ORIGINAL ARTICLE

Erythropoietin Restores C-Fiber Function and Prevents Pressure Ulcer Formation in Diabetic Mice

Claire Demiot[1], Vincent Sarrazy[1], James Javellaud[1], Loriane Gourloi[1], Laurent Botelle[1], Nicole Oudart[1] and Jean-Michel Achard[1]

Pressure-induced vasodilatation (PIV), a cutaneous physiological neurovascular (C-fiber/endothelium) mechanism, is altered in diabetes and could possibly contribute to pressure ulcer development. We wanted to determine whether recombinant human erythropoietin (rhEPO), which has protective neurovascular effects, could prevent PIV alteration and pressure ulcer formation. We developed a skin pressure ulcer model in mice by applying two magnetic plates to the dorsal skin. This induced significant stage 2 ulcers (assessed visually and histologically) in streptozotocin-treated mice with 8 weeks of diabetes compared with very few in controls. Control and streptozotocin mice received either no treatment or systematic rhEPO (3,000 UI kg^{-1} intraperitoneally, twice a week) during the last 2 weeks of diabetes. After 8 weeks of diabetes, we assessed ulcer development, PIV, endothelium-dependent vasodilation, C-fiber-mediated nociception threshold, and skin innervation density. Pretreatment with rhEPO fully prevented ulcer development in streptozotocin mice and also fully restored C-fiber nociception, skin innervation density, and significantly improved PIV, but had no effect on endothelium-dependent vasodilation. Our finding that rhEPO treatment protects the skin against pressure-induced ulcers in diabetic mice encourages evaluation of the therapeutic potential for non-hematopoietic analogs of EPO in preventing neuropathic diabetic ulcers.

INTRODUCTION

Rigorous control of glycemia and blood pressure, and lowering of cholesterol, has failed to reduce the risk of diabetic feet (Urbancic-Rovan, 2005; Rajamani et al., 2009). Owing to the devastating consequences in diabetic patients and the major burden on health-care systems, any therapeutic option able to decrease the necessity of amputation is highly desirable.

Pressure-induced vasodilation (PIV) has been described as being a cutaneous physiological neurovascular interaction (Fromy et al., 1998, 2000b), delaying the occurrence of tissue ischemia, and thus protecting the skin against pressure. The increase in cutaneous blood flow in response to local non-nociceptive pressure involves pressure sensing by unmyelinated C-fibers, which function on the endothelium to synthesize and release endothelial factors, such as nitric oxide (Fromy et al., 2000b), inducing smooth muscle relaxation. PIV is altered in diabetic humans (Koitka et al., 2004) and diabetic mice with established neurovascular

dysfunctions (Demiot et al., 2006), which could favor diabetic ulcer occurrence.

There are non-hematopoietic cellular targets of erythropoietin (EPO) in the skin, and the existence of EPO receptors on endothelial cells (Anagnostou et al., 1994) and neurons (Chen et al., 2010) has been described. There is also experimental evidence that demonstrates that EPO has an important function in skin wound healing (Galeano et al., 2004, 2006; Hamed et al., 2010). Human recombinant EPO (rhEPO) and non-hematopoietic analogs have been shown to prevent and reverse diabetic neuropathy in rats and mice (Bianchi et al., 2004; Leist et al., 2004). In addition, EPO increases endothelial nitric oxide synthase expression and stimulates nitric oxide release in cultured human endothelial cells (Beleslin-Cokic et al., 2004). The dual neurovascular nature of the defect responsible for PIV alteration in long-term diabetes (Demiot et al., 2006) points to EPO as a potential therapeutic tool for protecting skin from PIV alteration and preventing pressure ulcer formation.

The objectives of this study were to determine whether rhEPO could restore PIV in long-term diabetic mice with established neurovascular dysfunction and prevent pressure-induced skin ulcer formation.

RESULTS

At the time of experimentation (after 8 weeks of diabetes), all diabetic mice lost significant weight and had significant hyperglycemia compared with the respective control mice (Table 1). Treatment with rhEPO during the last 2 weeks had

[1]EA 3842, Faculté de Médecine et de Pharmacie, Institut Fédératif de Recherche, Université de Limoges, Limoges, France

Correspondence: Claire Demiot, Laboratory of Pharmacology, EA 3842, Medical-Pharmacist Schools, University of Limoges, 87025 Limoges Cedex, France. E-mail: claire.demiot@unilim.fr

Abbreviations: LDF, laser Doppler flowmetry; PGP 9.5, protein gene product 9.5; PIV, pressure-induced vasodilation; rhEPO, recombinant human erythropoietin

Table 1. Effects of EPO on body weight, glycemia, hematocrit, and SABP in 8-week diabetic and control mice on the day of the experiment

Group	Body weight (g)	Glycemia (mmol l^{-1})	Hematocrit (%)	SABP (mm Hg)
Control mice	43 ± 1	7.2 ± 0.4	51 ± 1.5	64 ± 3.5
Diabetic mice	28 ± 1***	25.9 ± 2.1***	39.5 ± 2.5***	73 ± 6
Control mice+EPO	40 ± 1	6.1 ± 0.4	54 ± 1	65 ± 4.5
Diabetic mice+EPO	27 ± 1***	25.8 ± 2.1***	53 ± 2†	65 ± 5

Abbreviations: EPO, erythropoietin; rhEPO, recombinant human erythropoietin; SABP, systolic arterial blood pressure.
Control and 8-week diabetic mice were untreated or treated during the last 2 weeks with rhEPO.
***$P<0.001$ versus respective control mice.
$^†P<0.05$ versus diabetic mice.
n=10 in each group.

no effect on weight and glycemia. The hematocrit of diabetic animals was decreased compared with controls. RhEPO increased the hematocrit in treated diabetic mice, but had no effect in control mice. Neither the treatments nor diabetes induction changed the systolic arterial blood pressure.

