Estradiol accelerates endothelial healing through the retrograde commitment of uninjured endothelium

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Am J Physiol Heart Circ Physiol 294: H2822–H2830, 2008. First published April 25, 2008; doi:10.1152/ajpheart.00129.2008.—Although the accelerative effect of 17β-estradiol (E2) on endothelial regrowth has been clearly demonstrated, the local cellular events accounting for this beneficial vascular action are still uncertain. In the present work, we compared the kinetics of endothelial healing of mouse carotid arteries after endovascular and perivascular injury. Both basal reendothelialization as well as the accelerative effect of E2 were similar in the two models. Three days after endothelial denudation, a regenerative area was observed in both models, characterized by similar changes in gene expression after injury, visualized by en face confocal microscopy (EFCM). A precise definition of the injury limits was only possible with the perivascular model, since it causes a complete and lasting decellularization of the media. Using this model, we demonstrated that the migration of uninjured endothelial cells proceeds proliferation (bromodeoxyuridine incorporation) and that these events occur at earlier time points with E2 treatment. We have also identified an uninjured retrograde zone as an intimate component of the endothelial regeneration process. Thus, in the perivascular model, the regenerative area can be subdivided into a retrograde zone and a reendothelialized area. Importantly, both areas are significantly enlarged by E2. In conclusion, the combination of the electric perivascular injury model and EFCM is well adapted to the visualization of the endothelial monolayer and to investigate cellular events involved in reendothelialization. This process is accelerated by E2 as a consequence of the retrograde commitment of an uninjured endothelial zone to migrate and proliferate, contributing to an enlargement of the regenerative area.

endothelial regeneration; arterial injury; estrogen; carotid injury model; en face confocal microscopy

THE INTEGRITY AND FUNCTIONALITY of the arterial endothelium, the very thin cell monolayer positioned at the interface between the blood and the vessel wall, play a crucial role in the physiology of circulation. Deendothelialization is the consequence of the treatment of coronary atherosclerosis disease by percutaneous transluminal coronary angioplasty, most often associated with intracoronary stents. One major drawback of this therapeutic approach is the residual in-stent restenosis, due to smooth muscle cell proliferation and intimal extracellular matrix accumulation. Drug-eluting stents releasing antimitotics (sirolimus and paclitaxel) efficiently prevent smooth muscle cell proliferation and, thereby, in-stent restenosis but increase the risk of late-stent thrombosis. This very serious event, commonly associated with sudden death or acute myocardial infarction, is at least partly the consequence of the inhibition of the reendothelialization of the stent struts by the antimitotics (13, 25).

The endothelial healing after the injury process has previously and mostly been studied in larger animals. More than 20 years ago, a series of pioneer studies unraveled several cellular mechanisms involved in endothelial regrowth after injury in rats and rabbits (19, 37, 38, 42). To further gain insight into the molecular mechanisms, the possibility to use transgenic mice provides its obvious advantage. However, the endovascular injury model in mice, proposed by Lindner et al. (24), is laborious due to the very small size of the mouse carotid artery. As an alternative method, we more recently proposed a model of perivascular electric injury of the carotid artery (9), based on a femoral artery injury model developed by Carmeliet and colleagues (10). Even though the perivascular carotid model has been used in several published studies (8, 15, 41, 45), no systematic comparison with the endovascular injury has yet been reported, although both models behave similarly in terms of accelerated endothelial healing by 17β-estradiol (E2) estimated 3 days postinjury by Evans blue staining (8).

Indeed, it is now clear that the physiological and pathophysiological role of E2 is not limited to reproductive organs but involves many other tissues, in particular those belonging to the cardiovascular system. In particular, E2 exerts several beneficial effects at the level of the endothelium; it promotes endothelial nitric oxide (eNO) production and prevents VCAM-1 expression and endothelial apoptosis (4, 28). In addition, E2 stimulates endothelial regrowth after endothelial denudation in rats (23) and in mice (9, 20, 40). Interestingly, the inhibition of neointimal proliferation and acceleration of reendothelialization with the local administration of E2 following balloon angioplasty in a pig model has been recently reported (11, 30). Moreover, the implantation of E2-eluting stents appears feasible and safe in humans (1).

