Prevention of Skin Flap Necrosis by Estradiol Involves Reperfusion of a Protected Vascular Network

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Abstract—Although 17β-estradiol (E2) is protective in experimental models of myocardial and brain ischemia, its effect on skin ischemia remains unknown. Here, we assessed the protective effect of E2 in a mouse model of skin ischemia, mimicking the surgery of skin flaps. Whereas necrosis appeared in the half portion of the skin flap within 1 week after surgery in ovariecтомized mice, it was reduced up to 10-fold when mice were pretreated with E2, at least 3 days before the surgery. The beneficial effect of E2 appeared to be attributable to an increase in skin survival, revealed by measuring viability of ex vivo explants and enhancement of the antiapoptotic Bcl-2 protein expression in vivo. This protective effect on the skin contributed to the protection of the vascular network and facilitated reperfusion, which was found to be accelerated in ovariecтомized E2-treated mice, whereas hemorrhages were observed in untreated mice. E2 also increased expression of fibroblast growth factor-2 isoforms in the skin and circulating vascular endothelial growth factor in the serum. Finally, this protective effect of E2 was abolished in estrogen receptor–deficient mice (ERα−/−) but maintained in chimeric mice reconstituted with ERα-deficient bone marrow, indicating dispensable action of E2 in bone marrow–derived cells. This protective effect of E2 was mimicked by treatment with tamoxifen, a selective estrogen receptor modulator. In conclusion, we have demonstrated for the first time that E2 exerts a major preventive effect of skin flap necrosis through a prevention of ischemic-induced skin lesions, including those of the vascular network, which contributes to accelerate the reperfusion of the skin flap. (Circ Res. 2009;104:245-254.)

Key Words: estradiol ■ skin flap model ■ ischemia

Skin flaps are frequently used in plastic and reconstructive surgery. However, necrosis represents a major complication that may require secondary surgical interventions, generate multiple infections, and delay future treatments. Necrosis is caused by severe ischemia, resulting from impaired arterial inflow, especially in the distal part of the flap. Therapeutic angiogenesis by local administration of angiogenic growth factors fibroblast growth factor (FGF)-21 and vascular endothelial growth factor (VEGF),2 has been successfully tested to enhance blood perfusion in affected tissues, decreasing the extent of flap necrosis. However, the safety of this approach remains controversial, and no efficient therapy is currently available.

Estrogens appear to be attractive candidates, because 17β-estradiol (E2) exerts protective effects in various animal models of cardiac, brain, and hindlimb ischemia by favoring angiogenesis, limiting endothelial dysfunction, and exerting inflammatory and antiapoptotic effects.8,9 In elderly patients, E2 supplementation accelerates cutaneous wound healing.10 The mechanisms underlying these changes involve an increase in transforming growth factor (TGF)-β secretion11 but also inhibition of the local inflammatory response by downregulating migration inhibitory factor, which contributes to enhance matrix deposition.12

In the present work, we used a mouse model of cutaneous ischemia to investigate whether E2 can prevent necrosis after severe blood flow impairment. Whereas necrosis appeared in the distal portion of the skin flap within 1 week after surgery in ovariecтомized mice, it was largely reduced or even totally prevented in E2-treated animals. We then analyzed the E2-induced mechanisms accounting for this protection, such as skin viability and kinetics of revascularization.
Materials and Methods

Animal Studies
Female C57BL/6, FVB, hairless (SKH1) (Charles River Laboratories, Saint-Germain sur l’Arbresle, France), nude (NMRI) (Janvier, Le Genest St Isle, France) mice, and estrogen receptor α knockout (ERαKO) (described elsewhere)13 mice were ovariectomized at 5 weeks of age to suppress endogenous production of estrogens and implanted or not with pellets releasing either E2 (0.1 mg or 0.25 mg; 60-day release) or tamoxifen (5 mg; 60-day release) (Innovative Research of America, Sarasota, Fla). Skin flap surgery was performed 2 weeks later (Figure 1A). All mice were maintained under specific pathogen-free conditions in our animal facilities. Procedures were performed in accordance with the recommendations of the European Accreditation of Laboratory Animal Care and guidelines established by the National Institute of Medical Research (INSERM).

Administration of the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) (Sigma, Saint Quentin, France) (50 mg/kg per day in drinking water) was initiated 1 week before surgery.