Effect of EPO on mechanical pressure algesia and skin innervation

Untreated diabetic mice had mechanical hypoalgesia detected using the tail pressure Randall–Sellito test (Figure 1a). The tail pressure nociceptive threshold was significantly increased by 57% in diabetic mice compared with control mice (21.9 ± 3 vs. 12.5 ± 1 g, $P<0.01$). The mechanical algesia response observed in the rhEPO-treated diabetic group was restored to the same level as its respective control group. In control mice treated or untreated with rhEPO, protein gene product 9.5 (PGP 9.5)-positive axons were evident in both the dermis and epidermis of flank skin. In contrast, analysis of PGP 9.5-positive axons in diabetic skin revealed an obvious decrease in the general innervation of the skin. Visual examination showed that rhEPO prevented this decrease (Figure 1b). Quantification of PGP 9.5-positive axons in the skin using axon tracing confirmed the visual observation (Figure 1c). The skin areas occupied by labeled axons were significantly reduced in diabetic mice compared with the untreated control group. RhEPO treatment restored these areas to the same level of labeled axons as the respective controls.

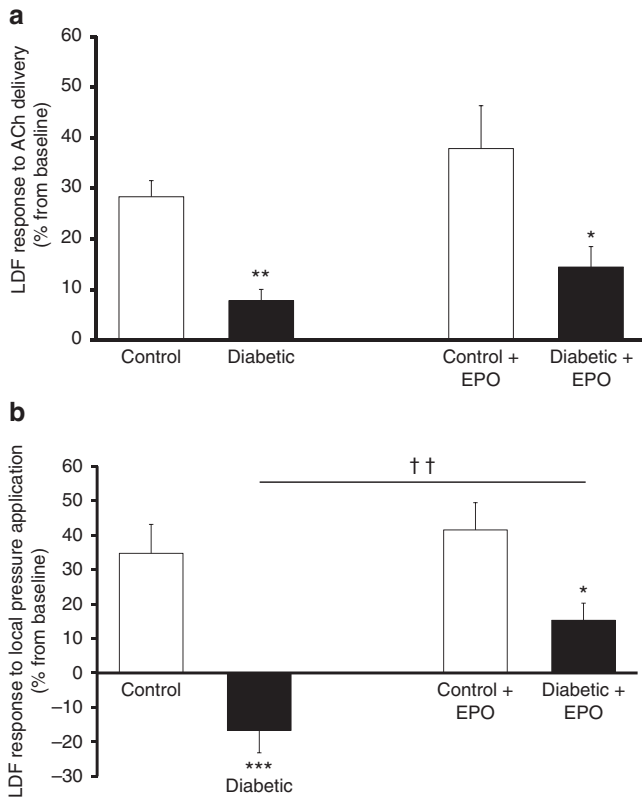### Effect of EPO on skin microcirculation reactivity

