. wrapping a band around the extremity) and it may cause iatrogenic ischemia-reperfusion (IR) injury. The affected tissues undergo the typical IR-induced biochemical and microcirculatory changes, the periosteal microcirculation primarily being affected, which may lead to further systemic inflammatory reactions (i.e. activation of circulatory polymorphonuclear leukocytes; PMNs). Ischemic tolerance of the tissues can be increased via application of ischemic preconditioning (IPC), an approach when transient, brief periods of ischemia are followed by short intervals of reperfusion. 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. In experimental settings, osteoporosis/osteopenia is often induced via bilateral ovariectomy (OVX) in animals. In previous studies, the positive effect of prolonged estrogen (E2) substitution on the PMN reactions and TNF-alpha release was demonstrated suggesting that 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 the release of inflammatory mediators.

Bisphosphonates (BISs) are widely used for the treatment of osteoporosis and tumors with bone metastasis to inhibit osteoclast activity and bone resorption, and ameliorate the osteoporosis-induced decrease of bone mineral density. BIS treatment, however, exerts site-specific, differential effects during the early healing processes of tibial and mandibular fractures by causing delayed callus formation and bone remodeling and defective

angiogenesis. Furthermore, unwanted, necrotic reactions induced by BIS (particularly by the nitrogen-containing compounds such as zoledronate, ZOL) may also be present in the skeletal system such as osteonecrotic complications of the jaw bones. Previously, our research group also showed that BISs can induce significant inflammatory reactions in the mandibular periosteum after tooth extractions, while the microcirculation in the tibial region remained unaffected.

2. MAIN GOALS OF THE STUDIES

The major aim was to examine and modulate the local (periosteal) microcirculatory and the systemic inflammatory consequences of transient lower limb ischemia. We addressed the following clinical problems, both of which affect the elderly population with osteoporosis:

- IPC has been shown to provide protection against the deleterious consequences of IR induced by limb ischemia. We aimed to examine whether IPC exerts its potentially positive anti-inflammatory effects on limb IR injury with chronic estrogen deficiency. We also sought to examine whether the periosteal microcirculatory reactions are modulated by exogenous estrogen supplementation. Therefore, we characterized 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.
- There is a wide range of clinical indications for BIS treatment including treatment for osteoporosis, but this compound causes serious complications at axial bones (such as osteonecrosis of the jawbones). Our next aim was to assess the effects of chronic BIS treatment on the consequences of tourniquet ischemia (on the postischemic periosteal microcirculation and systemic inflammatory reactions) in a clinically relevant model of osteoporosis, where anesthetized rats were challenged with standardized limb IR in the presence or absence of chronic ZOL treatment.

3. MATERIALS AND METHODS

The experiments were performed in two studies. In the first Study, the microcirculatory effects of chronic estrogen deprivation (elicited by OVX) and estrogen supplementation were examined on the efficacy of IPC in a tourniquet ischemia model. In the second Study, the effects of chronic BIS treatment on the tibial periosteal microcirculatory consequences of limbs ischemia were examined in a shorter-term OVX model. In both studies, 12-week-old female rats (weighing 180-200 g) were randomly allocated to ovariectomized (OVX) or sham-operated groups, where OVX was performed using a standard procedure under

anaesthesia with combination of ketamine and xylazine (25 mg kg^{-1} and 75 mg kg^{-1}, respectively).

3.1. Experimental series

Study 1

In the first Study, 8 weeks after OVX, chronic estrogen therapy was initiated in some of the OVX animals for 5 days/week with 20 μg kg^{-1} subcutaneous 17beta-estradiol (E2, Sigma, St. Louis, MO, USA), and it was continued for 5 weeks (i.e. until the end of the experimental protocol). The remaining OVX and sham animals received the vehicle for E2 (100% ethanol diluted in corn oil) in the same volume.

In vivo experiments were performed in two major series 13 weeks after the OVX and sham operations (in week 25) after allocation of animals to one of the following 5 groups (see later). Among the vehicle-treated animals, a 60-min complete hindlimb 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 9 sham-operated animals (sham+IR group) and 11 OVX animals (OVX+IR groups). Two other vehicle-treated groups were also subjected to 2 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. 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 hrs 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.

