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ORIGINAL ARTICLE

Selective blockade of endothelin-B receptor improves survival of critically perfused musculocutaneous flaps

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Abstract
Background and aims Insufficient perfusion of distal flap areas, which may lead to partial necrosis, still represents a challenge in reconstructive surgery. In the process of microvascular and endothelial dysfunction, endothelins (ETs) and their receptors may play an important role. Therefore, the aim of the study was to investigate in a chronic in vivo model the effect of various ET-receptor antagonists in critically perfused flap tissue.
Materials and methods A random pattern musculocutaneous flap was elevated in the back of 25 C57BL/6 mice and fixed into a dorsal skinfold chamber. Repetitive intravital fluorescence microscopy was performed over a 10-day observation period, assessing arteriolar diameter, arteriolar blood flow (aBF), functional capillary density (FCD), the area of tissue necrosis, and the development of newly formed blood vessels. ET-receptor blockers were administrated intraperitoneally 30 min before induction of ischemia, as well as daily during the subsequent 4-day period, including (1) BQ-123, a specific ET-A-receptor antagonist (ET-A=1 mg/kg), (2) BQ-788, a selective ET-B-receptor antagonist (ET-B=1 mg/kg), and (3) PD-142893, a nonselective ET-AB-receptor antagonist (ET-AB=0.5 mg/kg). Animals receiving saline only served as controls (n=7).
Results Despite an increase in aBF during the 10-day observation period (day 1=1.92±0.29 nl/s; day 10=4.70±1.64 nl/s), the flaps of saline-treated controls showed a distinct decrease in FCD (94±12 cm/cm^2). This perfusion failure resulted in flap necrosis of 52±3%. Selective blockade of the ET-B receptor caused a further increase in aBF already at day 1 (2.97±0.42 nl/s), which persisted during the following 10-day observation period (day 10=5.74±0.69 nl/s). Accordingly, adequate FCD could be maintained (day 10=215±8 cm/cm^2; p<0.05 vs control), resulting in a significant reduction in flap necrosis (day 10=25±4%; p<0,05). In contrast, neither selective blockade of the ET-A receptor nor nonselective ET-A- and ET-B-receptor blockade were able to significantly affect aBF when compared to controls (day 1=ET-A=1.39±0.10 nl/s; ET-AB=1.53±0.80 nl/s; n.s.). Accordingly, flap necrosis after ET-A- and ET-AB-receptor inhibition did not differ from that of controls (day 10=ET-A: 46±10%; ET-AB=51±7%).
Conclusion Our data show that only selective ET-B-receptor inhibition is capable of maintaining nutritive perfusion and, hence, reducing necrosis in critically perfused flap tissue. Accordingly, administration of ET-B-receptor antagonists may be considered in the treatment of critically perfused flaps.

Keywords Chronic ischemia · Endothelin-receptor blockade · Intravital fluorescence microscopy · Microcirculation · Random pattern flap

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Introduction

The endothelin (ET) system plays a fundamental role in cardiovascular regulation. Therefore, ET, a peptide pro-

duced in endothelial cells, has been implicated in various disease mechanisms including ischemic tissue damage in flap surgery [1–5]. Ischemic tissue damage is one of the most dreadful complications in flap surgery. It leads to delayed wound healing or loss of tissue, which both may be associated with increased morbidity, prolonged hospital stay, and an increased frequency of reoperations.

ET is one of the most potent vasoconstrictors, acting on both arteries and veins [6, 7]. The vasoconstrictor effect of ET is thought to be mediated via the ET-A receptor, but also via the ET-B receptor expressed on vascular smooth muscle cells [8–10]. In addition, ET-B receptors have been identified on endothelial cells and seem to have vasodilative properties [11–13]. Therefore, it has been postulated that ET-related vasoconstriction is predominantly mediated by the ET-A receptor, whereas the contribution of ET-B receptors seems to depend on a balance between dilative and constrictive effects.