Cutaneous temperature and systolic arterial blood pressure were stable throughout the experiment. Resting blood flow was not significantly different between each group. In the untreated control group, we observed an increase in laser Doppler flowmetry (LDF) in response to iontophoretic delivery of acetylcholine, corresponding to a maximal percentage endothelium-dependent vasodilation of 28.5 ± 3% from baseline (Figure 2a). Diabetes caused a significant decrease in acetylcholine-induced vasodilation that was not significantly improved with EPO. Moreover, in all groups, LDF
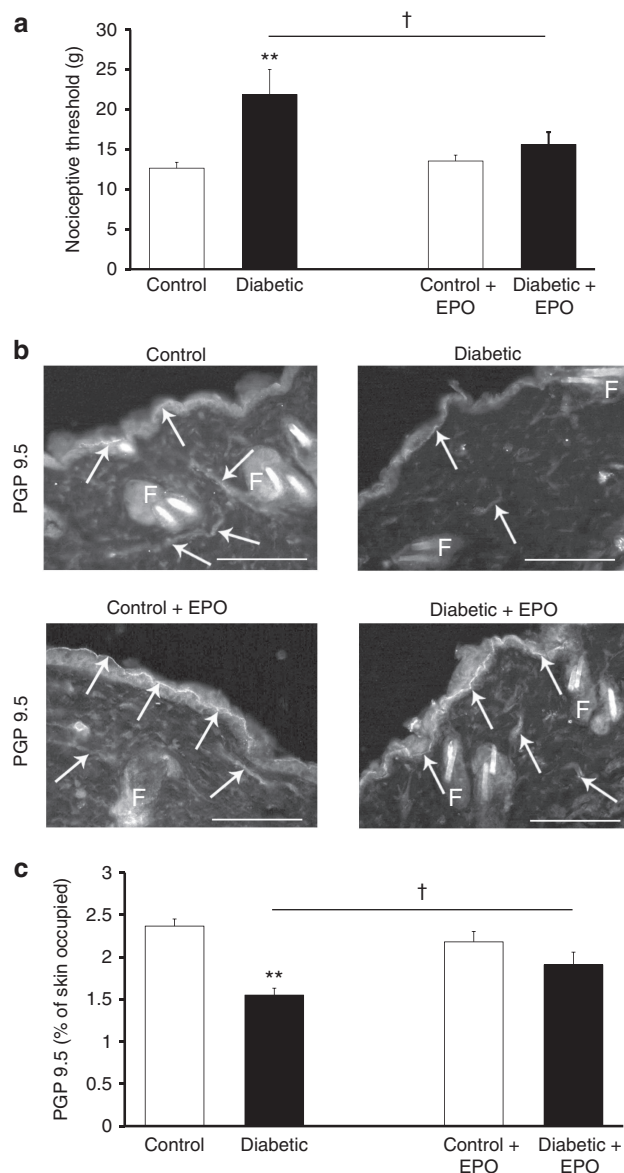
Figure 1. Effect of erythropoietin (EPO) on skin neuropathy.
(**a**) Randall–Sellito tail pressure test. Mechanical withdrawal thresholds in control and diabetic mice treated or not treated with recombinant human erythropoietin (rhEPO). **$P<0.01$ versus respective control mice; $^†P<0.05$ versus diabetic mice; $n=10$ in each group. (**b**) Immunohistochemical labeling of protein gene product 9.5 (PGP 9.5)-positive cutaneous axons (white arrows) from the flank region. The dermis of control skin contains horizontal PGP 9.5-positive nerve bundles. In contrast, diabetes causes an obvious decrease in dermal and epidermal innervations. RhEPO restores general innervation. Original magnification ×200; bar (white)=100 μm; ''F'' denotes hair follicles. (**c**) Abundance of PGP 9.5-positive axons in the skin from the flank. The percentage of skin area occupied by immunopositive axons is shown. **$P<0.01$ versus respective control mice; $^†P<0.05$ versus diabetic mice; $n=12$ in each group.

increased in response to iontophoretic delivery of sodium nitroprusside, and we did not observe any difference in the endothelium-independent vasodilation between groups (control 41.5 ± 7%, diabetic 65 ± 8%, EPO-treated control 40 ± 7.5%, EPO-treated diabetic 52.5 ± 10.5%; $n=8$ in each group).

Figure 2. **Effect of erythropoietin (EPO) on skin microcirculation reactivity alteration.** (**a**) Endothelium-dependent vasodilation. Maximal percentage of vasodilation from baseline in response to iontophoretic delivery of acetylcholine (ACh) in control and diabetic mice treated or not treated with recombinant human erythropoietin (rhEPO), $n = 8$ in each group. (**b**) Pressure-induced vasodilation. Maximal percentage of vasodilation from baseline during local pressure application in control and diabetic mice treated or not treated with rhEPO, $n = 10$ in each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus respective control mice; $^{††}P < 0.01$ versus diabetic mice. LDF, laser Doppler flowmetry.

Effect of EPO on PIV

Cutaneous temperature and systolic arterial blood pressure were stable throughout the experiment. In the untreated control group, LDF increased in response to increasing local pressure that reached a maximal value at 0.4 kPa corresponding to a PIV of $+34.7 \pm 8.5\%$ from baseline. In contrast, in the untreated diabetic group, LDF decreased in response to local pressure applied at 0.4 kPa (Figure 2b). RhEPO treatment had a significantly beneficial effect on PIV in diabetic mice compared with untreated diabetic mice, but failed to restore a normal PIV in diabetic mice compared with the treated control group.

Effect of EPO on macroscopic and histological findings in the pressure ulcer model

3H pressure. In untreated control mice, the mean percentage of stage 2 ulcer areas at days 2 and 3 (Figure 3e) and histological scores were low (Figure 3f). In contrast, daily progression of a superficial pressure ulcer (stage $\geqslant 2$) with partial-thickness skin loss involving the epidermis and dermis

was observed in the center of the compressed area in diabetic mice (Figure 3a). Stage 2 ulcer surface reached a maximum at days 2 and 3 (Figure 3e). Histopathology showed epidermal and superficial dermis defects with necrotic areas in the dermis (Figure 3b), giving a histological score of 2.75 ± 0.3 at day 1 and 2.9 ± 0.35 at day 3 (Figure 3f). Treatment with rhEPO in control mice had no effect. The mean percentage of stage 2 ulcer areas at days 2 and 3 (Figure 3e) and histological scores (Figure 3f) were not different from that of untreated control mice. In diabetic mice, however, treatment with rhEPO markedly and significantly reduced ulcer development (Figure 3c). Histological examination showed the presence of three skin layers, and sometimes a thickening of the epithelium and disruption of fibers (Figure 3d), with a score of 0.91 ± 0.2 at day 1 and 0.83 ± 0.1 at day 3 (Figure 3f).

12H pressure in non-diabetic mice. Stage 2 ulcer area development in control mice after 12 hours of pressure was similar to that observed in diabetic mice after 3 hours of pressure stage 2 ulcer surfaces (% in compressed area): at day 1, day 2, and day 3, they were $19.0 \pm 3.7\%$, $29.1 \pm 3.0\%$, and $26.5 \pm 3.3\%$, respectively, and were not different from the respective values observed in the group of mice pretreated with rhEPO during 2 weeks $(11.9 \pm 2.9\%,$ $23.7 \pm 3.4\%,$ and $33.8 \pm 3.2\%; n = 10$ in each group).