In a second series of Study 1, identical protocols for the same groups were applied to detect changes in the pro-inflammatory 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). It was necessary to separate the two series to avoid any interference between the fluorescent dyes used for IVM and the 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. At the end of the protocol, periosteal specimens were harvested under RNase- and DNase-free conditions to detect periosteal estrogen receptor (ER) expressions, and then the samples were stored at –80°C until assay.

Study 2

In the second Study, a chronic ZOL treatment was initiated in 16 animals (OVX+BIS group) 5 weeks after OVX (i.e. at 17 weeks of age) with 14 of the sham-operated animals serving as negative controls (sham+BIS group). ZOL (80 μg kg^{-1} Zometa®, Novartis Europharm, Budapest, Hungary) was administered once a week intravenously into the tail vein under light ether anesthesia. The remaining OVX and sham-operated animals received physiological saline in the same volume (OVX+vehicle and sham+vehicle groups, n=16 each). These weekly injections were continued for 4 weeks. At the end of the experimental protocol (in week 21), all of 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 placing a miniclip on the femoral artery. The experiments were performed in two experimental series. In series 1, the periosteal microcirculation was examined using IVM at baseline and every 60 min during the 180-min reperfusion period (n=7–9 per group) (see below). In the second experimental series, blood samples from the carotid artery were taken at baseline and during the reperfusion period to detect changes in the plasma concentrations of TNF-alpha and in the expression of the adhesion molecule CD11b (n=7 in each group).

3.2. Microcirculatory measurements

Intravital microscopy (IVM)

Microscope: Zeiss Axiotech Vario 100HD, 100W HBO mercury lamp, Acroplan 20× water immersion objective, Carl Zeiss GmbH, Jena, Germany, Camera: Teli CS8320Bi (Toshiba Teli Corporation, Osaka, Japan)

Labelling: fluorescein isothiocyanate-labeled erythrocytes (0.2 ml iv), rhodamine 6G-labelled PMNs (Sigma, St. Louis, MO, USA, 0.2%, 0.1 ml iv). Analysis: off line using the IVM software package (Pictron Ltd., Budapest, Hungary)

Parameters: Adherent leukocytes (stickers) number of adherent cells per mm^2 of endothelial surface. Rolling leukocytes: the number of rolling cells/vessel circumference in millimeters.

3.3. Detection of systemic inflammatory reactions

CD11b expression of PMNs

Labelling: with fluorescein isothiocyanate-conjugated mouse anti-rat monoclonal antibody (clone OX-42, AbD Serotec, Kidlington, UK)

Flow cytometry: CyFlow ML (Partec GmbH, Münster, Germany)

Determination of plasma TNF-alpha levels

ELISA: Quantikine Ultrasensitive ELISA kit for rat TNF-alpha; R&D Systems, Minneapolis, MN, USA).

Free radical-producing capacity of the blood

Based on zymosan-induced increase in chemiluminescence of whole blood (FB12 Single Tube Luminometer (Berthold Detection Systems GmbH, Bad Wildbad, Germany), normalized for leukocyte counts in the peripheral blood.

Determination of plasma E2 levels

Endogenous E2 levels were determined using the Elecsys Estradiol III kit 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

RNA purification: NucleoSpin® RNA XS kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the protocol provided by the manufacturer.

Real Time PCR: 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, USA). 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.

3.4. Statistical analyses

Data analysis was performed with the SigmaStat statistical software package (Jandel Corporation, San Rafael, CA, USA). Based on the normality of the data sets, one of the following tests was used: 1) two-way repeated measures ANOVA test followed by the Holm–Sidak test (Study 1), 2) Kruskal–Wallis test, followed by the Dunnett test (Study 1, PCR data); 3) two-way RM ANOVA was used followed by the Holm–Sidak and Dunn's tests (Study 2).

4. RESULTS

Study 1.

4.1. Effects of E2 on limb IPC-related periosteal microcirculatory changes

When compared with the baseline values, the values for the 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. 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 min 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 the OVX+E2+IPC+IR group).