Increased ET concentrations were found locally [4, 14–17] but not systemically [2, 3] after surgical trauma inflicted by flap elevation. Therefore, the administration of ET-receptor antagonists might be ideal to improve local flap microcirculation by vasodilation without causing systemic hypotension, detrimental to the flap's perfusion pressure. ET-A- and combined ET-A- and ET-B-receptor antagonists proved to be beneficial in the prevention of ischemic tissue damage in acute [1] and chronic [5–15] experimental settings. However, no direct examination of the effect of ET-receptor blockade on the microvasculature and, hence, the microcirculation, i.e., the target of ETs, has been performed in these previous studies. In addition, the relative contribution of the two receptor subtypes remains to be determined yet.

The aim of the present study was therefore to investigate in vivo the effect of ET-A-, ET-B-, and combined ET-A- and ET-B-receptor blockade on the development of microcirculatory dysfunction and tissue necrosis using a chronic musculocutaneous flap model. To this end, we used the previously modified dorsal skinfold chamber that includes a random pattern musculocutaneous flap, which develops a 50% necrosis if untreated [18].

Materials and methods

Animals All experiments were performed according to the guiding principles for research involving animals and the German legislation on protection of animals. The experiments were approved by the local governmental animal care committee. A total of 25 mice (C57BL/6; 12–24 weeks; 24–26 g body weight [bw]; Charles River Laboratories GmbH; Sulzfeld, Germany) were included in the study. The animals were housed in single cages at a room temperature of 22–24°C and a relative humidity of 60–65% with a 12-h day–night cycle. The animals were allowed free access to drinking water and standard laboratory chow (Altromin, Lage, Germany).

Anesthesia For surgery and repetitive intravital fluorescence microscopy, the animals were anesthetized by intraperitoneal injection of 90 mg/kg bw ketamine hydrochloride (Ketavet®, Parke Davis, Freiburg, Germany) and 25 mg/kg bw xylazine hydrochloride (Rompun®, Bayer, Leverkusen, Germany).

Flap preparation The dorsal skinfold chamber preparation was used for the experiments [19]. Ischemia in musculocutaneous tissue was induced by the creation of a random pattern flap. The inclusion of the flap into the dorsal skinfold chamber results in necrosis of ~50% of the distal tissue as previously described in detail [18]. Briefly, after depilation of the back of the animal, a random pattern flap including skin and panniculus carnosus was elevated perpendicular to the spine. To this end, two incisions perpendicular to the spine were performed, thereby creating a bipedicled flap with bilaterally transected main blood supply. The incisions are joined 2–3 mm across the spine to the intact backside of the skinfold to completely mobilize the flap. Then the flap is sutured back to the surrounding skin laterally, resulting in a rectangular, laterally based flap measuring 15 mm in width and 11 mm in length. One of two identical titanium frames of the window chamber is then fixed to the skinfold. To achieve direct view onto the deep surface of the flap, an area of ~90 mm^2—which corresponds to the window in the titanium frame—of the backside of the skinfold was removed. Finally the frame's counterpart was mounted and the observation window was sealed with a coverglass for subsequent in vivo microscopy [18]. Chambers (total weight ~3 g) were well tolerated by the animals, which showed no changes in sleeping and feeding habits.

Intravital fluorescence microscopy For in vivo microscopic analysis of the microcirculation, anesthetized mice were placed on a plexiglas pad and received an intravenous injection (retrobulbar) of 0.05 ml 5% fluorescein isothiocyanate (FITC)-labeled dextran (molecular weight 150,000; Sigma-Aldrich, Taufkirchen, Germany). Subsequently, the mice were positioned under a Zeiss Axiotech microscope (Zeiss, Oberkocchen, Germany). The epi-illumination setup included a 100-W mercury lamp and filter sets for blue (450–490 nm excitation, >520 nm emission wavelength) and ultraviolet (330–390 nm, >430 nm) light. Microscopic images were captured by a charge-coupled device video camera (FK6990, Pieper, Schwerte, Germany) and recorded on video tape (Panasonic AG-7350-SVHS, Matsushita,

Tokyo, Japan). All parameters were analyzed offline using a computer-assisted image analysis system (Cap-Image, Zeintl Software, Heidelberg, Germany) [20]. The microscopic procedures for analysis of the microcirculation were performed at constant room temperature of 23°C. Different objectives (5×, NA (numerical aperture) = 0.16; 10×, 0.30, and 20×, 0.32) were used for recordings.

