

Ischemia Reperfusion Injury of the Skeletal Muscle after Selective Deafferentation

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Received October 23, 2003

Accepted January 27, 2004

Summary

The present study analyzes the effect of selective deafferentation on the reperfusion injury of the skeletal muscle when nociceptive sensory fibers of the left sciatic nerve are selectively damaged by capsaicin pretreatment in a rat model following tourniquet ischemia (ISC) applied for 30 min, 1 h, and 2 h on the left hind limb. The isometric tetanic contractile force of the extensor digitorum longus (EDL) muscle was measured after 1 h, and 1, 3, or 7 days of reperfusion. Contractile force of the damaged muscle was compared to the intact contralateral muscle. In another group, ISC was used without capsaicin pre-treatment. After 30 min of ISC, there was no difference between deafferented and non-pretreated groups. Following 1 h ISC, with the exception of 1 h reperfusion, the non-pretreated group produced stronger contractions than the deafferented group. After 2 h ISC, the contractile force of the deafferented muscle was significantly stronger compared to the non-deafferented muscle force at all reperfusion times. In conclusions, it was found that the absence of peptidergic sensory fibers after long-lasting (2 h) ischemia is beneficial in reperfusion injury, whereas the absence of vasodilator peptides has unfavorable effects if tissue damage is milder (after 1 h ischemia).

Key words

Capsaicin • Rat • Sensory afferents • Muscle contraction • Neurogenic inflammation

Introduction

Neurological deficits in the limbs, in particular the absence of sensory afferent fibers, have deleterious effects on wound healing and inflammation (Kjartansson *et al.* 1987, Maggi *et al.* 1987).

Cutaneous vasodilatation has been described when the peripheral end of the cut dorsal root fibers was stimulated (Uvnäs 1954, Pintér and Szolcsányi 1988). This phenomenon was similar to the axon reflex first described by Bayliss (1901). It has been proved in the last

decades that retrograde stimulation of A- δ -mechano-heat sensitive nociceptor and C-polymodal nociceptor fibers is followed by characteristic signs of local inflammation: arteriolar vasodilatation, increased venular permeability, protein exudation, and cellular emigration (McDonald 1988, Szolcsányi 1988, Pintér and Szolcsányi 1995). Together, these phenomena are also known as neurogenic inflammation (Jancsó *et al.* 1967, Szolcsányi 1988). All these symptoms could be abolished selectively by capsaicin pretreatment (Szolcsányi *et al.* 1992). As there are inflammatory components in ischemia reperfusion

injury, we presumed that this pathological situation of the rat hind limb can be influenced by capsaicin pretreatment of the sciatic nerve.

In orthopedic surgery, two hours are the maximal normothermic ischemia time allowed. It is true that traditional histology has not provided any definite evidence of tissue destruction following 2-hour tourniquet ischemia (Rácz *et al.* 1996, Steinau 1987). However, functional changes such as reduction of the contractile force have been found under these conditions (Suzuki *et al.* 1995, Rácz *et al.* 1996, 1997, Joneschild *et al.* 1999). These studies have shown that longer ischemia leads to more serious tissue damage. In addition, the time period needed for regeneration is directly proportional to the duration of ischemia (Fish *et al.* 1989, Gardner *et al.* 1984). Our particular interest was therefore focused on the 2 h ischemia, which is the maximally allowed tourniquet time in surgical practice during leg operations.

In the present study, we examined the difference between contractile force of the extensor digitorum longus muscle of capsaicin pretreated and non-pretreated animals after tourniquet ischemia of different duration. The changes were studied up to 7 days after reperfusion.

Methods

Animals and anesthesia

Altogether 180 male Wistar rats (250-350 g) were used in the experiments in accordance with the Hungarian Law of Animal Protection. Animals were fed with rat chow and water *ad libitum*. All phases of the experiments were done under pentobarbital (Nembotal, Sanofi) anesthesia (35 mg/kg i.p. with maintenance doses

given when necessary).