E2 effects can be mediated by estrogen receptor (ER)-α and ER-β, also named NR3A1 and NR3A2, referring to their classification within the nuclear receptor superfamily (30a). The accelerative effect of E2 on reendothelialization is medi-
ated by ER-α (9), and eNO synthase (eNOS) appears absolutely required for this effect (8, 20). Interestingly, the effect of E2 on endothelial healing remains unaltered upon the inhibition of NOS activity by Nω-nitro-L-arginine methyl ester (8). In addition, ER-α also mediates the beneficial effect of E2 on medial hyperplasia (33). However, at least in mice, ER-β is not necessary for both these E2 effects on arterial healing (9, 22).

To further study the cellular mechanisms accounting for the accelerative effect of E2 on reendothelialization, we have compared the kinetics of reendothelialization in the conventional endovascular and a perivascular electric injury model using Evans blue staining. Since the endothelial cell monolayer is rather poorly visualized by immunohistochemical analyses on frozen transversal sections, we also applied en face confocal microscopy (EFCM) to more precisely visualize the regenerating endothelium, as well as the expression of several molecular key actors. We confirmed, in agreement with previous work in a rat (38), the appearance of the regenerative area (Regen) characterized by endothelial cell proliferation and gene expression changes. At variance with the endovascular model, the injury limit can be precisely defined in the perivascular model by EFCM analysis. This feature revealed the implication of the retrograde zone in the adjacent uninjured endothelium in the regenerative process. The commitment of this zone, named retrograde proliferating zone (RetroP), could be of profound importance in the beneficial action of E2, since its size is increased upon E2 treatment.

MATERIALS AND METHODS

**Mice.** All procedures involving experimental animals were performed in accordance with the principles and guidelines established by the National Institute of Medical Research (Institut National de la Santé et de la Recherche Médicale (INSERM)) and approved by the Institutional Animal Care and Use Committee. They were housed in cages in groups of five, kept in a temperature-controlled facility on a 12-h:12-h light-dark cycle, and fed normal laboratory mouse chow. At 4 wk of age, C57BL/6J mice (Charles River) were ovariectomized, and pellets releasing either placebo or E2 (0.1 mg E2, 60 days release, i.e., 80 µg·kg⁻¹·day⁻¹; Innovative Research of America, Sarasota, FL) were implanted subcutaneously in the back of the animals. C57BL/6-Tg(NACTbEGFP)1Os transgenic mice (GFP-Tg; Jackson, Bar Harbor, ME) (31), with at least eight backcrosses with C57BL/6J mice, were used in bone marrow (BM) grafting experiments. Two weeks after ovariectomy, recipient C57BL/6 mice were lethally irradiated (9 Gy and γ-source) and received an intravenous injection of 4 × 10⁶ donor BM cells 24 h later. One month later, mice were implanted with either a placebo or an E2 pellet. Chimeric mice were euthanized 3 days postinjury.

Mice were exposed to a placebo or E2 for 2 wk before artery injury and until euthanization as previously described (9). We systematically checked that placebo-treated ovariectomized mice had an atrophied uterus (<20 mg) and nondetectable (<5 pg/ml; i.e., 20 × 10⁻¹² M) circulating levels of E2, whereas those implanted with an E2-releasing pellet had a significant increase in uterine weight and serum E2 concentrations (100 to 150 pg/ml; not shown).

**Mouse carotid injury.** Endovascular injury was performed mechanically by a modification of the protocol described by Lindner and coworkers (24). Briefly, animals were anesthetized by an intraperitoneal injection of a mix of ketamine and xylazine (1% and 0.1% wt/vol, respectively). The right common carotid artery was exposed from bifurcation, via an anterior incision of the neck. The left external carotid was ligated distally and looped proximally with a 7-0 silk suture. Silk loops were placed around internal and common carotids to temporally restrict blood flow in the area of surgical manipulation. The external carotid was incised. The injury was performed using a 0.3-mm-diameter swab built by loosely winding and sticking an 8-0 silk suture around 0.16-mm-diameter blunted guide wire. The swab was introduced, advanced through the common left carotid artery, and withdrawn three times. A 4-mm-length denudation from bifurcation of the common carotid artery was performed using the silk suture on the common carotid as the injury. The device was removed, and the external carotid was ligated proximally. Blood flow was then restored in internal and common carotids. The skin incision was closed, and the animals were allowed to wake up under warm conditions.