Microcirculatory analysis At each observation time point, the tissue within the window of the chamber was first scanned using the 5× objective to determine the surface of nonperfused, respectively necrotic tissue. The area of necrosis, which was microscopically defined as tissue with complete lack of perfusion, was determined planimetrically and is given in percent of the total flap size.

Microcirculatory parameters were measured in the perfused areas of the flap, including the proximal, central, and distal part of the flap. For repetitive measurements of the same vessels over the 10-day observation period, easily identifiable branching patterns of second- or third-order arterioles, accompanying collecting venules, and capillary fields were selected. Using the 10× and 20× objective, video printouts of the areas described above were made to relocate the same vessels for repetitive measurements. Functional capillary density (FCD, given as cm/cm^2) was defined as the length of all red blood cell (RBC)-perfused capillaries per observation field. Arteriolar diameters (μm) were measured perpendicularly to the vessel path. Arteriolar RBC velocity (μm/s) was analyzed using the line shift method. This method is based on the measurement of the shift (mm) of an individual intravascular gray level pattern over time (seconds) [20]. Volumetric blood flow (nl/s) was calculated in arterioles and venules from RBC velocity and vessel cross-sectional area ($\pi \times r^2$) according to the equation of Gross and Aroesty, i.e., $Q = V \times \pi \times r^2$ assuming a cylindrical vessel shape [21].

All three zones of the flap were examined under 20× magnification for the development of signs of angiogenesis, such as microvascular sprouting and bud formation.

ET-receptor antagonists Three ET-receptor antagonists were used, i.e., (1) BQ-123, a specific ET-A-receptor antagonist (1 mg/kg), (2) BQ-788, a selective ET-B-receptor antagonist (1 mg/kg), and (3) PD-142893, a nonselective ET-AB-receptor antagonist (0.5 mg/kg). The antagonists were dissolved in 5% dimethyl sulfoxide. The solution was stored at a maximum temperature of 4°C.

Experimental protocol A total of 25 animals were assigned to four groups. In the control group ($n=7$), animals received the same amount of saline at the corresponding time points as the experimental groups ($n=6$ in all groups), which received intraperitoneally an ET-A-, ET-B-, or ET-AB-receptor blocker. The substances were first administered 30 min before surgery and then, to functionally block the activity of ET during the progress of necrosis, every 24 h during the following 4 days. Repetitive microscopic observations were performed at day 1, 3, 5, 7, and 10 after surgery. At the end of the experiments, the animals were killed by injection of an overdose of anesthetics.

Statistical analysis All values are expressed as mean ± standard error of the mean (SEM). For comparison between individual time points within each group, analysis of variance (ANOVA) for repeated measures was performed. This was followed by the appropriate post-hoc test, including the correction of the alpha-error according to Bonferroni probabilities. For comparison between the groups, ANOVA and the appropriate post-hoc test to compensate for multiple comparisons were used (SigmaStat, Jandel, San Rafael, CA). A value of $p<0.05$ was taken to indicate statistical significance.

Results

Flap necrosis Macroscopic analysis of the skinfold chambers at day 10 after surgery demonstrated morphological changes of the microvasculature within the zone of demarcation of the flaps (Fig. 1). A clear demarcation of vital tissue from distal necrosis, separated by a hyperemic red zone that indicates microvascular remodeling, was found. This line of demarcation was located within the center of the window of the chamber in controls, as well as in animals receiving the ET-A- and ET-AB-receptor blocker (Fig. 1a,b,d). In comparison, flaps treated with the selective ET-B-receptor antagonist developed this zone of demarcation more distally, thus presenting with reduced flap necrosis (Fig. 1c).

