Protective effect of the endothelin antagonist Bosentan against ischemic skeletal muscle necrosis

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We tested the hypothesis that blocking of the endothelin system by Bosentan, a combined endothelin-A and -B receptor antagonist (Hoffmann La Roche, Basel, Switzerland), improves postischemic skeletal muscle reperfusion and reduces tissue damage.

16 Wistar rats were subjected to 3 h and 15 min hindlimb tourniquet ischemia at 27 °C. Perfusion was continuously measured with Laser Doppler Flowmetry (LDF) in the anterior tibial muscle during ischemia and the first 2 h of reperfusion. Perfusion indices were calculated for each 15 min, by dividing each LDF value by the preischemic LDF value of the leg. The areas under the perfusion index curves were compared. 72h after ischemia, histomorphometry of necrosis and no-reflow zones, and counting of neutrophils were done in cross-sections of the muscles.

The animals were randomized into two groups. The treatment group received an injection of Bosentan 15 mg/kg 10 min before ischemia, and this dose was repeated 5 min before reperfusion of the hindlimbs. The treatment group obtained an improved reperfusion (4.48 vs. 1.72, p = 0.02). The median cross-sectional area of necrosis was smaller in the treatment group, 70% vs. 93% (p = 0.02), while neither the areas of no-reflow nor the neutrophil counts in reperfused necrotic areas were different.

This study supports the hypothesis that Bosentan seems to improve reperfusion and reduces postischemic skeletal muscle damage.

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Acute, transient circulatory arrest caused by thromboembolism or trauma may lead to severe skeletal muscle necrosis in the limbs. Several methods have been tested to reduce this injury, but none is in clinical use. In a rat tourniquet model developed in our laboratory (Skjeldal et al. 1994), we showed that hypothermia or a vasodilating drug may limit ischemic necrosis (Skjeldal et al. 1992, 1993).

Endothelin-1, a 21-amino acid peptide first isolated from porcine endothelial cells (Yanagisawa et al. 1988), is one of the most potent and long-lasting vasoconstrictors known. In a previous study, we found a fourfold upregulation of mRNA encoding for endothelin-1 in skeletal muscle after severe ischemia (Hvaal et al. 1998). Administered locally, exogenous endothelin-1 produces a dose-dependent constriction of skeletal muscle arterioles (Joshua 1990), and increases postischemic damage (Chang et al. 1992).

In this study, we tested the hypothesis that blocking the endothelin system by Bosentan, a combined endothelin-A and -B receptor antagonist (Clozel et al. 1994), improves postischemic perfusion and reduces tissue damage. We also investigated whether this intervention would change the no-reflow area and neutrophil infiltration.

Material and methods

16 male Wistar rats (Møllegaard, Copenhagen, Denmark) weighing 300 g were randomized into 2 groups. The animals were anesthetized with a combination of Hypnorm and Dormicum. 1 mL of the anesthetic contained 0.05 mg fentanyl, 2.5 mg fluanison and 1.25 mg midazolam. The initial dose was 2.3 mL/kg body weight s. c. followed by a continuous infusion of 1.7 mL/kg body weight per hour. Postoperatively, the animals were given

buprenorphine, 0.2 mg/kg b.w. at 12-hour intervals. The experiments conformed to the Norwegian Council of Animal Research Code for the Care and Use of Animals for Experimental Purposes.

Induction of ischemia

Complete left-sided hindlimb ischemia was induced by a tourniquet technique previously developed in our laboratory (Skjeldal et al. 1991). During the period of ischemia and reperfusion, the animals were kept in an infant incubator (Air-Shields Europe, Shannon, Ireland) at a constant temperature of 27 °C. Ischemia was maintained for 3 h 15 min. The tourniquets were removed and the hindlimbs reperfused for 72 h. The animals were killed with CO_2, and the anterior tibial muscles dissected. All animals were given 2 mL/h anesthetic, diluted in 0.9% NaCl in the carotid artery (see below) for the duration of the anesthesia, and, finally, 5 mL 0.9% NaCl intraperitoneally to prevent dehydration in the recovery period. 1 mL 0.5% lidocaine chloride was applied locally immediately before reperfusion, to prevent spasm of the vessels.