The carotid electric injury was performed essentially as previously described (9). Briefly, the left common carotid artery was exposed as described above. The electric injury was applied to the distal part (4 mm precisely) of the common carotid artery with a bipolar microregulator.

One to seven days later, the endothelial regeneration process was evaluated by staining the denuded areas with Evans blue dye as previously described (9). Briefly, 50 µl of solution containing 5% Evans blue diluted in saline were injected into the tail vein 10 min before euthanasia, followed by fixation with a perfusion of 4% phosphate-buffered Formalin (pH 7.0) for 5 min. The left common carotid artery was dissected from the aortic arch to the carotid bifurcation. The artery was then opened longitudinally. The total and stained carotid artery areas were planimetered after image digitalization. The percentage of reendothelialization was calculated by 1 − (Adays/Aday0) × 100, where Adays represents the remaining deendothelialized area at the day of euthanization and Aday0 represents the initially deendothelialized area.

**EFCM.** Intravascular blood was first removed with an intracardiac injection of PBS 1×. The carotids were carefully dissected and fixed for 20 min in PBS containing 4% paraformaldehyde. The carotids were longitudinally opened, and the fixation was quenched with 100 mM glycine (pH 7.4), permeabilized for 10 min in 0.1% Triton X-100, and washed in PBS. Fixed tissues were blocked with a solution containing 75 mM NaCl, 18 mM Na3citrate, 2% goat serum, 1% BSA, and 0.05% Triton X-100, with fluorophore-conjugated antibodies (Molecular Probes) diluted in solution I and incubated with carotids during 48 h at 4°C. Tissues were then washed with solution II containing 75 mM NaCl, 18 mM Nacitrate, and 0.05% Triton X-100 for 2 h and rinsed in PBS. The carotids were incubated, 1 h at room temperature, with fluorophore-conjugated antibodies (Molecular Probes) diluted in solution I. Finally, the tissues were washed in solution I for 1 h at room temperature. Primary antibodies used were as follows: CD31 (01951D; Pharmingen), eNOS (sc-654; Santa Cruz Biotechnologies), and von Willebrand factor (vWF; A0082; Dako). Secondary antibodies (Invitrogen) were all conjugated with Alexa solution I for 1 h at room temperature. Primary antibodies used were as follows: CD31 (01951D; Pharmingen), eNOS (sc-654; Santa Cruz Biotechnologies), and von Willebrand factor (vWF; A0082; Dako). Secondary antibodies (Invitrogen) were all conjugated with Alexa Fluor 633. To label the nuclei, propidium iodide was used (Sigma-Aldrich). All preparations were mounted with Kaiser’s glycerol gelatin (Merck). Microscopy imagery was performed on a LEICA SP2 confocal microscope, and quantification was performed with ZEISS LSM 510 software. The lengths of the reendothelialized area (RE) and of the RetroP are means of at least 10 measures spanning the carotid injection of 100 µl bromodeoxyuridine (BrDU) solution (33 mg/ml; Roche Diagnostic) 15 h before euthanization. At earlier time points (24, 36, or 50 h postinjury), the mice received an injection at time 0 and then approximately every 16 h. After tissue recovery, samples were fixed in 100% methanol during 30 min and permeabilized with 2 N HCl during 7 min at 37°C. After three washes in PBS, the samples were treated with a borate solution (24.6 g/l Na2B4O7 and 4.36 g/l KH2PO4) during 15 min. The carotid arteries were subsequently