Quantitative analysis confirmed that in control animals, the initial microcirculatory perfusion failure at day 1 after flap elevation was associated with an area of nonperfused tissue of 44±7% (Fig. 1e). The persisting microcirculatory dysfunction resulted in necrosis of 52±3% of the flap tissue at day 10. Flap necrosis of animals pretreated with ET-A- and ET-AB-receptor antagonists did not differ from that observed in controls. In contrast, the administration of the ET-B-receptor blocker showed already at day 1 a markedly reduced microcirculatory perfusion failure (35±7% vs control=44±7%), which resulted in a significantly improved flap survival at the end of the 10-day observation period (25±4%; $p<0.05$ vs control; Fig. 1e).

Arteriolar perfusion In controls, analysis of the diameter of perfused arterioles of the flaps showed a ~25% dilation

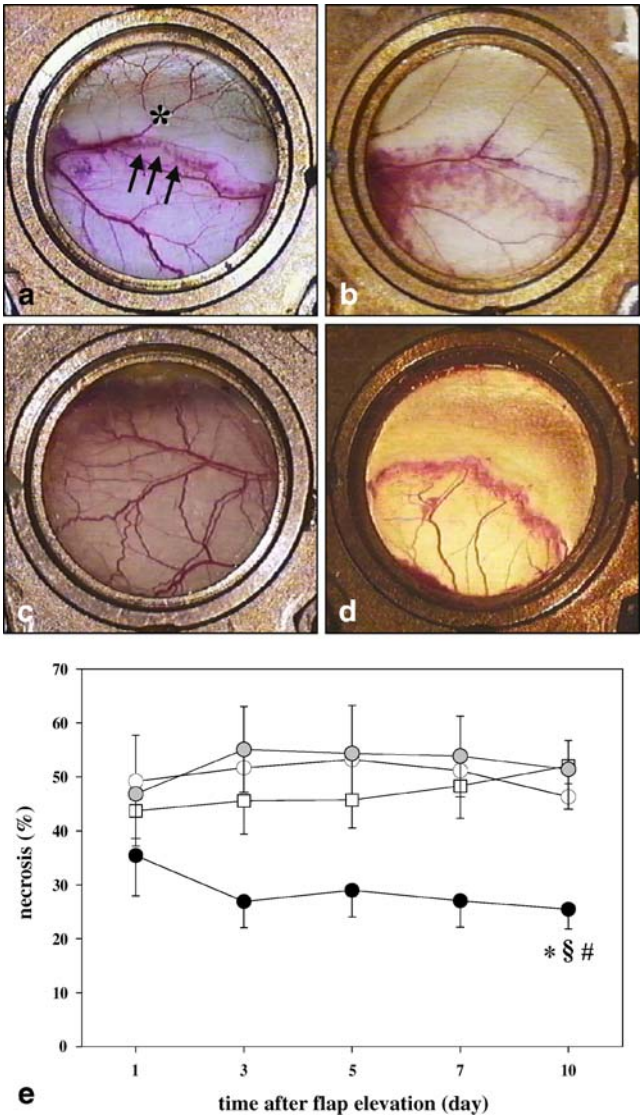

Fig. 1 Chamber tissue at day 10 after flap elevation. Sham-operated animals (**a**) and mice treated with the ET-A- (**b**) and the ET-AB-receptor antagonist (**d**) show a distinct area of demarcation including a red zone that indicates a hyperemic response and microvascular remodeling (*arrows*), separating surviving proximal tissue from distal necrosis (*asterisk*) (**a**). Flaps receiving the ET-B-receptor blocker develop this zone of demarcation more distally, indicating increased flap survival (**c**). Magnification 16×. Quantitative analysis of the area of necrosis (**e**), given in percent of the total flap area, confirmed significant protection from flap necrosis after ET-B-receptor antagonist treatment (*black circles*) when compared to controls (*white squares*) and flaps treated with the ET-A- (*white circles*) and the ET-AB-receptor antagonists (*gray circles*). Mean ± SEM; $*p<0.05$ vs control, $^{\#}p<0.05$ vs ET-A-receptor antagonist; $^{\S}p<0.05$ vs ET-AB-receptor antagonist