Anti–TGF-β1/β2/β3 or an isotype matched (IgG2b) control monoclonal antibody was injected 2 days before surgery and then twice a week.14

Ischemia Model and Necrosis Quantification
A U-shaped peninsular skin incision was created on the dorsal surface of anesthetized (ketamine [100 mg kg⁻¹] and xylazine [10 mg kg⁻¹]) mice.15,16 The skin was elevated from the underlying muscular bed and the 2 vascular pedicles arising from the lateral thoracic arteries (T) were sectioned (Figure 1B). To quantify necrosis, mice were photographed and necrosis area was measured by planimetry, measuring the area of necrosis on the day of analysis (red area) divided by the area of the skin flap on day 0 (green area). The percentages of necrosis ± SEM on C57BL/6 (C) and FVB (D) mice are indicated with representative photographs on days 6 and 12. *P<0.05, **P<0.01, ***P<0.001.

Bone Marrow Transplantation
Recipient C57BL/6 mice were lethally irradiated (9Gy, γ-source) and reconstituted with bone marrow from either ERαKO or ERαWT donor 24 hours later.

Ex Vivo Viability Assays
Hairless female mice were ovariectomized and implanted or not with a pellet of E2. Two weeks later, dorsal skin samples were maintained in culture in DMEM medium (pH 7.4) supplemented with nonessential amino acids, 10% calf serum, 50 µg/mL gentamicin, and E2 (Sigma-Aldrich; concentration, 10⁻¹⁵ mol/L) or DMSO vehicle as control. Skin explants were incubated for 24 or 48 hours at 37°C in a 5% CO2 atmosphere. Viability of skin samples was determined by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described.17

Skin Blood Perfusion and Angiography
Skin flap blood perfusion was assessed with a Laser Doppler Perfusion Imaging system (PeriScan PIM II Imager, Perimed AB, Järfalla,
Swedish. Perfused skin vessels were visualized by angiography after intracardiac injection of fluorescein isothiocyanate dextran.

Information regarding histological analysis, Western blot analysis and ELISA, transmission electron microscopy, and statistical analysis is available in the expanded Materials and Methods section in the online data supplement at http://circres.ahajournals.org.

Results

Protective Effect of E2 on Skin Flap Necrosis

To test the protective effect of E2 on skin flap necrosis, we standardized a model to induce severe ischemia. In ovariectomized untreated C57BL/6 mice, severe and extended necrosis in the distal part of the flap (desquamation, alopecia, and scab formation) appeared maximal within 1 week after surgery (Figure 1C). In ovariectomized E2-treated mice, necrosis was largely reduced or even totally absent and wound healing at the incisions was accelerated. Quantitative analyses showed that necrosis in the untreated group accounted for 53.7±3.8% of the total flap area at its maximum on day 6, whereas in the E2-treated group, it represented only 3.8±2.6% (P<0.001). Similar results were obtained in FVB mice (48.1±8.2% in the untreated group versus 7.7±3.4% in E2-treated mice) (P<0.001) (Figure 1D). Mice were euthanized at the end of the protocol (day 21). Ovariectomized mice showed atrophied uteri (<20 mg), whereas E2-treated mice showed a significant increase in uterine weight (115±5 mg).

Effect of E2 Treatment on Skin Structure and Microscopic Lesions

Histological analysis was performed to qualify ischemic lesions. Ovariectomy increased both density and size of pilosebaceous units, which were the predominant proliferative structure in the absence of endogenous E2 (intense nuclear Ki-67 staining; Figure 2A). E2 decreased skin thickness and favored a mucinous layer rich in glycosaminoglycans below the platysma muscle (detected by acid Alcian blue coloration; data not shown), as well as an arrest of follicle proliferation, as reported. Necrosis was detected by microscopic examination earlier than by macroscopic examination (Figure 2B, day 2 versus day 6). In E2-treated animals, necrosis extended to one-tenth the length of the flap on the distal part, whereas it affected not only the distal but also the medial portion of the flap in ovariectomized animals. The progression of degeneration toward necrosis was prominent in hair follicle cells (see Figure I in the online data supplement). The protective effect of E2 persisted in nude and hairless SKH1 mice treated or not with E2. **P<0.001.

Figure 2. Effect of E2 on skin structure and microscopic lesions. A, The skin structure of intact female mice was assessed by H&E staining (×100 magnification) and compared to ovariectomized (OVX) mice treated or not with E2. The insets show Ki-67 immunostaining (×400 magnification). PM indicates platysma muscle. B, Extent of necrosis observed after H&E staining of skin flap longitudinal sections from ovariectomized mice, treated or not with E2 at different times postsurgery. *indicates position of the suture; bars, extent of necrosis. C, Percentages of skin flap necrosis in ovariectomized nude and hairless SKH1 mice treated or not with E2. **P<0.001.
shown). Even if TGF-β1 was shown to be involved in increase of wound healing by E2, we showed that administration of TGF-β-neutralizing antibody did not have any major effect on necrosis extent (supplemental Figure II, A). However, as previously reported,19 blocking TGF-β delayed wound healing in ovariectomized mice (detectable from days 12 to 21).