Capsaicin pretreatment

Crystalline capsaicin (trans-N[4'-hydroxy-3'-methoxyl-benzyl]-8-methyl-6-nonenamid, Sigma, 50 mg) was dissolved in 0.5 ml 96 % ethanol + 0.5 ml Tween-80 (Sigma). The final volume was made up to 4 ml with distilled water. Dilutions up to 1 % from this stock solution were diluted with physiological saline. A fibrin sponge cuff (10x10x5 mm) soaked with 1 % capsaicin solution was put around the exposed sciatic nerve at a length of 10 mm in the proximal third of the left thigh. The average capsaicin solution intake of the sponge was 0.3 mg, i.e. the dose of capsaicin applied was 9-10 µg/kg. The surrounding connective tissues were protected by a polyethylene film. After 30-min exposure, the capsaicin was washed out thoroughly with saline and the incision was sutured. The sham group was prepared in the same way, but the sciatic nerve was treated only with the solvent. The procedure was similar to the method described by Jancsó *et al.* (1967) and Scott *et al.* (2000). The wound healing was uneventful. Further preparations and measurements were made 7 days after pretreatments.

Tourniquet ischemia and reperfusion

Tourniquet ischemia lasting for 30 min, and 1 or 2 h was applied after various pretreatments (Table 1). The animals were placed on a temperature-controlled pad designed for the measurements (Experimetria Ltd, Hungary). A standard A30-32 elastic rubber band was turned around three times below the hip joint. Reperfusion periods lasted for 1 h, and 1, 3, or 7 days.

Table 1. Measurement of muscle contraction force (experimental groups).

Experimental group	ischemia (h)	reperfusion	number of animals
<i>controls</i>	0	0	6
<i>capsaicin alone</i>	0	0	6
<i>I/R + vehicle (sham)</i>	1, 2	1 h, 1, 3, 7 days	2 x 4 x 3 = 24
<i>I/R non-pretreated</i>	1/2, 1, 2	1 h, 1, 3, 7 days	3 x 4 x 6 = 72
<i>I/R + capsaicin</i>	1/2, 1, 2	1 h, 1, 3, 7 days	3 x 4 x 6 = 72

I – ischemia, R – reperfusion

Contraction force measurements

Surgical preparations for contraction force measurements started either at the end of 1-hour

reperfusion or at the above indicated reperfusion times in pentobarbital anesthesia as described before. The sciatic nerves were isolated through a 1-cm long incision in the

middle third of the thigh on both sides. Each nerve was proximally ligatured and placed on a bipolar silver electrode. The muscles were indirectly stimulated through the sciatic nerve. The EDL muscles were exposed along their central parts through a 1-cm-long incision, and a silver bipolar electrode was placed on the surface of the muscle for the direct stimulation. The respective distal tendons of EDL muscle under the level of intact extensor retinaculum were also prepared. The preparation was performed carefully to avoid retinaculum injury, because the measured contraction force was much (>30-50 %) higher if the retinaculum was not intact. The skin incisions were wrapped with a plastic sheet to avoid heat losses and evaporation of the tissues. The rats were immobilized on a temperature-controlled pad in the supine position. The knee and the ankle joints were immobilized using fixed clamps. The distal ends of the tendons were attached to a strain gauge (type FSG-01, Experimetria Ltd. Hungary). The electrodes (for direct and indirect stimulations) were also attached to the pad. The muscles were preloaded (approximately 20 g) to reach their original physiological lengths.

Stimulations were carried out with a type ST-02 stimulator (Experimetria Ltd, Hungary) with an intensity double of the rheobase. Optimal parameters for supramaximal stimuli were previously determined in our laboratory: frequency 75 Hz, amplitude 9 mA, impulse width 1 ms, train duration 3 s. This pattern of stimulation elicited tetanic isometric contractions which were recorded by the Isosys computer software (Experimetria Ltd, Hungary) after amplification of the strain gauge signals. Only isometric contractions were measured. Direct and indirect stimulations were randomly applied. Both left and right sides were stimulated simultaneously. Contractile forces of the experimental EDL muscles were compared to the contractions of the contralateral side in the same animal.