**Bromodeoxyuridine incorporation and staining.** To label proliferating cells 3 or 5 days postinjury, the mice received an intraperitoneal injection of 100 µl bromodeoxyuridine (BrDU) solution (33 mg/ml; Roche Diagnostic) 15 h before euthanization. At earlier time points (24, 36, or 50 h postinjury), the mice received an injection at time 0 and then approximately every 16 h. After tissue recovery, samples were fixed in 100% methanol during 30 min and permeabilized with 2 N HCl during 7 min at 37°C. After three washes in PBS, the samples were treated with a borate solution (24.6 g/l Na2B4O7 and 4.36 g/l KH2PO4) during 15 min. The carotid arteries were subsequently
incubated with solution I (previously described in EFCM) for 2 h at room temperature. Finally, the tissues were incubated with anti-BrdU antibody conjugated with Alexa Fluor 633 (Invitrogen) during 1 h at room temperature and rinsed in solution II (described in EFCM). A mean length of each zone (Regen, RE, and RetroP) was measured and obtained as described in EFCM.

Statistics. Results are expressed as means $\pm$ SE. To test the role of E2 treatment on reendothelialization, a one-factor ANOVA was performed. To test the respective role of E2 treatment and the type of injury or time on reendothelialization, a two-factor ANOVA was performed. When an interaction was observed between the two factors, the four groups were compared by a one-factor ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Endovascular and perivascular electric injury models exhibit similar kinetics of both basal and E2-stimulated reendothelialization. To compare the reendothelialization process after endovascular and perivascular electric carotid injury, we performed an injury of equal length (4 mm) in both models in ovariectomized mice treated with either a placebo or E2. Under these conditions, the reendothelialization is completed 7 days after both types of injury (data not shown), as previously observed (9). Therefore, the kinetics of reendothelialization was analyzed at 3 and 5 days postinjury. Interestingly, no difference in the kinetics of the endothelial healing was observed between both models (Fig. 1). To test the respective role of the injury type and E2 treatment on reendothelialization at each time point, a two-factor ANOVA was performed and revealed a significant effect of E2 on the acceleration of reendothelialization at days 3 and 5 (both $P < 0.05$) but no effect of the type of injury and no interaction between the two parameters. Thus no difference in the rate of the endothelial healing could be detected between both models.

En face confocal immunohistochemical analysis of endovascular and perivascular electric carotid injury reveals similarities between the two models in terms of reendothelialization. We next wanted to gain further insight into the molecular and cellular events during reendothelialization in both injury models at 3 days postinjury. Since the visualization of the endothelial cell monolayer by transversal sections is limited, we employed EFCM on longitudinally opened and flattened mouse carotid arteries. Since the endothelial regeneration in both models progresses mainly from the proximal edge of the lesion, in the direction of the blood flow, we focused on this region for further studies. Staining by propidium iodide confirmed that the endothelial and smooth muscle cell nuclei could be distinguished by their orientation, size, and shape. Indeed, in contrast with smooth muscle, endothelial cell nuclei were oriented in the direction of the blood flow and were larger and rounder in shape than smooth muscle cell nuclei. When compared with that of the intact endothelium, EFCM analysis revealed a decreased expression of vWF and platelet endothelial cell adhesion molecule (CD31) and an increased expression of eNOS in the injured area, irrespective of the injury model (Fig. 2, A–C and A’–C’).

We then determined the proliferative index of the endothelial cells following a peritoneal injection of BrdU 15 h before euthanization. The fraction of BrdU-positive cells after endovascular (38.6 $\pm$ 4.6%) and perivascular (44.3 $\pm$ 3.9%) injury did not differ significantly ($P = 0.35$; Fig. 2, D and D’).

In agreement with previous reports (24), endovascular carotid injury using a thin swab preserves most of the vascular smooth muscle cells (Fig. 2E). In contrast, perivascular electric carotid injury provokes a complete decellularization of both the intima and the media (Fig. 2E’), without any cellular recolonization of the latter 1 wk after injury. Thus, at variance with the endovascular model, the electric model permits the precise definition of the injury limit using EFCM.

The electric injury model analyzed by EFCM was also allowed to determine two distinct areas: the RE, corresponding to the part of the injured intima that has been recolonized with endothelial cells, and the Regen, corresponding to the zone of proliferating cells. In these ovariectomized mice, the Regen was only slightly larger than the RE. Interestingly, the overexpression of eNOS and the lowered expression of vWF and CD31 were overlapping with the Regen both in the endovascular (Fig. 2, A–D) and in the perivascular (Fig. 2, A’–D’) model.