E2 Improves Skin Survival
Tissue viability was assessed in ex vivo skin samples by following the activity of mitochondrial dehydrogenases (MTT assay) (Figure 3A).17 Samples from ovariectomized mice showed 59.4%±5.4% of MTT conversion after 24 hours of culture, whereas samples from E2-treated or intact female mice were still close to the maximum of viability (88.7%±2.4% and 91.2%±4.4%, respectively). After 48 hours of culture, skin viability was reduced in all groups but remained significantly higher in the E2-treated group (62.8%±3.7%) and intact female skin (63.9%±5.5%) compared to the untreated ovariectomized mice (31.3%±4.6%). Addition of 10−9 mol/L E2 in the culture medium increased not only skin samples viability from ovariectomized mice but also skin viability from both E2-treated and intact mice.

The expression of pro- and antiapoptotic molecules was detected in vivo in C57BL/6 skin flaps. As shown in Figure 3B, the expression of the antiapoptotic Bcl-2 protein was always low in ovariectomized mice as compared to E2-treated mice. It decreased slowly from day 0 to day 4 on the former, whereas a transient decrease was observed on day 2 on the latter. The procaspase-3 and Bcl-XL proteins were not affected by E2 treatment or by ischemia duration. Furthermore, the activated form of caspase-3 was not detected (data not shown).

E2 Accelerates Reperfusion of a Preserved Vascular Network
Histopathology indicated that vasodilatation occurs shortly after surgery (visible 4 hours after surgery; see supplemental Figure III, A). Because E2 is known to increase NO bioavailability, we investigated whether its protective effect against necrosis might be blunted by nitric oxide synthase inhibition. Pharmacological inhibition with N^G-nitro-L-arginine methyl ester (L-NAME) had no effect on necrosis prevention by E2 (see supplemental Figure III, B).

Evaluation of skin flap perfusion using color laser Doppler imaging showed that the severe ischemia induced by surgery was followed by a blood flow restoration starting from the proximal part of the flap (Figure 4A). In untreated mice, skin perfusion remained very low, even on days 6 and 8 and impaired perfusion was associated with necrosis. A quicker and more intense perfusion was observed in E2-treated mice, with an almost complete reperfusion on day 6. Quantification of blood flow then showed a significant higher reperfusion in the E2-treated group as compared to ovariectomized mice (Figure 4B).

Figure 3. Viability of the skin. A, Evaluation of ex vivo skin viability from hairless mice in culture: MTT assay was performed on dorsal skin samples harvested from intact female and ovariectomized mice treated or not with E2 immediately after animals were euthanized and after 24 or 48 hours of culture in DMEM supplemented or not with 10−9 mol/L E2. Skin viability was expressed as a percentage of viability measured at time 0 (considered as 100%), as previously described.17 Values are means±SEM from 4 skin samples. *P<0.05, **P<0.001. B, Evaluation of in vivo expression of pro- and antiapoptotic factors in C57BL/6 mice. Bcl-2, Bcl-XL, and procaspase-3 protein expressions in the proximal area of skin flaps before surgery and at days 2 and 4 after surgery were determined by immunoblotting on protein extracts. β-Actin was used as a loading control.
Figure 4. Analysis of blood flow perfusion of the skin flap. A, Color laser Doppler analysis of the skin flap in ovariectomized FVB mice treated or not with E2 on day 0 (on preoperative and postoperative surgery) and on days 4, 6, and 8 postsurgery. The color scale illustrates variations in blood flow from maximal (red) to minimal perfusion (dark blue). B, Blood flow perfusion of the total flap area was quantified and reported in perfusion units ± SEM. **P<0.01, ***P<0.001. C, Major pedicles were sectioned by the surgery (arrows). Angiography showed growth of functional collaterals from preexisting anastomoses on the top of the flap. D, Representative analysis of the vascularization of the skin on day 4 using angiography (left), color laser Doppler (middle), and visual observation of the reverse side of the skin flap (right). E, Histological analysis by H&E staining (×400 magnification) and Ki-67 immunohistochemistry (×1000 magnification) in the skin flaps of ovariectomized mice treated or not with E2. Arrows point the presence of extra vascular erythrocytes. F, Transmission electronic microscopy of vessels in the most distal part of the skin flaps in nonoperated mice (intact vessel) and in ovariectomized C57BL/6 mice treated or not with E2, on day 4. The arrows indicate erythrocytes. EC indicates endothelial cells. The scale bar represents 0.4 μm. G, Immunoblotting experiments show that expression of FGF-2 isoforms is enhanced in E2-treated C57BL/6 mice. H, Circulating VEGF concentration is enhanced on day 2 after the surgery in both groups of mice, but the increase is more important in E2-treated animals. **P<0.01.
Angiograms fitted well to laser Doppler images. On day 2, skin flap vascularization was generally absent, with no obvious difference between the 2 groups (data not shown). On day 4, examination of the vascular network indicated that remodeling of numerous arteriolar anastomoses present in the skin occurred on the top of the flap, in both groups, where pedicles were sectioned (Figure 4C). This remodeling allowed blow flow restoration, especially in E2-treated mice, in which reperfusion was almost complete. In untreated mice, perfusion was still missing in the most distal part of the flap and flap elevation showed severe hemorrhages (Figure 4D). Hemalum/eosin (H&E) staining (Figure 4E) clearly showed vessel thrombosis and erythrocyte extravasations in the dermis, especially in the middle zone of skin flaps. In contrast, a slight vascular congestion with minor hemorrhages was observed in E2-treated animals. Ki-67 immunohistochemistry indicated abundant proliferation of endothelial cells on day 4, without any significant difference between the 2 groups, even then TGF-β blocking antibody was administrated. Transmission electron microscopy confirmed the loss of vessel integrity in ovariecotomized mice with presence of free erythrocytes in the tissue, whereas erythrocytes remained intravascular in E2-treated animals even if endothelial cells were damaged (Figure 4F).