Groups of experimental animals

Table 1 summarizes the different types of experimental groups indicating the time of ischemia and reperfusion and also the number of animals used. In the baseline control group, the contraction force of both sides (left and right) was compared. In the capsaicin-treated group, one side was pretreated with capsaicin and the contractions forces of the two sides were compared. In I/R non-treated group, ischemia-reperfusion (I/R) was carried out without any pretreatment, while in the I/R + vehicle (sham) and the I/R + capsaicin group vehicle and

capsaicin pretreatment were carried out before I/R, respectively (Table 1). All animals used in the experiments survived the surgical preparation procedures and the ischemia reperfusion period.

Statistical analysis

Statistical analysis was carried out by Statistica software (version 6.0) using the Anova/Manova test. Independent variables were the capsaicin pretreatment (0 or +), duration of ischemia (30 min, 1 or 2 h), duration of reperfusion (1 h, and 1, 3 or 7 days), the type of the stimulation (direct or indirect). The dependent variable was the contractile forces of the left (experimental) side expressed in percentages of the right (non-treated, contralateral) side. Tukey's HSD test was used for evaluation. The differences between the types of pretreatment (capsaicin vs. solvent or nothing) or type of stimulation (direct vs. indirect) were considered to be significant at $p < 0.05$ value. Results are expressed as mean values \pm S.E.M.

Results

Contractile force of the EDL muscle of both left and right side in the control group was 65 ± 2.5 g (above the 20 g preload) to both direct and indirect stimulations. Capsaicin pretreatment without ischemia had no effect on the contractile force of the muscles stimulated either directly or indirectly. Changes of contractile force of the EDL muscle were identical in the I/R + vehicle and the I/R non-pretreated groups.

Influence of capsaicin pretreatment and ischemia reperfusion

Thirty-min ischemia

Contractile force after direct and indirect stimulation of ischemia reperfused EDL gradually decreased maximally to around 80 % in both capsaicin pretreated and non-pretreated groups during the first 3 days of reperfusion, but became normal by the end of the first week. There were no major differences among the groups based on type of stimulation (direct or indirect).

One-hour ischemia

The directly stimulated EDL muscle in the capsaicin pretreated groups was weaker by approximately 30 % after 3 and 7 days of reperfusion, while a similar

weakness could be measured through indirect stimulation after 1 and 7 days of reperfusion.

Indirect stimulations compared to direct stimulation resulted in weaker contractions at 1 h and 3 days of reperfusion in the non-treated group, or 1 and 3 days of reperfusion in the capsaicin pre-treated groups, respectively.

Two-hour ischemia

The contractile force of the EDL muscle of capsaicin pretreated groups was significantly stronger

with the exception of the direct stimulation at 1 day of reperfusion. The indirectly stimulated muscles compared to those directly stimulated, contracted more weakly at all reperfusion times in the non-pretreated groups, while similar results were observed in the capsaicin pretreated group only on the third day of reperfusion.

The contraction force of the EDL muscle in non-pretreated and capsaicin pretreated animals after 30 min to 2 h of ischemia and 1 h to 7 days of reperfusion are summarized in Table 2.

Table 2. Contraction force of the EDL muscle: the left, affected I/R side in percentage of the contralateral, non-ischemic right side.