from day 1 to 10 after flap elevation. Treatment with the ET-A- and ET-AB-receptor blockers showed a slightly increased arteriolar diameter compared to controls at day 1 after flap creation and a comparable dilation until day 10 (Fig. 2). In contrast, ET-B-receptor antagonist-treated flaps showed significantly increased arteriolar diameters compared to controls at day 1 and a ~40% further dilation until day 10 (Fig. 2). Accordingly, arteriolar blood flow (aBF) was found to have increased during the first days after flap elevation by 1.5- to 2-fold after blockade of the ET-B receptor when compared with controls and flaps treated with ET-A- and ET-AB-receptor antagonists (Table 1).

Capillary perfusion In control animals, FCD was found reduced already at day 1 after flap elevation (149±12 cm/cm^2) and further decreased during the 10-day observation period (94±12 cm/cm^2, Figs. 3 and 4). ET-A- and ET-AB-receptor antagonist treatment could not improve capillary perfusion at day 1. However, the decrease in FCD during the following 10 days was less pronounced, resulting in slightly but significantly higher capillary density values compared to controls at day 7 and 10, respectively (Figs. 3 and 4). In contrast, ET-B-receptor antagonist blockade was effective to significantly protect capillary perfusion compared to controls already at day 1 (199±7 cm/cm^2; $p<0.05$) and to maintain this protection of nutritive perfusion over the entire 10-day observation period (215±8 cm/cm^2; $p<0.05$; Figs. 3 and 4).

Venular perfusion In all groups studied, venular blood flow (vBF) showed a progressive increase in perfusion during the 10-day observation period. However, flaps treated with the ET-B-receptor antagonist presented a massive venular outflow already at day 1, whereas ET-A- and ET-AB-

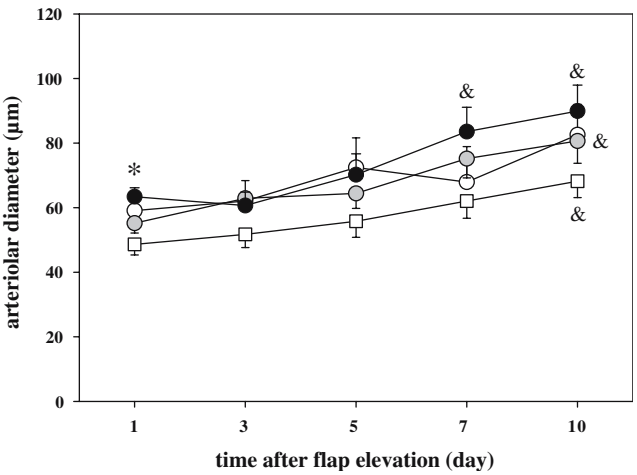

Fig. 2 Arteriolar diameters over the 10-day observation period within perfused arterioles of control flaps (*white squares*), as well as flaps treated with the ET-A- (*white circles*), the ET-B- (*black circles*), and the ET-AB-receptor antagonists (*gray circles*). Only flaps pretreated with ET-B-receptor antagonist revealed a significantly increased arteriolar dilation when compared to controls already at day 1. Over the 10-day observation period, the dilation response of ~40% was more pronounced in these animals when compared with controls as well as the ET-A- and ET-AB-receptor antagonist pretreated animals. Mean ± SEM; $*p<0.05$ vs control; $^{\&}p<0.05$ vs day 1

Table 1 Individual volumetric blood flow (nl/s) of perfused arterioles of the flaps at day 1, 3, 5, 7, and 10 after flap elevation

Group	1 days	3 days	5 days	7 days	10 days
Control	1.92±0.29	2.81±0.70	3.58±0.23	3.81±0.22	4.70±1.64
ET-A-RA	1.39±0.08	3.07±0.49	2.16±0.42	4.60±0.54*	5.76±0.61*
ET-B-RA	2.97±0.42	4.22±0.77	4.04±0.27	7.10±1.45	5.74±0.69
ET-AB-RA	1.53±0.75	1.74±1.06	3.33±0.63	4.06±0.79*	5.89±0.29*

Control (saline-treated flaps); ET-A-, ET-B-, and ET-AB-receptor antagonist (RA)-treated flaps. Values are means ± SEM
*$p<0,05$ vs day 1

receptor blockade induced a significant increase in vBF only from day 7 onwards (Table 2).