Detection of cutaneous FGF-2 isoforms and plasmatic VEGF (Figure 4G and 4H) indicated higher expression of both FGF-2 isoforms (all times) and VEGF (day 2) in E2-treated mice as compared to untreated mice. Additionally, in E2-treated animals, secreted FGF-2 isoform (supplemental Figure III, C) and circulating VEGF were further increased after surgery on day 2. Therefore, prevention of skin necrosis by E2 was associated with earlier reperfusion of a better preserved vascular network.

**Influence and Duration of Dose of E2 Pretreatment**

We evaluated exposure to different doses of E2, using untreated intact (endogenous E2) or treated intact mice (0.1-mg pellet) and ovariecotomized mice given either a 0.1- or 0.25-mg pellet (releasing 80 and 200 μg/kg per day, respectively), compared to untreated ovariecotomized mice (dose, 0). There was no significant effect of the dose among the four groups impregnated with E2 (Figure 5B) (11.3±4.5% and 15.2±4.0% of necrosis in intact female mice treated or not with E2, respectively, compared to 11.9±2.6% and 9.3±2.3% in mice receiving 0.1 or 0.25 mg, respectively on day 8).

To reduce exposure to E2, the minimal time required to prevent skin necrosis was also evaluated. E2 treatment initiated 3 days before flap elevation conserved the protective effect, whereas abolition was observed when treatment was initiated the same day of surgery (Figure 5A) (8.3±3.9%, and 41.1±5.6%, respectively, compared to 38.2±3.5% in ovariecotomized animals).

These data indicated that a physiological estrogen exposure (ie, estrus cycle) or an exogenous E2 short treatment initiated 3 days before flap elevation were sufficient to obtain an optimal protection against necrosis.

**Figure 5.** Effect of timing and dose of E2 treatment on the prevention of necrosis. A, Effect of E2 dose on necrosis. Flap surgery was performed on intact and ovariecotomized female C57BL/6 mice treated or not with E2. The pellet of E2 released either 0.1 mg or 0.25 mg for 60 days (80 and 200 μg kg⁻¹ per day), respectively. B, Effect of E2 pretreatment duration on necrosis. E2 treatment was started 14 days or 3 days before the surgery or the day of the surgery (day 0). Percentages of skin necrosis±SEM were given on day 8. **P<0.01; ***P<0.001; ns, not significant vs O VX-E2.

**Estrogen Receptor α Mediates the Protective Effect of E2 but Is Dispensable in Bone Marrow–Derived Cells**

Estrogens exert their biological effects through 2 different nuclear estrogen receptors (ERs), namely ERα and ERβ, which are expressed in various cell types, including endothelial cells, immune cells, and skin. ERα is known to mediate most of the E2 effects in the vascular system.