Taken together, we show here that even though the media exhibits major differences in the two injury models, the endothelial cells of the Regen had similar characteristics in both models. Since both injury models exhibited similar kinetics of reendothelialization (Fig. 1), but only the electric model in combination with EFCM allowed a precise localization of the injury limit due to the absence of the cellular recolonization of the media (Fig. 2E’), this latter model was chosen for most of the subsequent experiments.

En face confocal immunohistochemical analysis of perivascular electric carotid injury reveals the absence of medial smooth muscle regeneration within the first week postinjury. One of the surprising features of the model of perivascular electric carotid injury was the total absence of regeneration of the media at day 7, at a time when the reendothelialization was completed. We therefore decided to explore the regeneration of the medial layer at days 1, 3, and 5 postinjury. We first observed that, at these different time points, the limit of injury remained abrupt, with no visible evidence of migrating cells and with no change in smooth muscle cell density (Fig. 2E’). Second, we determined the proliferative index of the smooth muscle cells following a peritoneal injection of BrdU 15 h
always before euthanization. The fraction of BrdU-positive cells was 1% of the cells examined at the edge of the injury, i.e., less than one medial cell per carotid artery examined (not shown). Third, the en face technique also offers the opportunity to precisely determine the abundance of BM-derived cells in regions of reendothelialization as well as the blood flow. Fourth, E2 treatment elicited two main modifications of reendothelialization at day 3 after perivascular injury reveals the commitment of a retrograde zone. E2 treatment elicited two main modifications at day 3 postinjury. First, EFCM confirmed that E2 accelerates the reendothelialization process (Figs. 3 and 4), in agreement with data obtained through Evans blue staining (Fig. 1). Second, E2 induces the enlargement of Regen in the adjacent uninjured area (Fig. 3B). BrdU labeling enabled the visualization of this RetroP, defined as the area with a density $\geq 10\%$ of BrdU-positive cells. RetroP was present in placebo-treated mice but was impressively increased by E2 (Figs. 3B and 4). Changes in expression levels, such as increased eNOS, decreased CD31, and vWF, observed in RE also overlapped with RetroP (Fig. 3, B and C).

The quantification showed that E2 increased RE by 32.9% and Regen by 43.5% (both $P < 0.05$; Fig. 4A). Moreover, the combination of EFCM and BrdU labeling allowed us to define the proliferation index as well as the total number of endothelial cells. When compared with the placebo, E2 increased in the RE the total number of BrdU-positive cells by 93.4% and the total cell number by 86.1% (both $P < 0.05$; Fig. 4, B and C).
Since the density of BrdU-positive cells in RE was not significantly increased by E2, the increase in the total number of BrdU-positive cells was mainly due to the increased size of RE. The proliferative effect of E2 was even more spectacular in the RetroP. Indeed, E2 increased not only the size of RetroP (from 75 ± 7 to 163 ± 29 μm, P < 0.05) but also the percentage of BrdU-positive cells in this zone (from 16.0 ± 1.3% to 42.4 ± 1.2%, P < 0.05), this latter value being similar to that found in the RE. Altogether, E2 drastically (10-fold) increased the total number of BrdU-positive cells in the RetroP (Fig. 4C).

In the endovascular injury model 3 days postinjury, where only Regen could be defined due to the lack of a precise determination of the injury limit, E2 increased the Regen (+37.4%, P < 0.05) to a similar extent as in the perivascular injury model (not shown). Indeed, a two-way ANOVA did not reveal any interaction between the E2 effect and the model used. Furthermore, the percentage of BrdU-positive cells in Regen was very similar to that in E2- and placebo-treated mice (35.7 ± 3.2% and 36.0 ± 2.1%, respectively, P = 0.87; not shown), which is very close to the observations made in the perivascular injury model.