		Duration of ischemia (h)		Pretreatment		
Duration of reperfusion				1 h	1 day	
Type of stimulation		direct	indirect	direct	indirect	
1/2	capsaicin 0	97 ± 0.2	96 ± 0.3	91 ± 0.6	87 ± 0.3	
	capsaicin +	96 ± 2.0	93 ± 1.4	88 ± 1.6	97 ± 1.3* [#]	
	1	capsaicin 0	71 ± 4.3	50 ± 4.6 [#]	65 ± 3.6	64 ± 4.1
		capsaicin +	71 ± 1.5	66 ± 2.7	67 ± 1.7	44 ± 1.7* [#]
	2	capsaicin 0	6 ± 1.7	1 ± 0.2 [#]	11 ± 1.1	1 ± 0.3 [#]
		capsaicin +	22 ± 1.3*	24 ± 2.6*	15 ± 1.9	16 ± 1.8*
Duration of reperfusion		3 days		7 days		
Type of stimulation		direct	indirect	direct	indirect	
1/2	capsaicin 0	85 ± 1.6	79 ± 3.0	97 ± 0.2	97 ± 0.5	
	capsaicin +	81 ± 1.7	83 ± 1.2	92 ± 0.9*	96 ± 1.2 [#]	
1	capsaicin 0	57 ± 2.3	32 ± 3.9 [#]	51 ± 3.2	51 ± 3.1	
	capsaicin +	42 ± 2.3*	30 ± 1.2 [#]	31 ± 1.6*	32 ± 1.5*	
2	capsaicin 0	16 ± 1.2	0 ± 0 [#]	11 ± 1.5	2 ± 0.3 [#]	
	capsaicin +	28 ± 1.7*	14 ± 1.0* [#]	38 ± 4.3*	35 ± 2.8*	

Capsaicin and/or I/R were applied to the left hind limb. Values represent the means ± S.E.M. of contraction forces of the left, affected EDL muscle, expressed in the percent of the non-treated, non-ischemic contralateral right side. capsaicin 0: I/R on non-pretreated animals, capsaicin +: I/R on capsaicin-pretreated animals, [#] significant differences (p<0.05) between direct and indirect stimulation, * significant differences (p<0.05) between capsaicin-pretreated and non-pretreated animals.

Discussion

To our knowledge, the effect of capsaicin pretreatment on the contractile force of skeletal muscle under physiological conditions and in reperfusion injury has been reported for the first time in this study.

There are data in the literature about the contralateral effects of unilateral hind limb ischemia or denervation (Hickey *et al.* 1993, Liu *et al.* 1999, Scott *et al.* 2000). However, we did not find any contralateral effect of ischemia-reperfusion on muscle contraction force in our experiments. In the studies mentioned, the

ischemia time was always longer, generally 4 h, which is significantly longer than 2-h ischemia applied in our experiments. Reperfusion injury following such long ischemia duration has already systemic consequences that could result in contralateral effects.

We investigated the effect of combined tissue injury on the neuromuscular functional unit that is known as a sensitive indicator of ischemia-reperfusion injury (Pórszász and Szolcsányi 1994, Rácz *et al.* 1996, 1997). Numerous structural and functional changes of the muscle tissue have been reported after ischemia and reperfusion injury (Patterson and Klenerman 1979, Gardner *et al.* 1984, Korthals *et al.* 1985, Chervu *et al.* 1989, Fish *et al.* 1989, Heppenstall *et al.* 1989, Suzuki *et al.* 1995, Tuncel *et al.* 1997), but it is difficult to differentiate between neurogenic, synaptogenic, and myogenic lesions during reperfusion.

The effect of ischemia-reperfusion injury on the contraction force is more pronounced after indirect than direct stimulation of the EDL muscle as we have found that the contraction force of the indirectly stimulated muscles was lower compared to the direct stimulation. This result is supported by our earlier results showing that the neuromuscular junction was seriously damaged by 2 h ischemia and 1 day of reperfusion, while the regeneration was evident at day 7, and was nearly complete by day 28 of reperfusion (Tömböl *et al.* 2002). As there is no histological evidence of nerve injury after 1 or 2 h of ischemia, it is probable that the damage of synapses can be responsible for the difference (Patterson and Klenerman 1979, Korthals *et al.* 1985, Tömböl *et al.* 2002). After 30 min of ischemia, the contractile force decreased to 80-85 % of the contralateral intact side, but there were no obvious differences between the directly or indirectly stimulated muscles, suggesting that the ischemia tolerance of the synapses lasts for about 30 min.