Surgery was performed in ERα⁻/⁻ mice to evaluate the role of ERα in the protective effect of E2 on necrosis. Similar high rates of necrosis were obtained in ovariecotomized ERα⁻/⁻ mice treated or not with E2 (32.9±5.7% and 36.9±2.3%, respectively, on day 8) (Figure 6A). To assess the contribution of bone marrow–derived cells, we grafted irradiated mice with ERα⁺/⁺ or ERα⁻/⁻ bone marrow. No significant difference was observed between the 2 groups of chimeric mice (ERα⁺/⁺ versus ERα⁻/⁻) (Figure 6B), but E2 treatment was as effective in ERα⁻/⁻ chimeric mice as in control ERα⁺/⁺ chimeric mice.

These data indicated that the protective effect of E2 requires ERα expression, independently of its expression in bone marrow.
Tamoxifen antagonizes the effect of E2 in breast cancer and is frequently administered at the same time as breast reconstructive surgery but has some agonist actions on other tissues, for instance, uterus. Tamoxifen treatment 14 days before flap surgery significantly reduced the area of necrosis to 9.3 ± 3.0% on day 8 compared to the placebo (38.2 ± 3.5%), this protective effect being similar to that of E2 (13.2 ± 2.9% on day 8, P > 0.05) (Figure 7A). Histological analyses at the time of surgery showed that tamoxifen involved similar structural modifications than E2 (Figure 7B).

Effect of the Selective Estrogen Receptor Modulator Tamoxifen

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Discussion

Using a standardized mouse model of skin flap, we demonstrated for the first time, to our knowledge, that E2 efficiently protects against skin necrosis. We showed that ovarian cycle E2 amounts are sufficient to mediate this protective effect. Maximal protection was maintained with a 3-day pretreatment but abolished when initiated the day of surgery. Therefore, functional and/or structural changes in skin and/or vasculature seemed to be a prerequisite for the protective effect of E2. The similarity of the results obtained in different genetic backgrounds, including hairless mice, demonstrated the robustness of E2 effect. The protective effect of E2 was abolished in ERα−/− mice, indicating an additional role for ERα in skin necrosis prevention, which adds to its effects on reproduction, bone remodeling, vascular system.

The effect of E2 against skin ischemia involves preservation of skin viability, as demonstrated in ex vivo explants. Antiapoptotic effect of E2 has been extensively studied in ischemic brain and heart. E2 inhibits apoptosis in cultured keratinocytes by promoting Bcl-2 expression. Our present data further indicated that E2 directly promotes skin survival, enhancing the antiapoptotic Bcl-2 expression in vivo. E2 treatment induces skin structural modifications, increasing the mucinous layer that might play a major function to improve skin survival. This mucinous layer plays an important role in tissue structural integrity and increased skin water content. Indeed, these modifications are important to stimulate connective tissue synthesis in normal and photodamaged skin and are involved in the wound-healing process.

Moreover, the present study indicates that the protective effect of E2 against necrosis is associated with an accelerated reperfusion of the flap, following collateral remodeling. Doppler analysis and angiography showed that E2 improved blood perfusion, from day 4 to 8 after ischemia. In ovariectomized animals, major hemorrhages were found within the tissue and extravascular erythrocytes were most abundant in the middle zone of skin flaps, where reperfusion occurred. Transmission electron microscopy revealed vascular leakage, with obvious endothelial disjunctions responsible for erythrocyte extravasations. In E2-treated animals, erythrocytes remained intravascular, even if some endothelial cells displayed ultrastructural damages, suggesting that the increase in skin survival induced by E2...
also benefits on vascular network preservation. This original result is in line with its antiapoptotic effect reported previously in vitro on different cultured endothelial cells\textsuperscript{9,32,33} but also on the decrease of vessel permeability observed in the brain following estrogen treatment in rats.\textsuperscript{34}

E2 is known to directly modulate endothelial migration, proliferation,\textsuperscript{8} and vascularization through induction of several growth factors, such as VEGF\textsuperscript{35} or FGF-2.\textsuperscript{36} Here, we found that both VEGF and FGF-2 levels were higher in E2-treated mice. These results strongly suggest that E2 probably contributes to accelerate vascular remodeling: arteriogenesis and angiogenesis. Collateral remodeling is known to be rapid in C57BL/6 mice, with functional collaterals already present 3 days after hindlimb ischemia.\textsuperscript{37} Because this remodeling occurred rapidly and because skin is rich in anastomoses, small differences in the timing of collateral remodeling between treated and untreated C57BL/6 mice were difficult to evaluate in our model. Because arteriogenesis was shown to be reduced in BALB/c mice and delayed to days 7 to 14 after femoral occlusion,\textsuperscript{37} skin flap necrosis was investigated in BALB/c mice (see supplemental Figure IV). The protective effect of E2 was found to be largely decreased on this genetic background but remained significant compared to ovariectomized mice. Indeed, high rates of necrosis (more than 30\%) were measured in E2-treated BALB/c mice, which developed the most extensive necrosis among all mice of strains tested. These are associated with no significant difference in blood perfusion following surgery between the 2 groups, suggesting that delayed arteriogenesis in BALB/c could have impaired the protective action of E2 in treated mice.