DISCUSSION

The main findings of our study are that rhEPO treatment administered for 2 weeks in mice with diabetes of 6 weeks duration with established neuropathy (1) fully restored the loss of unmyelinated C-fibers in the skin and restored functional hypoalgesia (2) significantly but incompletely improved PIV, and (3) had no effect on diabetes-induced endothelial dysfunction in skin microcirculation.

In addition, our study demonstrated that rhEPO was able to fully prevent ulcer development induced by one single high pressure in advanced diabetes with neuropathy.

Our study thus confirms the well-documented potent neuroprotective and neurorepairing effects of EPO. Similar to diabetic patients, mice with streptozotocin-induced diabetes develop peripheral diabetic neuropathy with alterations in nociceptive thermal and mechanical thresholds (Uehara et al., 2004; Vinik and Mehrabyan, 2004). The first fibers affected in diabetic neuropathy are the small sensory ones (Vinik and Mehrabyan, 2004). In Swiss mice, thermal hypoalgesia develops after only 2 weeks of streptozotocin-induced diabetes, and a measurable reduction in PGP 9.5-immunoreactive epidermal nerve fiber density is present after 4 weeks (Beiswenger et al., 2008). In our model, as anticipated, treatment with rhEPO for 2 weeks administered after 6 weeks of diabetes was sufficient to fully restore a normal skin innervation density that functionally correlated with the recovery of a normal nociceptive threshold in diabetic mice.

In contrast to its neuroprotective effect, rhEPO had no effect on the deleterious effect of long-lasting diabetes on endothelial dysfunction. As a result, rhEPO treatment significantly improved, but failed to fully restore PIV in diabetic
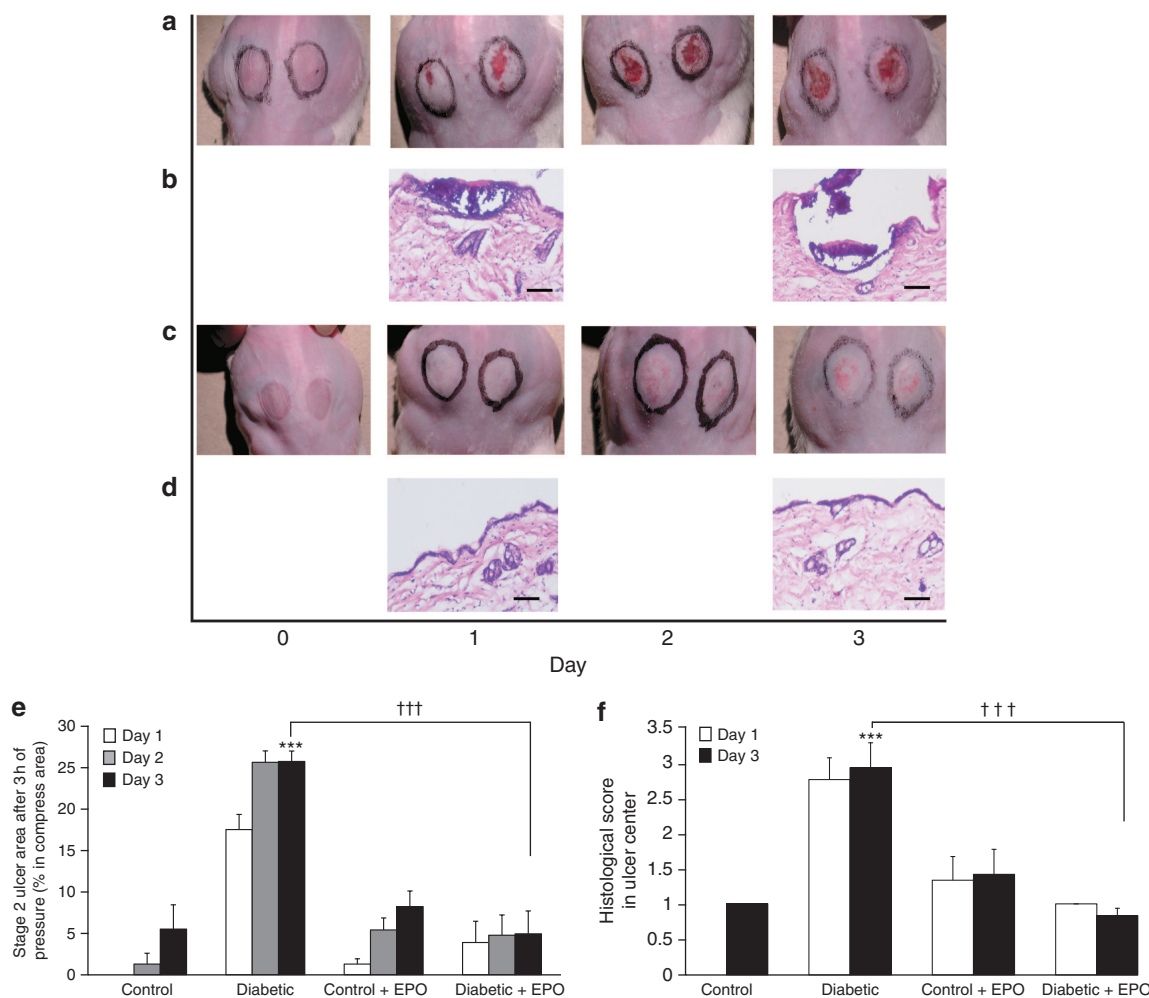
Figure 3. Effect of erythropoietin (EPO) on cutaneous macroscopic and histological findings following 3 hours of pressure. Representative photographs of skin compressed areas and central histological skin compressed sections in untreated (**a**, **b**) and in recombinant human erythropoietin (rhEPO)-treated diabetic (**c**, **d**) at days 1–3. Histological section: hematoxylin and eosin staining; original magnification $\times 100$; bar $= 100 \mu m$. (**e**) Time course of macroscopic stage 2 ulcer areas: control, $n = 13$; diabetic mice, $n = 12$; rhEPO-treated control, $n = 13$; rhEPO-treated diabetic mice, $n = 10$. ***$P < 0.001$ versus respective control mice; [†††]$P < 0.001$ versus diabetic mice. (**f**) Central histological pressure ulcer score in control and diabetic mice treated or not treated with rhEPO. ***$P < 0.001$ versus respective control mice; [†††]$P < 0.001$ versus diabetic mice; $n = 6$ in each group.