Early events in reendothelialization: endothelial migration precedes proliferation effect of E2. To characterize the initial cellular responses in endothelial regrowth with respect to cell migration and proliferation, we used the perivascular model followed by repeated intraperitoneal administrations of BrdU on average every 16 h. At 24 h postinjury, neither endothelial cell migration nor cell proliferation was observed in both E2- and placebo-treated mice (data not shown).

At 36 h after injury, the front of endothelial cells had clearly started to colonize the injured area, and the reendothelialized surface was significantly increased by E2 (+50.4%, P < 0.05; Fig. 5, A and B). However, as for the observation at 24 h, no BrdU-labeled cells were detected in any condition at 36 h postinjury (Fig. 5A). When the same analysis was performed 50 h postinjury, cellular proliferation was observed in half (3/6) of the E2-treated mice but never (0/6) in placebo-treated mice (Fig. 5C). Of note, in BrdU-positive E2-treated carotids, the percentage of labeled cells (39.2 ± 5.0%) was already close to that observed at day 3 or 5, indicating that the onset of proliferation quickly reached its maximal level.

The results in Fig. 5, A and B, clearly showed that the migration of endothelial cells precedes the proliferative response and that both of these processes appeared earlier under E2 treatment. The fact that the RE at 36 h was larger in E2-treated than in control mice in the absence of proliferation could be explained by several mechanisms. One possibility is that an equal number of cells had migrated into the injured area in both placebo- and E2-treated mice but that the cells in the latter group had an increased size and, therefore, covered a larger surface. To test this hypothesis, the cellular density in RE was measured by the counting of propidium iodide-stained nuclei in the intima of carotids 36 and 50 h postinjury. Since we did not observe any significant difference in cellular density in the two conditions, this would imply that more cells are recruited in the healing process under E2 treatment. These cells could derive from either circulating cells and/or from the increased migration of surrounding uninjured endothelial cells. To determine whether the recruitment and incorporation of
bone marrow-derived cells could account for the observed difference, we performed the transplantation of GFP-positive bone marrow into wild-type mice. The EFCM technique offers the opportunity to precisely determine the percentage of bone marrow-derived cells at different levels in the artery wall. Unexpectedly, E2 did not increase the density of GFP-positive cells in the RE (3.4 ± 1.2%) compared with the placebo (4.2 ± 0.9%), not significant (data not shown), although it increased the total number of GFP-positive cells in the Regen in proportion to the increase in its size, as previously reported (20, 40).

However, we found that E2 engages the migration of more endothelial cells from the uninjured area into the injured area. Indeed, as shown in Fig. 5C, with the depiction of data from carotids 50 h postinjury, E2 decreased the cell density further upstream of the injury limit (in the retrograde-uninjured endothelium) than did the placebo. Noteworthy, the decrease in the cell number in this region coincided with the increase in cells observed downstream of the injury limit (in the RE) in E2-treated mice. Thus E2 mobilized a larger retrograde area and, thereby, a larger number of endothelial cells to migrate.

Taken together, these results demonstrate that endothelial cell migration constitutes an early cellular response to injury, which precedes endothelial proliferation, and that E2 generated an earlier and enhanced action of both of these processes through the recruitment of a larger area of uninjured endothelium. Since the surface of uninjured committed endothelium, with decreased cellular density, was considerably larger than that of the RetroP at both days 2 (Fig. 5C) and 3 (not shown), we next sought to examine the evolution of RetroP at a later time point.

Accentuation of the retrograde proliferation at day 5 postinjury in placebo- and E2-treated mice. The size of RetroP at day 5 was significantly larger than at day 3 both in placebo- and E2-treated mice (Fig. 6) and corresponded well with the size of the committed zone, exhibiting a decreased cell density at day 3 (not shown). The size of both RE and RetroP in both placebo- and E2-treated mice increased progressively and significantly from day 2 to 3 and from day 3 to 5 (P < 0.05; Fig. 6B). Moreover, both RE and RetroP were significantly larger under the E2 treatment compared with the placebo at all time points (Fig. 6B).
These analyses by EFCM confirmed the effect of E2 on the reendothelialization observed by Evans blue staining (Fig. 1). At day 30, when reendothelialization was completed for about 3 wk, no proliferation was observed in both placebo- and E2-treated mice (not shown).