4.2. Effects of E2 on limb IPC-related systemic inflammatory changes

An increased expression of the adhesion molecule CD11b on the PMN surface was observed after 120 min and 180 min of reperfusion. Subsequently, 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 the sham-operated animals (sham+IPC+IR). 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 accounted for 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. Free radical production did not reveal any more differences between the different experimental groups at later time points.

We found that IR brought about a significant increase in TNF-alpha levels in the plasma in all of the groups. 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^{-1}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^{-1}). 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^{-1}, $p < 0.05$).

4.3. Effects of estrogen supplementation on OVX-induced ER expression

In the periosteum, a similar level of ER-beta transcription was observed in the sham-operated and OVX animals, and the highest transcription level was noted after chronic E2 supplementation. 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.

4.4. Effects of chronic BIS treatment on limb IR-induced periosteal microcirculatory changes

Chronic ZOL treatment did not influence baseline values of leukocyte-endothelial interactions in the periosteal microcirculation. IR, however, induced significant increases in both PMN rolling and adhesion during the entire reperfusion period, and these changes reached a similar level in sham-operated and ovariectomized rats. BIS treatment caused a temporary increase in leukocyte rolling in OVX+IR animals and, similarly, an earlier rise in PMN adhesion in both sham+IR and OVX+IR animals at 60 min of reperfusion but did not influence PMN–endothelial interactions in later stages of reperfusion.

4.5. Effects of chronic BIS treatment on limb IR-induced systemic inflammatory changes

As compared to baseline, TNF-alpha values showed marked increases during the reperfusion period under examination. No differences could be traced among the different experimental groups. Compared to baseline values, the quantity of adhesion molecule CD11b on the PMN surface significantly increased in saline-treated sham-operated and OVX rats during reperfusion. In animals that received chronic BIS treatment, however, this elevation reached a significantly lower level.

5. DISCUSSION

5.1. Effects of estrogen supplementation on the efficacy of IPC in reducing local postischemic periosteal microcirculatory injury

Previously, our group examined the periosteal microcirculatory consequences of tourniquet-induced ischemia in a clinically relevant, long-term follow-up study with osteoporotic rats. We showed that OVX did not enhance IR-induced periosteal microcirculation dysfunction, but chronic estrogen supplementation ameliorated 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. It appears that IPC mostly influences the second stage of IR-induced periosteal PMN–endothelial interactions (sticking) both here

in females and in males, which might be explained by the effect of IPC on adhesion molecule expression responsible for leukocyte adhesion to the postischemic endothelium. 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, 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. 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, and E2 has also been shown to have beneficial microcirculatory effects in numerous other models of IR. 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.

In this study, the microcirculatory manifestations of reduced efficacy of IPC were demonstrated for the first time, but similar reactions were observed by others with other manifestations of postischemic tissue injury in other organs (i.e. cardiac dysfunction). The consequences of E2 supplementation in these scenarios, however, are not at all clear. As such, it was possible to restore the OVX-related loss of IPC-induced protection in cardiac functions with E2 in certain studies with rats. The results are somewhat controversial, as the protective effects of IPC were present in OVX rabbits. Also, E2 exerted no alleviating effects in other studies, where IPC was combined with OVX. Furthermore, long- and short-term estrogen administration produced different effects, and inter-species and inter-organ differences and dissimilarities cannot be ruled out either. The reason for the differences between endogenous and exogenous estrogen effects in different experimental models is not well understood.