mice. These mixed results contrast with the fact that rhEPO, nonetheless, fully prevented skin ulceration in response to pressure. It is possible that the improvement in PIV, although incomplete, was nonetheless sufficient to provide diabetic skin with the ability to resist pressure. Conversely, these results may suggest that the ability of rhEPO to prevent ulcer formation is independent of PIV in our specific experimental conditions. Indeed, the idea that failure of a physiological adaptation to pressure, increasing skin blood flow, could be central to the pathogenesis of diabetic ulcers is intuitively compelling. However, it is worth noting that the pressure threshold that elicited the maximal increase in flow (0.4 kPa, i.e. 3 mm Hg) was almost three orders of magnitude lower than the pressure applied to skin with magnets (estimated about 2,000 mm Hg). Under such conditions, blood flow necessarily stopped within the compressed skin areas, whether PIV was present or not. This suggests that the protective effect of rhEPO was the consequence of its ability to

restore effective mechanisms in diabetic mice, enabling the deleterious effects of ischemia and/or reperfusion in skin cells to be overcome. However, this protective effect of rhEPO most certainly did not result from a hemodynamic mechanism, which preserved some degree of tissue perfusion during exposure to pressure.

It is nonetheless possible that rhEPO may have exerted its protective effects by increasing the hematocrit, through increasing tissue oxygenation during reperfusion, once pressure had been released. The ability of systemic administration of EPO to accelerate wound closure in various models of skin excision (Galeano *et al.*, 2004), such as burns (Galeano *et al.*, 2006) and ischemic wounds (Buemi *et al.*, 2004), has been reported repeatedly. This effect appears to be independent of hematocrit changes as the non-erythropoietic, tissue-protective derivatives, carbamylated EPO and ARA 290, are highly effective in facilitating wound healing (Erbayraktar *et al.*, 2009), and as topical administration of

rhEPO, while devoid of a systemic erythropoietic effect, improves wound repair in the skin of diabetic rats and mice (Hamed *et al.*, 2010, 2011). However, wound repair and formation of ulcers in response to pressure are clearly distinct processes, so that the beneficial mechanisms of EPO in both settings are not necessarily the same. The observed regression of diabetic neuropathy together with protection against ulcer formation with EPO is in good agreement with the well-established clinical risk of skin ulceration in pressure-exposed areas in patients with sensitive neuropathy (including diabetic neuropathy; Urbancic-Rovan, 2005). Interestingly, the common understanding is that sensory neuropathy renders the foot ''deaf and blind'' to stimuli, normally provoking pain or discomfort, therefore exposing the foot to repetitive trauma, which may then go unnoticed until ulceration occurs. However, in our experimental conditions, the mice had no way at escaping the pressure stimuli. This suggests that, in addition to impairing nociception, the alteration of skin nerve fibers may impede the normal protective response of the skin to ischemia.

However, our results do not enable us to formally conclude that there is a causal relationship between the neuroprotective effect and pressure ulcer prevention induced by rhEPO. We decided to test the hypothesis that pressure ulcer prevention in the skin could reflect the general property of rhEPO to improve tissue tolerance to ischemia–reperfusion, which has been demonstrated in numerous other organs such as the heart (Shen *et al.*, 2010), kidney (Ishii *et al.*, 2010), testis (Ergur *et al.*, 2008), and intestine (Sayan *et al.*, 2009), independently of its neurotrophic action. We therefore conducted an additional set of experiments in normal non-diabetic mice. We increased the time of skin exposure to pressure to 12 hours in order to induce skin ulcers in non-diabetic mice comparable to that induced in diabetic mice after only 3 hours. Pretreatment with rhEPO had no effect on the severity of ulcer development in non-diabetic skin. Taken together, our findings suggest that rhEPO has a specific protective effect on pressure-induced ulcers in diabetic skin only. Although consistent with the hypothesis that the protection against pressure ulcer is the consequence of the functional neuroprotection, a firm conclusion is, however, prevented by the fact that, under our experimental conditions, treatment with rhEPO normalized hematocrit in anemic diabetic mice, but unexpectedly failed to significantly increase it in control mice. Our findings thus stress the need for further studies to determine whether non-erythropoietic analogs of EPO share similar protective effects in diabetic mice.