Recruitment of bone marrow cells has been demonstrated to be involved in neovascularization of ischemic tissues.\textsuperscript{38,39} Otherwise, transplantation of bone marrow from ER\textsuperscript{α−/−} mice did not have any impact on the protective effect of E2, indicating that the protective targets of E2 are not bone marrow--derived cells.\textsuperscript{40} All of these data suggest that, rather than neovascularization involving endothelial progenitor cells, the early collateral remodeling is critical for future survival of the skin and consequently for the protective effect by E2.

We propose the following mechanism of protection by E2 (Figure 8). (1) E2 modifies the structure–function characteristics of the skin, which (2) favors skin survival including dermis and vessels integrity. In the meantime, (3) collateral remodeling occurs and allows skin reperfusion. Because the vascular network is protected in E2-treated mice, reperfusion induces revascularization of the skin flap. Conversely, in the absence of E2, the vascular network is damaged, and this is followed by vessel leakage and strong damage. Thus, preservation of the vascular network associated with arteriogenesis appeared as the prominent mechanism accounting for the protective effect of E2 against ischemia-associated necrosis. In addition, E2 was reported to accelerate cutaneous wound healing through TGF-β secretion.\textsuperscript{11} TGF-β is important at the final healing phase to resolve inflammation and reconstitute the extracellular matrix. We confirmed this effect using a TGF-β–neutralizing antibody, which delayed wound healing.

Tamoxifen has partial agonist or antagonist estrogenic activities in different tissues\textsuperscript{41} and has been recently shown to accelerate cutaneous wound healing.\textsuperscript{42} We report here that tamoxifen is as efficient as E2 to prevent skin flap necrosis, providing a new perspective in patients undergoing reconstructive surgery after breast cancer. Moreover, the effects on skin structure are close to those of E2, suggesting that tamoxifen acts as an estrogen-like molecule in this tissue. Recent data showing that this selective estrogen receptor
modulator confers estrogenic effect in various vascular systems are in agreement with the vascular protection evidenced in the present model.

In summary, we demonstrated here for the first time that a short administration of E2 prevents ischemia-induced skin necrosis. E2 thus delayed progression of the wavefront of ischemic cell death described by Reimer et al, preserving the vascular network that facilitates reperfusion. These observational data are important for the different models of ischemia/reperfusion.

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Disclosures
None.

References


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SUPPLEMENT MATERIAL

Materials and Methods:

Histological analysis

The whole skin flap was harvested, fixed in 10% buffered formalin for 24 hours, divided in 5 longitudinal sections before embedding in paraffin to obtain different views of the skin flap. 4-µm sections were then stained with hematoxylin and eosin (H&E) or for Ki-67 (TEC-3, Dako Cytomation, France, revealed with AEC substrate).

Western blot analysis and ELISA

Frozen skin samples placed in Lysing Matrix A tube (Q-BIOgene, Carlsbad, CA, USA) were homogenized in 500 µl of lysis buffer (20 mM Tris-HCl, 1 mM NaCl, 2 mM EGTA, 1 mM DTT, pH 7.6 containing 1 % Triton X100, 100 µg/ml PMSF, and Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals)) using FastPrep® Sample Preparation system (Q-BIOgene, Carlsbad, CA). The homogenate was centrifuged at 10 000 g for 20 min at 4°C. Proteins were then electrophoresed in 12 % SDS-PAGE and transferred onto nitrocellulose membrane before immunoblotting using the Bcl-2 polyclonal, the Bcl-XL(54H6) and caspase-3 (8G10) rabbit monoclonal antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) and the β-actin antibody for normalization (clone AC-15, Sigma-Aldrich, USA). For FGF-2 immunoblotting, frozen skin samples placed in Lysing Matrix A tube (Q-BIOgene, Carlsbad, CA) were homogenized in 500 µl of SDS 2 % lysis buffer at 95°C using FastPrep® Sample Preparation system. FGF-2 protein was immunodetected as previously described ¹. An enhanced chemiluminescence system was used as the detection method (ECL Plus, Amersham).
Sera were analyzed for circulating levels of murine VEGF by means of an enzyme-linked immunosorbent assay (DuoSet ELISA, R&D Systems), according to the manufacturer’s instructions.