In summary, these data suggest that the decreased endothelial cell density observed in the retrograde uninjured endothelium at earlier time points gives rise to a subsequent proliferative response covering a similar surface. E2 not only increased the RE corresponding to the previously reported acceleration of reendothelialization but clearly promoted the progressive recruitment of a retrograde zone in the adjacent uninjured endothelium (RetroP), which substantially contributed to the regenerative capacity of the Regen.

E2 does not alter medial smooth muscle regeneration. We also explored the regeneration of the medial layer at days 1, 3, and 5 postinjury in response to E2. As with that in placebo-treated mice, the limit of injury remained abrupt, with no visible migrating cells and with no change in smooth muscle cell density, <1% of proliferating smooth muscle cells, and no GFP-positive cells could be detected in the injured media at variance with the intima and adventitia (not shown). Altogether, these data confirm that the limit of injury can be reliably defined by the front of the smooth muscle cells.

**DISCUSSION**

The main achievements of the present study are as follows. First, the conventional endovascular and the perivascular electric injury models were found to be indistinguishable in terms of basal and E2-elicited reendothelialization. Second, a Regen characterized by endothelial cell proliferation and gene expression changes is similar in both of the mouse models, and E2 elicits an enlargement of this Regen. Third, in contrast to the endovascular model, the underlying smooth muscle cells are destroyed in the injured area of the perivascular electric model and do not regenerate during the first week, allowing to precisely define the injury limit. This permitted to subdivide Regen into a RE (in the injured area) and a RetroP (in the adjacent uninjured endothelium). Finally, E2 strongly increased the retrograde commitment of adjacent uninjured endothelium, which is first distinguished by the mobilization of endothelial cells and a decrease in cellular density, followed by a proliferative response. This increase of RetroP, in addition to the increase of RE, contributed to the enlargement of the Regen, which is likely to represent key events in the acceleration of reendothelialization.

Endovascular and electric perivascular injuries can be similar in their efficiency to destroy the endothelium, but they differ significantly in their effects on the underlying cell populations in the vessel wall. Endovascular injury preserves most of the adventitial cells as well as most of the medial smooth muscle cells (24). In both models the intima is regenerated after 1 wk; however, in the perivascular model, the media does not show any sign of regeneration during the time of reendothelialization. Since both models had similar characteristics with respect to basal and E2-elicited reendothelialization (evaluated by Evans Blue staining), changes in gene expression, and proliferative response (see below), this would suggest a minor, if any, role of the smooth muscle cells in the injured area in the reendothelialization process. However, smooth muscle cells in the RetroP area might play an important role (see below). The perivascular model offers various major advantages, such as no modification of the flow in the carotid artery associated to the ligation of external carotid artery, rapidity, and reliability (thrombosis rate, 5–10% vs. 30% in the endovascular). Moreover, perivascular electric injury can be easily transferred from one experimenter to another and requires less operator skill than the endovascular injury. Taken together, all these similarities allowed us to conclude that perivascular electric injury represents a valid and alternative model to endovascular injury.

More than two decades ago several researchers, such as Reidy, Clowes, Schwartz, Spagnoli, and others (12, 19, 37–39), beautifully described different cellular events involved in reendothelialization models of endovascular artery injury in the rat or the rabbit. In concordance with our data, Schwartz and colleagues (38) showed that the proliferation (using 3H-thymidine incorporation) of endothelial cells from each side of the adjacent uninjured area was not restricted to the front edge but involved up to 100 rows of endothelial cells, contributing to a regeneration zone (38). Using BrdU incorporation, we observed here in the mouse a similar Regen. At variance with the study by Schwartz et al. (38), we did not observe any proliferating cells 24 or even 36 h postinjury. It is possible that this divergence could in part be due to differences between blood vessels (carotid vs. aorta) or species (mouse vs. rat). Species-dependent differences in regenerative capacity have indeed been reported. The endothelium of the rabbit carotid is, for instance, less prone to heal than that of rats (36).