Prevention is fundamental in avoiding diabetic foot ulcers and amputation. To date, this strategy is primarily based on identifying high-risk patients, providing education about appropriate foot care, and implementing measures to prevent ulceration such as protective footwear and podiatry. The FIELD (fenofibrate intervention and event lowering in diabetes) study has recently provided a breakthrough in amputation prevention (Rajamani *et al.*, 2009). This randomized controlled trial performed in Type 2 diabetic patients showed that patients receiving fenofibrate had a significantly reduced amputation risk compared with patients receiving placebo. The reduction was particularly substantial (hazard ratio 0.53) for minor amputation risk in patients without large-vessel disease, and was most likely explained by non-lipid mechanisms. Beyond establishing the potential benefit of fenofibrate *per se*, this cornerstone study demonstrates for the first time that reducing the risk of amputation in diabetic patients using a pharmacological approach is a reachable goal. This should encourage the search for other drugs that may also be effective, in order to go even further in amputation prevention. Our preclinical study identifies rhEPO as such a candidate drug. Much caution is, however, required before implementing a clinical study to examine whether this experimental protective effect of rhEPO translates into efficient protection in diabetic patients. Chronic use of rhEPO in non-anemic patients would expose them to an undesirable increase in hematocrit. Furthermore, the TREAT (Trial to Reduce Cardiovascular Events With Aranesp Therapy) trial recently demonstrated that even in diabetic patients with mild anemia, the deleterious side effects of rhEPO may overcome its benefits (Solomon *et al.*, 2010). This has prompted the discovery of the new derivatives that retain the tissue-protective effects of rhEPO, but are devoid of hematopoietic properties and their related side effects such as venous thromboembolisms (Hand and Brines, 2010). Our results thus strongly encourage evaluation of the therapeutical potential of these new compounds in the prevention of neuropathic diabetic ulceration.

MATERIALS AND METHODS
Male Swiss mice aged 5–6 weeks (20–30 g) were randomly assigned to four weight-matched experimental groups—untreated controls, rhEPO-treated controls, untreated diabetic mice, and rhEPO-treated diabetic mice–and maintained on a 12-h light/dark cycle with food and water available *ad libitum*. Diabetes was induced by a single intraperitoneal injection of streptozotocin (200 mg kg^{-1}; Sigma-Aldrich, Lyon, France). Hyperglycemia occurred 2 days after streptozotocin injection and was verified using an Accu-Check Active glucometer (Roche, Lyon, France). Mice included in the diabetic groups were excluded when blood glucose was <16 mmol l^{-1} 2 days after injection. Treatment with rhEPO (Roche; 3,000 UI kg^{-1} intraperitoneally, twice a week, or saline) was implemented after 6 weeks of diabetes duration for two additional weeks. Assessment of skin neurovascular alterations and exposure to pressure were performed after 8 weeks of diabetes.

The current investigation conformed to the guidelines for ethical care of experimental animals of the European Community and was approved by the French Agriculture Ministry (authorization no. B-00889).

Mechanical pressure algesia
Tail pressure thresholds were registered with the Paw/Tail Pressure Analgesia meter for the Randal–Sellito test (Bioseb, Vitrolles, France). Pressure increasing at a linear rate of 16 g s^{-1}, with a cutoff at 250 g to avoid tissue injury, was applied to the base of the tail. The applied tail pressure that evoked biting or licking behavior was registered and expressed in grams. Three tests separated by at least 15 minutes were performed for each animal, and the mean value of these tests was calculated.

Skin innervation density

Flank skin was dissected, fixed overnight in buffered 4% formaldehyde solution, embedded in Finetek Tissue-Tek Compound (Sakura, Chikuma, Japan), and then frozen at −20 °C. Sections were cut on a cryostat at 20 µm and incubated overnight with primary antibodies to PGP 9.5 (1:3,000; Chemicon, Temecula, CA), which labels all sensory and autonomic axons in skin biopsies from a variety of species, including both normal and diabetic rats and mice (Dalsgaard *et al.*, 1989; Wilkinson *et al.*, 1989). After washing, sections were incubated with secondary antibody Alexa Fluor 594 (goat anti-rabbit from Dako, Cergy Pontoise, France; 1:4,000), followed by 5 minutes of incubation with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) to stain the cell nucleus. Quantification of skin innervation density was performed using previously published protocols by Christianson *et al.* (2003). Briefly, sequential slides from each animal were stained and then coded to ensure that the observer was unaware of the identity of the animal. Fluorescent-labeled sections were viewed using a Leica microscope and analyzed by the Leica IM-500 software (Nanterre, France). Percentage of skin area (mm²) occupied by axons was measured with an image analyzer (Perfect-Image, Clara Vision, Orsay, France). Four sections were traced for each animal from all four groups.