**Transmission electron microscopy (TEM)**

Skin was fixed with 2% glutaraldehyde in Sorensen buffer (0.1 M, pH = 7.4) for 1 hour, washed with the Sorensen phosphate buffer (0.1 M) for 12 hours. Then, skin was post-fixed with 1% OsO₄ in Sorensen buffer (Sorensen phosphate 0.05 M, glucose 0.25 M, OsO₄ 1%) for 1 hour. Samples were dehydrated in an ascending ethanol series until ethanol 100° and then with propylene oxide. Skin was then embedded in epoxy resin (Epon 812). After 24h of polymerisation at 60°C, ultra thin sections (70 nm) were mounted on 100 mesh collodion-coated copper grids and poststained with 3% uranyl acetate in 50% ethanol and with 8.5% lead citrate before being examined on a HU12A Hitachi electron microscope at an accelerating voltage 75 kV.

**Statistical analysis**

Results are expressed as means ± SEMs. The effect of E2 was tested with a 2-factor ANOVA model for repeated measurements followed by pairwise comparisons with Bonferroni post-hoc test using the software Prism (Prism®, GraphPad). *p<0.05, **p<0.01, ***p<0.001. A value less than 0.05 was considered statistically significant.

Online Figure I:

**Zooms on follicle cells after H&E staining on days 0, 2 and 6 from untreated or E2-treated ovariectomized animals.**

(A) In ovariectomized mice, progression of degeneration towards necrosis is prominent and easily evaluated on hair follicle cells which are the most susceptible to necrosis. The normal appearance of epithelial cells on follicle bulbs, on day 0, undergoes coagulative necrosis such as pycnosis on day 2, until absence of cell outlines and total nuclear absence with homogeneous eosinophilic cytoplasm on day 6 (B). In E2-treated mice, necrosis was observed only in the most distal part of the flap extending to 1/10 length of the flap, affecting slightly the deeper layers of the flap (mucinous layer and platysma muscle) and mostly preserving hair follicle cells from necrosis.
Online Figure II:

Percentages of necrosis were also quantified on female ovariectomized mice untreated (OVX) or treated with E2 (E2), and injected intraperitoneally with either an anti TGF–β1/β2/β3 monoclonal antibody (clone 2G7, provided by D. Fradelizi, INSERM U477, Paris, France) or an isotype matched (IgG2b) control mAb, devoid of neutralizing activity. The intraperitoneal injection of the antibody was initiated two days before the surgery and then pursued twice a week until complete healing. A delay in the healing was observed on the group of ovariectomized mice treated with the anti-TGF-β as opposed to the control antibody (visible from days 12 to 21). Values are means ± SEMs. **p<0.01; ***p<0.001
Online Figure III:

Vasodilatation and no effect of L-NAME treatment on prevention of necrosis.

(A) Histological analysis of skin 4h after the surgery (H&E staining (100X) ) indicated a marked diffuse vasodilatation of the large vessels and capillaries (indicated by the arrows), in ovariectomized mice treated or not with E2. (B) Percentages of necrosis were also quantified on female ovariectomized mice treated or not with E2, and receiving L-NAME in the drinking water (50mg/kg/day). L-NAME treatment was initiated one week before the surgery and pursued until complete healing of necrosis. Values are means ± SEMs. **p<0.01; ***p<0.001.  (C) Densitometric units of the FGF-2 Western Blot in Figure 4E, using the Image J software. LMW (low molecular weight) represents the secreted 18kDa isoform. HMW (high molecular weight) represents the 22 and 24kDa isoforms.
Online Figure IV:

Effect of E2 treatment on prevention of skin flap necrosis (A) and blood flow perfusion (B) in BALB/c mice.

(A) Percentages of skin flap necrosis in ovariectomized BALB/c mice treated or not with E2. Values are means ± SEMs. ***p<0.001.

(B) Color laser doppler analysis of the skin flap in ovariectomized BALB/c mice treated or not with E2, on day 0 (on preoperative and postoperative surgery) and on days 4, 6, 8 post- surgery was performed. Blood flow perfusion of the total flap area was quantified and reported in perfusion units ± SEMs. ns= not significant.
SUPPLEMENTARY DATA

Materials and Methods:

Histological analysis

The whole skin flap was harvested, fixed in 10% buffered formalin for 24 hours, divided in 5 longitudinal sections before embedding in paraffin to obtain different views of the skin flap. 4-µm sections were then stained with hematoxylin and eosin (H&E) or for Ki-67 (TEC-3, Dako Cytomation, France, revealed with AEC substrate).