Not surprisingly, we observed that the regenerative process was also characterized by marked alterations of gene expression. It is of interest to note that these changes appear over the entire Regen including both injured and uninjured areas. Increased eNOS expression was reported 10 years ago by Berk and coworkers (34) in the Regen after rat carotid injury. The expression of eNOS is also increased in proliferating cultured endothelial cells (6). More recently, Losordo and colleagues (20) demonstrated that the eNOS is also indispensable in the accelerative effect of E2 on reendothelialization even though it is independent of the NO-producing activity of eNOS (8).

We also explored the regeneration of the smooth muscle cells in the injured media in the perivascular electric model. Surprisingly, and in contrast with the rapid endothelial healing, we could not detect any significant features of migration or the proliferation of the adjacent uninjured smooth muscle cells during the first week, and no BM-derived cells were observed in the media. We cannot provide an explanation for the absence of rapid regeneration of the media. The high expression of eNOS in the regenerated endothelium could be one contributing factor, since elevated eNOS levels are known to inhibit smooth muscle cell proliferation and migration (21, 43). The absence of the underlying smooth muscle cells and the lack of their regeneration (at least during the first week) have two major consequences. First, only the electric injury model allows to precisely define the limit of the injury and, thereby, to further divide Regen into a RE and a RetroP. Second, it suggests, given the similarities between the results observed in the two models of reendothelialization, that the underlying smooth muscle cells in the injured area do not appear to be of importance in endothelial healing. However, it does not rule out that those in the RetroP could still play an important role.
The suggested lack of role of the smooth muscle cells in the injured zone for both basal and estradiol-induced reendothelialization (Fig. 1) should not be interpreted as a general conclusion concerning the mechanisms involved in other treatments that influence the kinetics of endothelial healing.

At day 3 postinjury, the enlargement of Regen by E2 (Fig. 4A) was the outcome of an increased surface of both the RE and the RetroP, a zone expanding the Regen into the adjacent uninjured area. E2 treatment did not alter the percentage of the BrdU-positive cells in the RE but increased this percentage in the RetroP to a level similar to that observed in the RE (Fig. 4B). Thus E2 recruited cells to proliferate mainly by increasing in the size of the proliferative cell area (Regen = RE + RetroP). These phenomena were magnified at day 5 postinjury, in particular the effect of E2 on RetroP (Fig. 6). Earlier time points revealed that E2 initially accelerated cell migration, leading to a decrease in cellular density in the upstream uninjured endothelium over a distance that was threefold longer under E2 (Fig. 5C). This is likely to be a directly contributing factor to the increased size of RetroP, defined by proliferating cells, observed after E2 treatment (Fig. 6). Thus the spatiotemporal commitment of the adjacent uninjured endothelium occurred earlier under E2, leading to a larger and precocious RetroP, which could represent a key event in reendothelialization and the emergence of Regen.

Whereas E2 is known to promote endothelial cell migration and proliferation in vitro (17, 18, 29), the present study is, to our knowledge, the first demonstration of a clear distinction between E2-stimulated migration and the proliferation of endothelial cells in vivo. It was initially believed that E2 accelerated reendothelialization mainly through a local effect on adjacent residing uninjured endothelium. However, several studies have shown that arterial healing can be significantly modulated by circulating BM-derived cells as monocyte-macrophages (16), platelets (27), or endothelial progenitor cells (EPCs) (3, 7, 35). E2 increases the number of circulating EPCs as well as the incorporation of BM-derived cells in the RE (20, 40). However, the present work does not suggest that the incorporation of these BM-derived cells substantially contribute in the endothelial regeneration process, since the percentage of GFP-positive cells was low (about 4%) and did not differ in E2- or placebo-treated mice. We cannot exclude a potential role of non-BM-derived progenitor cells, since the homing of these cells seems important in neovascularization after hind leg ischemia (2). Third, platelets are abundant in the homing of these cells important in neovascularization after hind leg ischemia (2). The suggested lack of role of the smooth muscle cells in the injured zone for both basal and estradiol-induced reendothelialization (Fig. 1) should not be interpreted as a general conclusion concerning the mechanisms involved in other treatments that influence the kinetics of endothelial healing.

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