Skin microcirculation reactivity

Hair of the animals was removed 2 days before experiments, with depilatory lotion to obtain a hairless area for skin LDF measurements, local pressure application, and iontophoretic delivery. For experiments, animals were anesthetized with thiopental sodium (65 mg kg^{-1} intraperitoneally) and then placed in an incubator (Mediprema, Tours, France) warmed to maintain a stable cutaneous temperature (35.0 ± 0.5 °C). Non-invasive blood pressure (Bionic Instruments, Tokyo, Japan) was recorded before and after experiments to verify systolic arterial blood pressure stability.

Endothelium-independent and -dependent responses.
Skin blood flow was recorded, using a laser Doppler multifiber probe (481-1; Perimed, Stockholm, Sweden), during transcutaneous iontophoresis applied to a 1.2-cm² area on the hairless back of animals. This method was described by Demiot *et al.* (2006), using cathodal sodium nitroprusside iontophoretic delivery (endothelium-independent assessment) and anodal acetylcholine iontophoretic delivery (endothelium-dependent assessment). We recorded skin blood flow baselines before endothelium-dependent and -independent response assessment. Vasodilator responses were reported as the maximal percentage increase from baseline in response to iontophoretic delivery of sodium nitroprusside and acetylcholine, respectively.

Pressure-induced vasodilation.
Skin blood flow in response to local pressure was measured by LDF. This method was described by Fromy *et al.* (2000a), using a weighbridge that was adapted to hold a laser Doppler probe at one end (PF 408, Periflux; Perimed). The probe was connected to a laser Doppler flowmeter (PF5000 Master, Periflux; Perimed). The weighbridge was carefully equilibrated, with the probe placed in the middle of the hairless skull and external pressure was increased progressively at 2.2 Pa s^{-1} through the laser Doppler probe, using a syringe pump. The LDF signal was digitized with a 20-Hz sampling frequency, using a computerized acquisition system (Biopac, Santa Barbara, CA). Data collection started with a 1-min control period before the onset of increasing pressure.

Pressure ulcer model

Pressure ulcers were created on the dorsum of mice as described by Stadler *et al.* (2004). The dorsal hair was shaved. After 24 hours, the skin was gently pulled up and placed between two round ceramic magnetic plates (10 mm diameter and 1 mm thick, with an average weight of 0.5 g and 10,000 Gauss magnetic force). Epidermis, dermis, and subcutaneous tissue layer, but not muscles, were pinched with the magnetic plates. This process created a compressive pressure of approximately 2,000 mm Hg between the two magnets. A pressure greater than 400 mm Hg has been estimated to be necessary to maintain microvascular closure in mouse dorsal skin (Tsuji *et al.*, 2005). In preliminary experiments, an exposure to pressure during 12 hours was shown to reproducibly elicit a skin ulcer in normal mice; a shorter exposure (3 hours) did not induce skin ulceration in normal mice, but was sufficient to induce an ulcer in diabetic mice.

Macroscopic and microscopic analysis of pressure ulcer

For the analysis of pressure ulcer, each compressed area was photographed using a 3.3 megapixel camera (Photo PC 3100Z; Epson, Nagano, Japan). Pressure ulcers were staged by visual assessment according to the standardized ulcer scale (Tsuji *et al.*, 2005). We delimited the compressed area and skin ulcer area percentage was calculated in the total compressed area using an image analyzer (Clara Vision, Orsay, France). After the mice were killed, six compressed tissue samples at days 1 and 3 were dissected from the flank, fixed overnight in buffered 4% formaldehyde solution, embedded in Finetek Tissue-Tek Compound, and then frozen at −20 °C. Sections of 12 µm were stained with hematoxylin and eosin. The samples were analyzed with an optical microscope (Leica). Histological examination was based on histological modification of the three skin layers and lack of tissue depth in the center of the sections (score 0: no histological modification of three skin layers; score 1: three skin layers with disruption of fibers and histological epidermis modification; score 2: epidermal defect with necrotic areas in dermis; score 3: superficial dermal defect with necrotic regions; and score 4: deep dermal defect).

Statistical analysis

Data were expressed as mean ± SE. They were first subjected to Bartlett's test for homogeneity of variances. One-way analysis of variance was followed by the Student–Newman–Keuls multiple range tests to estimate the significance of differences for between-group comparisons. Within a group (untreated, treated with rhEPO), control and diabetic mice were compared using an unpaired *t*-test. To study ulcer development, we used a paired *t*-test. Significance was defined at *P* < 0.05.

REFERENCES

Anagnostou A, Liu Z, Steiner M *et al.* (1994) Erythropoietin receptor mRNA expression in human endothelial cells. *Proc Natl Acad Sci USA* 91:3974–8

Beiswenger KK, Calcutt NA, Mizisin AP (2008) Epidermal nerve fiber quantification in the assessment of diabetic neuropathy. *Acta Histochem* 110:351–62

Beleslin-Cokic BB, Cokic VP, Yu X *et al.* (2004) Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells. *Blood* 104:2073–80

Bianchi R, Buyukakilli B, Brines M *et al.* (2004) Erythropoietin both protects from and reverses experimental diabetic neuropathy. *Proc Natl Acad Sci USA* 101:823–8

Buemi M, Galeano M, Sturiale A *et al.* (2004) Recombinant human erythropoietin stimulates angiogenesis and healing of ischemic skin wounds. *Shock* 22:169–73