Western blot analysis and ELISA

Frozen skin samples placed in Lysing Matrix A tube (Q-BIOgene, Carlsbad, CA, USA) were homogenized in 500 µl of lysis buffer (20 mM Tris-HCl, 1 mM NaCl, 2 mM EGTA, 1 mM DTT, pH 7.6 containing 1 % Triton X100, 100 µg/ml PMSF, and Complete Protease Inhibitor Cocktail (Roche Molecular Biochemicals)) using FastPrep® Sample Preparation system (Q-BIOgene, Carlsbad, CA). The homogenate was centrifuged at 10 000 g for 20 min at 4°C. Proteins were then electrophoresed in 12 % SDS-PAGE and transferred onto nitrocellulose membrane before immunoblotting using the Bcl-2 polyclonal, the Bcl-XL(54H6) and caspase-3 (8G10) rabbit monoclonal antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) and the β-actin antibody for normalization (clone AC-15, Sigma-Aldrich, USA). For FGF-2 immunoblotting, frozen skin samples placed in Lysing Matrix A tube (Q-BIOgene, Carlsbad, CA) were homogenized in 500 µl of SDS 2 % lysis buffer at 95°C using FastPrep® Sample Preparation system. FGF-2 protein was immunodetected as previously described 1. An enhanced chemiluminescence system was used as the detection method (ECL Plus, Amersham).
Sera were analyzed for circulating levels of murine VEGF by means of an enzyme-linked immunosorbent assay (DuoSet ELISA, R&D Systems), according to the manufacturer’s instructions.

Transmission electron microscopy (TEM)

Skin was fixed with 2% glutaeraldehyde in Sorensen buffer (0.1 M, pH = 7.4) for 1 hour, washed with the Sorensen phosphate buffer (0.1 M) for 12 hours. Then, skin was post-fixed with 1% OsO₄ in Sorensen buffer (Sorensen phosphate 0.05 M, glucose 0.25 M, OsO₄ 1%) for 1 hour. Samples were dehydrated in an ascending ethanol series until ethanol 100° and then with propylene oxide. Skin was then embedded in epoxy resin (Epon 812). After 24h of polymerisation at 60°C, ultra thin sections (70 nm) were mounted on 100 mesh collodion-coated copper grids and poststained with 3% uranyl acetate in 50% ethanol and with 8.5% lead citrate before being examined on a HU12A Hitachi electron microscope at an accelerating voltage 75 kV.

Statistical analysis

Results are expressed as means ± SEMs. The effect of E2 was tested with a 2-factor ANOVA model for repeated measurements followed by pairwise comparisons with Bonferroni post-hoc test using the software Prism (Prism®, GraphPad). *p<0.05, **p<0.01, ***p<0.001. A value less than 0.05 was considered statistically significant.

Supplementary Figure 1:
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Supplementary Figure 3:
**Vasodilatation and no effect of L-NAME treatment on prevention of necrosis.**
(A) Histological analysis of skin 4h after the surgery (H&E staining (100X) ) indicated a marked diffuse vasodilatation of the large vessels and capillaries (indicated by the arrows), in ovariectomized mice treated or not with E2. (B) Percentages of necrosis were also quantified on female ovariectomized mice treated or not with E2, and receiving L-NAME in the drinking water (50mg/kg/day). L-NAME treatment was initiated one week before the surgery and pursued until complete healing of necrosis. Values are means ± SEMs. **p<0.01; ***p<0.001. (C) Densitometric units of the FGF-2 Western Blot in Figure 4E, using the Image J software. LMW (low molecular weight) represents the secreted 18kDa isoform. HMW (high molecular weight) represents the 22 and 24kDa isoforms.
Supplementary Figure 4: Effect of E2 treatment on prevention of skin flap necrosis (A) and blood flow perfusion (B) in BALB/c mice.

(A) Percentages of skin flap necrosis in ovariectomized BALB/c mice treated or not with E2. Values are means ± SEMs. ***p<0.001.

(B) Color laser doppler analysis of the skin flap in ovariectomized BALB/c mice treated or not with E2, on day 0 (on pre-operative and post-operative surgery) and on days 4, 6, 8 post-surgery was performed. Blood flow perfusion of the total flap area was quantified and reported in perfusion units ± SEMs. ns= not significant.