Angiogenesis In vivo microscopic imaging during the 10-day observation period did not display any morphologic changes associated with angiogenesis, such as vascular sprouting or bud formation, neither within the zone of demarcation, nor within the well-perfused proximal area of the flaps (Fig. 3).

Discussion

The major finding of the present study is that the administration of the selective ET-B-receptor antagonist BQ-788 significantly protected from flap necrosis, whereas the specific ET-A- (BQ-123) and the nonselective ET-AB-receptor (PD-142893) antagonists were not capable of influencing the manifestation of necrosis when compared with controls. Of interest, blockade of the ET-B receptor protected from nutritive perfusion failure already at day 1 after flap creation, and only ET-B-receptor inhibition could further reduce the amount of the nonperfused and necrotic tissue. In all other groups, the initially nonperfused area of the flap finally also developed necrosis. This finding emphasizes the importance of early intervention in conditions of critical ischemia.

Because ET is one of the most potent vasoconstrictors, administration of receptor antagonists should result in vasodilation within the microcirculation. In the present study, however, there was no significant vasodilation after blockade of ET receptors when compared to controls except in ET-B-receptor-blocked animals, and no significant difference occurred among the study groups. Over the 10-day observation period, there was a trend towards dilation of

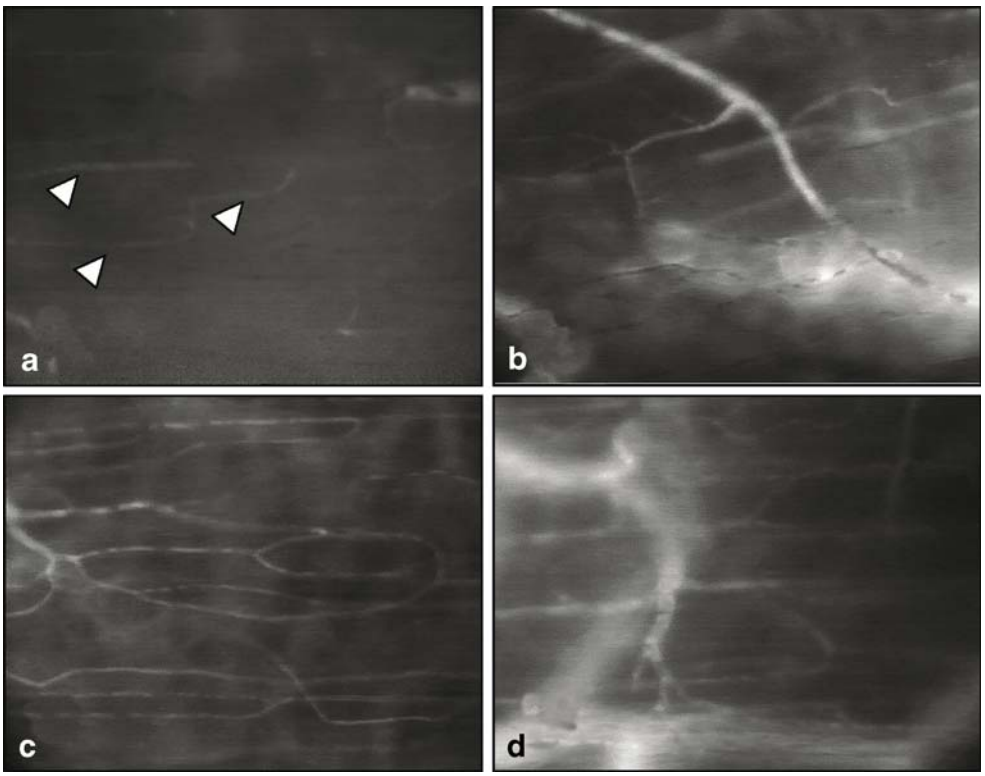

Fig. 3 Intravital fluorescence microscopy of capillary perfusion in the zone of demarcation of saline-treated controls (**a**) and animals treated with with ET-A- (**b**), the ET-B- (**c**), and the ET-AB-receptor antagonist (**d**). Note the maintained functional perfusion in capillaries pretreated with ET-B-receptor antagonist (**c**), whereas nutritive perfusion of capillaries was significantly decreased in the other groups (**a**, **b**, **d**), particularly in untreated animals (**a**, *arrow heads*) and mice receiving the ET-A-receptor antagonist (**b**). Of interest, no signs of angiogenesis, such as capillary budding and sprouting, were observed in either of the groups during the 10-day observation period. Contrast enhancement was achieved by FITC–dextran 150,000 administrated intravenously. Magnification 80×