Procedure for intervention

The treatment group was given Bosentan (Ro 47-0203, 4-tert-butyl-N-(6-(2-hydroxy-etoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidine-4-yl)-benzene sulfonamide). 15 mg/kg diluted in 0.5 mL water was given through the carotid catheter 10 min before ischemia and 5 min before reperfusion. The control animals received the same volume of 0.9% NaCl solution.

Monitoring

0.7 mm Single-fiber Laser Doppler probes (Perimed AB, Järfälla, Sweden) were inserted into the center of the left and right anterior tibial muscles and fixed to the muscle fascia by tissue glue. Laser Doppler Flux (LDF) values were measured at 780 nm with Periflux 4001 units (Perimed AB, Jarfalla, Sweden) and continuously recorded during ischemia and 2 h of reperfusion. Perfusion indices were calculated for each 15 min, starting 15 min before reperfusion by dividing the LDF value by the preischemic value of the leg. Blood pressure was recorded in the carotid artery.

Histological examination

The anterior tibial muscles were dissected and fixed in 4% phosphate-buffered formaldehyde. Transverse blocks were cut through the middle of the muscles, embedded in paraffin, and 5 μm sections were cut.

Histomorphometry

Sections for measurement of necrosis were stained with hematoxylin-eosin. The total cross-sectional area of the anterior tibial muscle, the overall necrotic area and the central no-reflow zone were measured by computerized morphometry. Areas of necrosis and the no-reflow zone were calculated in per cent of the total cross-section areas, as described by Skjeldal et al. (1993). The number of neutrophils in the reperfused, necrotic areas (see below) was counted by light microscopy, using an average number per mm^2 for 40 fields of 0.625 mm^2.

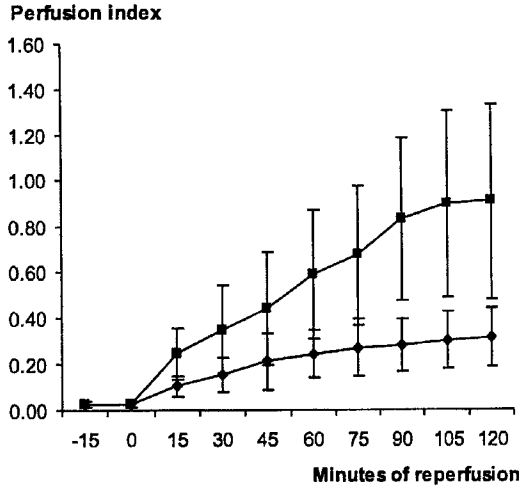
Statistics

Data from histomorphometry and the neutrophil counts were compared with the Mann-Whitney U-test. The areas under the perfusion index curves were compared with a t-test. $P < 0.05$ was considered significant.

Results

Blood pressure was unchanged during the experiments and no difference was found between the groups. Immediately after application of the tourniquets, LDF recordings decreased to a low and stable value. On reperfusion, the animals had increasing perfusion indices during the 2-h observation period (Figure). The LDF values increased more in the Bosentan group than in the control group. The areas under the perfusion index curves were higher in the treatment group, 4.48 v.s. 1.72 ($p = 0.02$).

Cross-sections of the muscles showed an outer brim of normal myocytes covering a circular zone of reperfused, necrotic cells invaded by leukocytes and macrophages. The core of the necrotic muscles was nearly acellular, representing an area of no-reflow. The number of neutrophils in the reperfused necrotic zone was similar in the two



Perfusion indices of anterior tibial muscles subjected to 3 h 15 min of ischemia and 2 h of reperfusion were calculated by dividing Laser Doppler Flowmetry values for each 15 min by the preischemic value of the leg, starting 15 min before removal of tourniquets. The areas under the index curves were larger in the Bosentan-treated animals (■) than i control animals (◆), 4.48 vs. 1.72 (p = 0.02). Error bars represent 95% confidence intervals.

groups. The median cross-sectional areas of necrosis were smaller in the treatment group, 70% vs. 93% (p = 0.02, Table), while areas of no reflow did not differ.

Areas of necrosis in cross-sections (%) from the anterior tibial muscle of rats subjected to 3 h 15 min of tourniquet ischemia and 72 h of reperfusion. Treatment with Bosentan reduced necrosis from 93% to 70% of the cross-sectional area (Mann-Whitney U-test, p = 0.02)

	Bosentan	Control
	97	74
	75	94
	38	97
	86	93
	70	76
	48	87
	56	93
	70	96
Median	70	93

Discussion

We found that treatment with Bosentan during ischemia and early reperfusion of skeletal muscle leads to improved initial reperfusion and a moderate reduction of necrosis.