Some of these differences might be due to the number and function of estrogen receptors within the affected tissue as well as 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 in different genes (e.g. nitric oxide synthase). In addition, the action of binding E2 to its (plasma and mitochondrial) membrane-associated receptors also mediates non-genomic events, including the prevention of injury/stress-induced apoptosis and cytochrome c release from myocardial mitochondria. In our investigations, the ER-beta expression in the periosteum did not vary in response to OVX; instead, it displayed an elevation in response to chronic E2 treatment (whereas the ER-alpha expression remained below the detector threshold). The upregulation 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. Moreover, the cardioprotective effects of E2 were attributable to the ER-beta receptor-related changes in the transcription of metabolic genes in another study. In all likelihood, ER-beta is involved in regulating the estrogen-related increase in nitric oxide synthase activation, and others have demonstrated the impact of ER-alpha as well. 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. 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. In our study, some of the inflammatory processes (the CD11b expression of PMNs and free radical content in the blood) were ameliorated with chronic E2, and the possible role of the upregulation 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, a glutathione-increasing effect and a direct modulatory action on NADPH activity. Antioxidant effects of E2 may also be related to its influence on NFκB signaling and the upregulation of Nrf2. As for the systemic effects, the involvement of ER-alpha-related actions of E2 also plays a role in heart IR without IPC, but a 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 others, the shorter-term effects of E2 may be caused by ER-alpha, whereas longer-term effects may be mediated mainly through ER-beta. Moreover, ER-independent effects of E2 in this study should not be ruled out either. It should be noted that the periosteal expression of ERs has yet to be 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. It appears that only the ER-beta mRNA expression was examined in the tibial periosteum in the rat, and here we were unable to detect any ER-alpha mRNA expression in the periosteum. This might mean that ER-alpha mRNA expression cannot be detected 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.

5.2. Effect of estrogen supplementation on the efficacy of IPC in reducing systemic inflammatory reactions

Systemic inflammatory parameters also displayed characteristic changes in Study 1. 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 and in trauma-hemorrhagic shock as well as in levels of other adhesion molecules, such as E-selectin. We are unaware of any studies that have 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; furthermore, it induced an earlier increase in this parameter in both sham-operated and OVX groups. It should also be noted 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. ER-beta has been shown elsewhere to be involved in reducing neutrophil activation and the free radical-reducing effect of E2 was also highlighted. Interestingly, levels of one of the central regulators of inflammation TNF-alpha were not influenced by IPC. Quite surprisingly, the phenomenon observed in humans 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) even in male patients. 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). Together with reduced CD11b expression and the slower postischemic increase in superoxide production, this parameter represents manifestations of the alleviated systemic inflammatory reactions after E2 supplementation.

Conclusions for Study 1

We found that the beneficial periosteal microcirculatory effects of local limb IPC vanished after OVX in rats. These observations suggest that during orthopaedic trauma interventions in postmenopausal females, 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.

5.3. Effects of chronic BIS treatment on limb IR-induced periosteal inflammatory reactions

BISs are effective medications for bone metastases and osteoporosis and promising treatment modalities for complex regional pain syndrome upon fracture healing. The use of ZOL has been shown to have a positive effect on spinal fusion and to promote osseointegration and fixation of dental implants in autologous bone grafts in osteoporosis. The periapical lesion-induced bone loss in the mandible was effectively ameliorated, and osseointegration of titanium implants in postmenopausal osteoporosis was promoted by ZOL. Furthermore, ZOL brought about periosteal bone formation after tooth extraction in osteopenic sheep. ZOL treatment, however, also induced reactive periosteal hypertrophy and even BIS-related osteonecrosis of the jaw in the same osteopenic sheep model. Nevertheless, the effect of BIS on IR-induced local and systemic inflammatory reactions has not been examined elsewhere in an osteopenic model.

It is noteworthy that both anti- and proinflammatory effects have been attributed to different BIS compounds. The anti-inflammatory aspects of BISs include upregulation of the number of inflammatory monocytes, modulation of the proliferation and the viability and apoptosis of monocytes and macrophages and downregulation of proinflammatory cytokines, such as

TNF-alpha, as well as other cytokines, such as IL-1, IL-6 and neurogenic growth factor. Similarly, inhibitory effects of BIS against neurogenic inflammation have also been reported. On the other hand, an acute phase response (<3 days) was induced by different BISs including ZOL with increased TNF-alpha release in patients, but tissue accumulation of PMNs, increased TNF-alpha release and marked oxidative stress were also demonstrated in other tissues, such as the gingiva and the liver, in animal models. Furthermore, priming of immunological reactions was also attributed to ZOL. BISs cause ocular inflammatory complications in some clinical cases and healing complications of the jawbones after invasive dental interventions, even leading to osteonecrosis. ZOL has been shown to aggravate kidney damage (by increasing cytokine production, metabolic acidosis and apoptosis) during IR injury in rats.