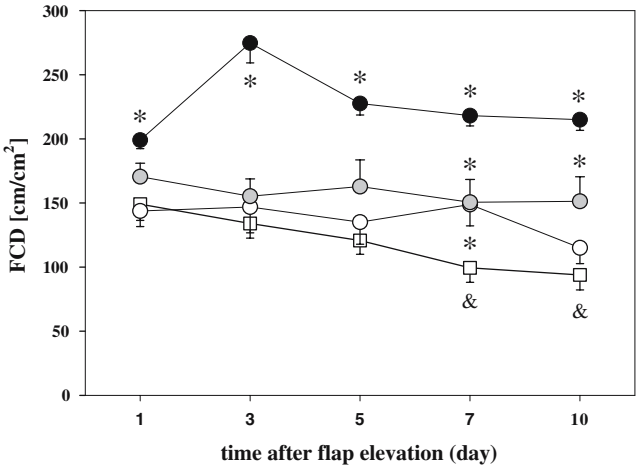

Fig. 4 Functional capillary density (*FCD*) over the 10-day observation period within perfused capillaries of control flaps (*white squares*), as well as of flaps treated with the ET-A- (*white circles*), the ET-B- (*black circles*), and the ET-AB-receptor antagonist (*gray circles*). Note the significantly higher FCD over the entire observation period in animals receiving the ET-B-receptor antagonist when compared to saline-treated controls. In contrast, ET-AB- and, in particular, ET-A-receptor blockade was not effective to significantly improve nutritive capillary perfusion. Mean ± SEM; *$p<0.05$ vs control; $^{\&}p<0.05$ vs day 1

arterioles and venules within the viable area of the flaps in all animals. This might rather be due to hypoxia, which is also a vasodilatory stimulus, or mechanisms of flow redistribution after flap elevation than due to the blockade of the ET system, because this tendency was also observed in critically ischemic tissue in controls.

The influence of ET receptor antagonists on aBF reflects the findings about arteriolar diameter to a certain extent with a generalized increase in blood flow over the observation period in all groups. After ET-B-receptor blockade, aBF at day 1 was approximately increased twofold when compared to the other study groups. This difference persisted for the first 7 days, whereas arteriolar flow in the other groups recovered only later. Accordingly, only after ET-B-receptor blockade capillary perfusion was found improved during the first days after flap elevation. In fact, the values of FCD after ET-B-receptor blockade were in the range of the physiologic values of intact tissue within dorsal skinfold chamber preparations [18, 22]. Assessment of FCD after blockade of the ET-A receptor or both the ET-A and ET-B receptor revealed that the late recovery of aBF was not anymore capable of improving capillary perfusion. At this late time point, the nonperfused area is most probably already beyond the possibilities of repair and destined to necrosis.