Bosentan is a non-peptide endothelin antagonist with high affinity for both endothelin-A and endothelin-B receptors (Clozel et al. 1994) in vasculature of skeletal muscle (Ekelund 1994). A dose of 5–30 mg given intravenously will block the effect of endothelin-1 (Clozel et al. 1994) and protect the myocardium from ischemic injury (Filep et al. 1995, Wang et al. 1995). The substance has a half-life of 3 h. Since no data regarding Bosentan in prolonged skeletal muscle ischemia were available, a relatively high dose, 30 mg/kg in total, was chosen. In a previous study, we found upregulation of endothelin-1 mRNA to increase during early reperfusion (Hvaal et al. 1998). 15 mg/kg was therefore given twice through the carotid catheter to the aorta, before the onset of ischemia and immediately before release of the tourniquets.

The spatial variations of LDF are explained by uneven distribution of vasculature in the muscle (Kvernebo et al. 1990). Single fiber probes were positioned centrally in the muscle, where the no-reflow zone appears. The tissue volume measured is approximately 1 mm3. Because of small variations in positioning of probes and the short distance to the outer part of the muscle, peripheral areas may also have influenced the recordings. To compensate for the spatial variation, the measured postischemic LDF value was divided by the preischemic PU value from the same probe 10 min after insertion.

Upregulation of endothelin-1 during ischemia and reperfusion of skeletal muscle probably originates from striated myocytes, as we have shown in a previous study (Hvaal et al. 1998). No data are available on a possible regulation of endothelin-2 and endothelin-3 after limb ischemia. All endothelin isoforms have been found to induce endothelin-B receptor-mediated vasodilation at low concentration and prolonged endothelin-A receptor-mediated vasoconstriction at higher concentration (Ekelund 1994). Blocking of endothelin receptors by Bosentan prior to the ischemic stimulus was therefore considered to be the most efficient way of abolishing endothelin effects.

Bosentan treatment improved initial perfusion, but did not reduce areas of no-reflow. Postischemic hypoperfusion may have reduced the access of

the second dose to the muscle core, where the no-reflow zones were found. These zones appear after severe ischemic trauma, and are characterized by capillary damage. Evidence of endothelin-1-mediated capillary endothelium damage has been found in lung, possibly by neutrophil activation (Helset et al. 1996, Khimenko et al. 1996). Data from the present study suggest that endothelin-1 is a less important mediator of ischemic necrosis in skeletal muscle, compared to lung and heart.

The similar neutrophil counts in both groups of our study do not indicate that endothelins stimulate leukotaxis in skeletal muscle. Data from other tissues suggest that endothelin-1 may cause accumulation of leukocytes (Helset et al. 1996) and increase leukotoxin dependent injury (Ishizaki et al. 1996), enhance the release of cytokines (Ishizaki et al. 1996) and facilitate leukocyte adhesion to capillary endothelium (McCarron et al. 1993). Migration of leukocytes may be endothelin-B receptor-modulated (Elferink and de Koster 1996). The reduction in the necrotic areas infiltrated by leukocytes in the treatment group suggests a reduced cytotoxity of these cells, and supports the assumption that endothelin has a pro-inflammatory effect on postischemic muscle.

No drop in blood pressure occurred after removal of tourniquets in either group, suggesting that endothelins do not influence systemic blood pressure after prolonged skeletal muscle ischemia. This is consistent with unchanged, low serum levels of endothelin-1 2 h after removal of tourniquets (Hvaal et al. 1998).

Increasing concentrations of endothelin-1 have been found to produce a biphasic modulation of the vascular tone in skeletal muscle (Ekelund et al. 1995). Vasodilation at low endothelin-1 concentration, thought to result from endothelin-B receptor, is replaced by vasoconstriction by endothelin-A receptor stimulation at higher dosage (Ekelund 1994). Administration of Bosentan in the present study, blocking both receptors, led to improved perfusion throughout the observed reperfusion period. Thus vasoconstriction by endothelin-A receptors is probably dominant during initial reperfusion of skeletal muscle.

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