Chen ZY, Wang L, Asavaritkrai P *et al.* (2010) Up-regulation of erythropoietin receptor by nitric oxide mediates hypoxia preconditioning. *J Neurosci Res* 88:3180–8

Christianson JA, Riekhof JT, Wright DE (2003) Restorative effects of neurotrophin treatment on diabetes-induced cutaneous axon loss in mice. *Exp Neurol* 179:188–99

Dalsgaard CJ, Rydh M, Haegerstrand A (1989) Cutaneous innervation in man visualized with protein gene product 9.5 (PGP 9.5) antibodies. *Histochemistry* 92:385–90

Demiot C, Tartas M, Fromy B *et al.* (2006) Aldose reductase pathway inhibition improved vascular and C-fiber functions, allowing for pressure-induced vasodilation restoration during severe diabetic neuropathy. *Diabetes* 55:1478–83

Erbayraktar Z, Erbayraktar S, Yilmaz O *et al.* (2009) Nonerythropoietic tissue protective compounds are highly effective facilitators of wound healing. *Mol Med* 15:235–41

Ergur BU, Kiray M, Pekcetin C *et al.* (2008) Protective effect of erythropoietin pretreatment in testicular ischemia–reperfusion injury in rats. *J Pediatr Surg* 43:722–8

Fromy B, Abraham P, Saumet JL (1998) Non-nociceptive capsaicin-sensitive nerve terminal stimulation allows for an original vasodilatory reflex in the human skin. *Brain Res* 811:166–8

Fromy B, Abraham P, Saumet JL (2000a) Progressive calibrated pressure device to measure cutaneous blood flow changes to external pressure strain. *Brain Res Brain Res Protoc* 5:198–203

Fromy B, Merzeau S, Abraham P *et al.* (2000b) Mechanisms of the cutaneous vasodilator response to local external pressure application in rats: involvement of CGRP, neurokinins, prostaglandins and NO. *Br J Pharmacol* 131:1161–71

Galeano M, Altavilla D, Bitto A *et al.* (2006) Recombinant human erythropoietin improves angiogenesis and wound healing in experimental burn wounds. *Crit Care Med* 34:1139–46

Galeano M, Altavilla D, Cucinotta D *et al.* (2004) Recombinant human erythropoietin stimulates angiogenesis and wound healing in the genetically diabetic mouse. *Diabetes* 53:2509–17

Hamed S, Ullmann Y, Egozi D *et al.* (2011) Fibronectin potentiates topical erythropoietin-induced wound repair in diabetic mice. *J Invest Dermatol* 131:1365–74

Hamed S, Ullmann Y, Masoud M *et al.* (2010) Topical erythropoietin promotes wound repair in diabetic rats. *J Invest Dermatol* 130:287–94

Hand CC, Brines M (2010) Promises and pitfalls in erythopoietin-mediated tissue protection: are nonerythropoietic derivatives a way forward? *J Invest Med*; e-pub ahead of print 30 July 2010

Ishii Y, Sawada T, Murakami T *et al.* (2010) Renoprotective effect of erythropoietin against ischaemia–reperfusion injury in a non-human primate model. *Nephrol Dial Transplant* 26:1157–62

Koitka A, Abraham P, Bouhanick B *et al.* (2004) Impaired pressure-induced vasodilation at the foot in young adults with type 1 diabetes. *Diabetes* 53:721–5

Leist M, Ghezzi P, Grasso G *et al.* (2004) Derivatives of erythropoietin that are tissue protective but not erythropoietic. *Science* 305:239–42

Rajamani K, Colman PG, Li LP *et al.* (2009) Effect of fenofibrate on amputation events in people with type 2 diabetes mellitus (FIELD study): a prespecified analysis of a randomised controlled trial. *Lancet* 373:1780–8

Sayan H, Ozacmak VH, Sen F *et al.* (2009) Pharmacological preconditioning with erythropoietin reduces ischemia–reperfusion injury in the small intestine of rats. *Life Sci* 84:364–71

Shen Y, Wang Y, Li D *et al.* (2010) Recombinant human erythropoietin pretreatment attenuates heart ischemia–reperfusion injury in rats by suppressing the systemic inflammatory response. *Transplant Proc* 42:1595–7

Solomon SD, Uno H, Lewis EF *et al.* (2010) Erythropoietic response and outcomes in kidney disease and type 2 diabetes. *N Engl J Med* 363:1146–55

Stadler I, Zhang RY, Oskoui P *et al.* (2004) Development of a simple, noninvasive, clinically relevant model of pressure ulcers in the mouse. *J Invest Surg* 17:221–7

Tsuji S, Ichioka S, Sekiya N *et al.* (2005) Analysis of ischemia–reperfusion injury in a microcirculatory model of pressure ulcers. *Wound Repair Regen* 13:209–15

Uehara K, Yamagishi S, Otsuki S *et al.* (2004) Effects of polyol pathway hyperactivity on protein kinase C activity, nociceptive peptide expression, and neuronal structure in dorsal root ganglia in diabetic mice. *Diabetes* 53:3239–47

Urbancic-Rovan V (2005) Causes of diabetic foot lesions. *Lancet* 366:1675–6

Vinik AI, Mehrabyan A (2004) Diabetic neuropathies. *Med Clin N Am* 88:947–99

Wilkinson KD, Lee KM, Deshpande S *et al.* (1989) The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. *Science* 246:670–3