Enhanced leukocyte-endothelial interactions have been demonstrated after BIS treatment in an arthritis model in mice, but little is known about ZOL-induced periosteal microcirculatory reactions. Previously, we demonstrated that chronic BIS treatment induces some level of microcirculatory inflammation in the mandible, but such effects were not observed in the tibial periosteum. Therefore, in this study, we tested the effect of chronic ZOL treatment in a tourniquet-induced limb ischemia model, where the role of PMN-endothelial interactions in the development of postischemic microcirculatory inflammatory reactions is well established. We have shown here that the reduced endogenous estrogen levels evoked by OVX do not predispose to enhanced periosteal microcirculatory complications per se, with the results also demonstrating that, apart from temporary exacerbation of PMN-endothelial interactions at the early stages of reperfusion, no major microcirculatory inflammatory risk could be detected after chronic ZOL treatment.

5.4. Effect of chronic BIS treatment on limb IR-induced systemic inflammatory reactions

Estrogen withdrawal induces a release of TNF-alpha, which is involved in the pathomechanism of osteoporotic bone loss in women, but, in this study, we did not demonstrate between-group differences in TNF-alpha levels in the postischemic phase. Nevertheless, unlike humans, where increased serum TNF-alpha levels have been observed after OVX, we detected no differences in baseline TNF-alpha levels between the different experimental groups. It should be noted that serum levels of TNF-alpha are rather low in rats and baseline values were close to the detection limit of the assay.

CD11b expression is a critical step for PMN adhesion to activated endothelial cells, and we detected a reduced IR-induced systemic PMN-derived CD11b expression after ZOL

administration. BISs have been shown to influence PMN functions, which manifested in impaired PMN chemotaxis and reactive oxygen species production capacity in vivo and reduced myeloperoxidase and NADPH oxidase activities in vitro. The inhibitory effect of BIS was also demonstrated in other immune cells, such as macrophages. In our study, ZOL reduced CD11b expression on the surface of circulating PMNs but did not influence the overall adhesion of PMNs in the periosteal postcapillary venules. This finding can only be explained by some degree of ZOL-induced endothelial activation and secondary endothelium-derived adhesion molecule expression. This possible ZOL-induced endothelial upregulation of adhesion molecules (the endothelial counterparts of CD11b), which might be responsible for the present results, should be investigated further.

Among other effects, BISs are known to inhibit vascular endothelial proliferation and to upregulate cellular apoptosis. Furthermore, BISs (alendronate) have also been shown to inhibit nitric oxide synthase expression, which is an important endogenous modulator of PMN–endothelial interactions. These ZOL-induced acute postischemic reactions affecting the endothelium may also warrant further in-depth investigations.

Conclusions for Study 2

BIS treatment exerted only a minor influence on limb IR-induced PMN rolling and adhesion in the periosteum, and the PMN-derived adhesion molecule (CD11b) expression on circulating PMNs was even reduced. Further, no effect on postischemic TNF-alpha release was demonstrated in ZOL-treated rats. These results suggest that, although some level of local endothelial activation might be attributable to the treatment, chronic ZOL administration has no major influence on the risk of postischemic inflammatory microcirculatory complications in the tibial periosteum.

6. SUMMARY OF NEW FINDINGS

1. The beneficial periosteal microcirculatory effects of experimental limb IPC vanished after OVX. Therefore, the efficacy of limb IPC in preventing the inflammatory complications of tourniquet ischemia might also be limited when orthopedic trauma interventions are performed on postmenopausal females.
2. Chronic extrogen supplementation reversed the local and systemic inflammatory reactions, but some of the alleviating effects of E2 might be independent of its effects on IPC and may be linked to those seen with periosteal ER-beta expression.
3. BIS does not cause systemic postischemic complications in terms of leukocyte activation, but moderately enhances the limb IR-induced periosteal microcirculatory reactions. This phenomenon might be explained by some level of local endothelial activation after chronic ZOL treatment.
4. When administered in osteoporosis, chronic ZOL treatment does not cause significant acute postoperative periosteal microcirculatory complications. This may be a relevant issue during tourniquet ischemia as part of trauma-orthopedic surgery.

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LIST OF FULL PAPERS RELATING TO THE SUBJECT OF THE THESIS

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ABSTRACT RELATING TO THE SUBJECT OF THE THESIS

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