The present study also showed a more than twofold elevated venular drainage after ET-B-receptor blockade when compared to the other study groups at day 1. In general, the course of venous outflow paralleled the findings on the arteriolar site. In contrast to the results obtained in pigs, in which a combined ET-AB blockade in musculocutaneous flaps attenuated only venous congestion but not the microcirculation [1], improved microcirculatory values were found at all levels in the present study, including the arteriolar inflow, the capillary perfusion, and the venous outflow. This difference might be explained by the fact that laser Doppler flowmetry but not direct intravital microscopy was used to assess the microcirculation in the previously performed studies. Because venous hypertension is more detrimental to flap survival than arterial insufficiency [23] and venules show an increased constriction upon ET [7, 24], it might be argued that improvements found in venules are more important or are established more rapidly in the course of the experiment than changes in arteriolar and capillary perfusion. However, the design of the study was not intended to solve this issue. Besides venous dilation, an additional potential mechanism is a possible anti-inflammatory property of ET [25] with a decrease in leukocyte–endothelium interaction and consequent improvement of rheologic conditions in the flap.

The finding that only ET-B-receptor blockade improves flap survival is somewhat intriguing. Previous studies have shown a benefit of an ET-A-receptor antagonist in rats [5, 15] and a nonselective combined ET-receptor antagonist in pigs [1]. However, no comparison with ET-B-receptor antagonists were preformed in those experiments. Thus, our data do not allow us to draw conclusions about the reasons for the differences observed in response to blockade of the two receptor subtypes. Nevertheless, one can speculate that a difference exists in receptor affinity and half-time of the blocker used. Furthermore, the literature is

Table 2 Individual volumetric blood flow (nl/s) of perfused venules of the flaps at day 1, 3, 5, 7, and 10 after flap elevation

Group	1 days	3 days	5 days	7 days	10 days
Control	2.84±0.39	3.92±0.12	4.73±0.54	7.79±1.26*	6.84±2.35*
ET-A-RA	1.21±0.46	3.60±0.59	3.68±0.17	9.35±2.00*	8.61±2.89*
ET-B-RA	4.76±0.49	6.24±1.64	6.84±0.78	8.87±0.77	11.37±0.47
ET-AB-RA	2.00±0.98	3.47±1.89	5.37±2.27	7.78±3.21	12.10±2.42*

Control (saline-treated flaps); ET-A-, ET-B-, and ET-AB-receptor antagonist (RA)-treated flaps. Values are means ± SEM
*$p<0,05$ vs day 1

controversial about the mechanism of ET-B receptors, which were almost exclusively identified on smooth muscle cells and endothelial cells, causing constriction and dilation [26]. In addition, it should be noted that there is a pronounced heterogeneity among species, organs, and vascular units with respect to both the distribution of receptors and the response to ET [27].

Another finding is that no signs of angiogenesis were observed during the entire observation period of 10 days. ET has been implicated in tumor angiogenesis [28] and ET-receptor antagonists therefore might have abolished all stimuli of angiogenesis. However, because there was also a lack of angiogenesis in the controls, this view cannot be substantiated by the results of the present study. The major stimulus for angiogenesis, i.e., hypoxia, is present in the vicinity of the zone of demarcation. The absence of signs of angiogenesis, which should be visible after ~day 3, might be due to desiccation of the tissue within the chamber. The transitory zone of demarcation between the perfused area of the flap proximally and the necrotic area distally adjacent revealed an increased microvascular stasis, arterio-venular dilation, and hence tortuosity but no signs of angiogenesis, representing a zone of compromised blood perfusion as previously shown [22].

Conclusion

The present study demonstrates the role of ET in the pathophysiology of chronic ischemia in randomly perfused flap tissue. ET-B-receptor blockade using the ET antagonist BQ-788 increases tissue survival significantly that, if untreated, suffers from ~50% necrosis. This is attributed to an increased arteriolar, capillary, and venous perfusion beginning already at day 1 after flap elevation. On the other hand, normalized blood flow values in groups treated with ET-A- and combined ET-AB-receptor blockers were found only at 5–7 days after flap elevation, which is too late to prevent necrosis in the distal flap tissue.

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