Reperfusion injury develops gradually. Following ischemia, oxygen free radicals play a major role at early periods of reperfusion injury (Suzuki *et al.* 1995, Tuncel *et al.* 1997, Hamar *et al.* 2003). The inflammatory reaction and a series of inflammatory mediators act later. The immune cells become activated during reoxygenation, and these reactions lead to secondary tissue damages (Steinau 1987).

Afferent fibers sensitive to capsaicin treatment release neuropeptides (like calcitonin gene-related peptide (CGRP) and tachykinines) responsible for the neurogenic inflammation (Szolcsányi 1996a). CGRP, for instance, is a strong vasodilator and substance P,

a tachykinine, can increase vascular exudation. Stimulation of these fibers with different frequencies can lead to different vascular effects. Depending on the type of stimulation vasodilatation only, or extravasation, with or without neurogenic inflammation, can be provoked (Szolcsányi 1996b). These peptides are responsible for increased microcirculation, which can be beneficial for tissue repair, whereas an overstimulation of the neural endings can lead to an uncontrolled inflammatory reaction. Inflammation also produces H⁺ ions, which are strong stimuli for the vanilloid receptors of the afferent fibers (Szolcsányi *et al.* 1992). CGRP is one of the responsible factors for increased microcirculation after skeletal muscle contraction (Yamada *et al.* 1997), but the absence of peptidergic fibers without ischemia did not affect muscle contraction force in our studies. The role of CGRP in post-occlusive reactive hyperemia is not documented clearly (Franco-Cereceda *et al.* 1989).

Capsaicin has powerful actions on peripheral sensory nerves, mainly on C and A δ fibers (Vyklícký and Knotková-Urbancová 1996, Vlachová and Vyklícký, 1993). The effects depend on the doses and the route of administration. Capsaicin (2 mg/kg intravenously or 50 mg/kg subcutaneously) has systemic effects, and measurable levels of capsaicin can be detected in the brain, but there is no evidence that capsaicin can affect the motor system (Fitzgerald 1983). As we used capsaicin topically and the dose was 10 μ g/kg, we assumed that the systemic and contralateral effects of capsaicin were negligible.

Only a moderate functional loss develops after 30 min of tourniquet ischemia. The effect of 30-min ischemia and 1-h reperfusion is quite negligible, but after one and 3 days of reperfusion, a mild but significant functional loss could be measured. The inflammatory component of reperfusion injury could be responsible for the muscle weakness at these times. We have no explanation for the bigger contraction force of the selectively denervated and indirectly stimulated EDL after 30 min of ischemia and one-day reperfusion.

If ischemia lasted for one hour, capsaicin pretreatment had generally a deleterious effect compared to the non-treated controls. This difference can be attributed to the absence of vasodilator peptides (most probable CGRP) which increase the microcirculation. On the other hand, shorter ischemia also produces a lesser inflammatory reaction compared to the group with 2 h of ischemia and the reduced microcirculation results in weaker contractions.

The ischemic-reperfused muscle performed much better after 2 h of ischemia following capsaicin pretreatment. In the absence of peptidergic fibers, the postischemic (neurogenic) inflammation in the muscles and nerves could not develop. This is thought to be one possible reason for the smaller functional loss, i.e. the stronger contraction at reperfusion in the capsaicin pretreated groups.

Acknowledgement

This work was supported by the Hungarian National Research Fond (OTKA N° 29561 and 42901). The scientific guidance and useful consultations with Dr. J. Szolcsányi throughout the studies and the preparation of the manuscript are gratefully acknowledged.

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