Structural Alterations of Skeletal Muscle Induced by Ischemia and Reperfusion

Hans J. Appell¹, ², Sabine Glöser², José M.C. Soares¹, ³ and José A. Duarte¹, ³

(1) The Muscle Atrophy Research Group (MARG), (2) Institute of Sport Orthopedics, German Sport University Cologne, Germany and (3) Department of Sport Biology, FCDEF, University of Porto, Portugal

Abstract
The purpose of the study was to undertake a series of experiments with varying time periods of ischemia followed by reperfusion to investigate the effects on muscle morphology. Charles River mice received a tourniquet at their right limb for 60, 90, and 120 minutes, respectively, under general anesthesia which was followed by a reperfusion period of 60 minutes (n = 5 in each group). Their soleus muscles were removed, the left one serving as contralateral control, and processed for light and electron microscopic examination. Muscle fiber cross sectional areas and the thickness of the capillaries basement membranes were evaluated morphometrically. Capillary endothelial ultrastructure showed considerable pathological alterations extending to intracellular edema, and the basement membrane was thickened by about 50%. The muscle fibers were reduced in size, which depended on the period of ischemia (15-23%). Skeletal muscle morphology showed mitochondrial disturbances, condensation of contractile material, presence of lysosomes, and focal fiber necrosis, which also seemed to be aggravated the longer the ischemic period had lasted. The interstitial space was widened speaking in favor of an intramuscular edema. It is concluded that skeletal muscle morphology is severely affected under conditions of ischemia/reperfusion and that the capillary endothelium is mainly involved in the pathophysiological mechanisms.

Key words: capillary endothelium, ischemia, muscle damage, reperfusion, skeletal muscle.

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When orthopedic surgeons administer a tourniquet allowing for surgery under bloodless conditions, the respective muscles suffer from ischemia and following reperfusion (I/R). This harmful situation has been studied predominantly with regard to functional [13, 17, 26] and metabolic [1, 6, 7, 8, 19, 27] alterations in muscle or focusing on the vascular bed [4, 9, 23, 28, 29, 32]. I/R is associated with capillary dysfunction that leads to an increased permeability during reperfusion thus allowing proteins to escape from the vascular bed; consequently the muscles become edematous [8, 9, 16]. These effects most probably originate from oxidative stress during reperfusion that is being triggered during the ischemic period by conversion of the endothelial enzyme xanthine dehydrogenase to xanthine oxidase [12, 22, 30].

Compared to other tissues, like cardiac and cerebral tissues, skeletal muscle has been described to be relatively resistant to ischemia because the maintenance of its metabolic capacity is assumed to cease only after five to seven hours [7, 15, 19, 27]. However, an ultrastructural study on biopsies obtained during surgery, i.e. after various periods (15 min to 90 min) of ischemia, but without reperfusion, clearly pointed out that already under such conditions skeletal muscle ultrastructure showed eventual pathological alterations, especially extending to metabolically important organelles [2]. It can be expected that these alterations will be aggravated during reperfusion. However, more recent studies dealing with the effects of I/R on skeletal muscle morphology did not bring about very detailed informations [10, 11, 24, 26, 31].

Since the harmful effects of reperfusion appear to be preconditioned during the ischemic phase, as outlined above, more reperfusion damage may be expected with prolonged periods of ischemia. The aim of the present set of experiments was to study various periods of ischemia (60, 90, 120 min) each followed by a 60 min period of reperfusion and to morphologically describe
skeletal muscle reactions. Moreover, some morphometric data on muscle fiber diameters and capillary wall architecture may shed some light on the role of edema development during I/R resembling a kind of compartment syndrome [9].

Materials and Methods
The experiments were performed on Charles-River mice (aged 8 weeks with a body weight of 35-40 g) under standardized conditions with regard to housing environment and feeding and following the institutional guidelines for the care and use of laboratory animals. Three groups of mice (n = 5) received a tourniquet (rubber cuff) at the proximal part of their right hindlimb taking several precautions into consideration [25] for 60, 90, or 120 minutes; respectively, and these periods of ischemia were followed by a 60 min. reperfusion period after removal of the cuff. All experiments were done under sodium-pentobarbital anesthesia (i.p. 50 mg/kg b.w.). One group of mice (n = 5) without any experimental intervention served as the overall control group. The animals were finally sacrificed by cervical dislocation after both soleus muscles had been completely removed, the right one serving as experimental and the left one as intragroup control muscle.

Each muscle was divided into three pieces maintaining constant muscle length and these were transferred to 2.5% glutaraldehyde for two hours. The specimen were postfixed with 1% osmiumtetroxide, dehydrated in graded alcohol, contrasted with 0.5% uranylacetate, and embedded in Epon. Semithin sections for light microscopy (Leitz) were stained with toluidine blue, ultrathin sections for electron microscopy (Zeiss EM 10A) were contrasted with 0.2% lead citrate.

Morphometry was performed using the MOP Videoplan (Kontron). From every specimen, at least 300 fibers were measured for fiber area in cross section at a light microscopic magnification of x250. To ensure the evaluation of proper cross sections, two fiber diameters were also measured (Feret X, Feret Y); the area measurements were only considered for further calculation, if Feret X and Feret Y did not differ more than 10%. Electron micrographs from cross sectioned capillaries (20 capillaries per sample) were used for estimation of the thickness of the vascular basement membrane at 4 randomly chosen capillary wall sites at primary magnifications of x10,000.

All quantitative data were expressed as means with standard deviations after transformation into percentages from controls. For statistical analysis of percentage differences from controls the Student t-test was applied. For differences between the groups, the one-way ANOVA for repeated measurements was used with post hoc comparison using the Scheffe F-test. The level of significance was set at an alpha of 5%.

Results
Regarding the morphometric data, there were no differences between the overall controls and the contralateral controls of the respective experimental groups. The quantitative data for muscle fiber cross sectional area and capillary basement membrane thickness are shown in Table 1.

The fiber cross sectional areas underwent a progressive reduction with prolonged periods of ischemia. The small standard deviations speak in favor of a homogeneous fiber size as well as a homogeneous reaction of the whole fiber population. This reduction amounted to 15 to 23% of the control fiber size. Basement membrane thickness of the capillary wall increased by more than half, with some tendency for more thickening with prolonged experimental periods, which, however, could not be substantiated statistically.

The microscopic specimens of the overall controls and all contralateral control muscles completely showed a normal appearance which also holds true for the microvascular bed. However, the capillary ultrastructure was conspicuously altered in all experimental groups. Beyond the thickening of the endothelial basement membrane (Tab. 1), the endothelial cells appeared swollen and contained large vacuoles resembling confluent vesicles. In some cases, the endothelial cells contained large edematous blebs (Fig. 1). They sometimes showed pseudopodia-like flaps protruding into the lumen. The capillary lumen frequently appeared narrowed or collapsed, and fenestrations of the (normally) continuous endothelium occurred (Fig. 2). Even endothelial gaps or breaks could be observed. These alterations were already present in the 60 I / 60 R group and did not seem to increase in severity or frequency with prolonged periods of ischemia.

The pathological alterations of the muscle fibers appeared to become more severe the longer the muscles had been ischemic, as far as could be judged by the frequency and conspicuousness of their occurrence. Some muscle fibers showed a moderate intrafiber edema, which, however, stood back against an edematous widening of the interstitial space (Figs. 3, 4, 5, 6). This in-

Table 1. Means and standard deviations of the percentage changes as compared to the contralateral controls (= 100) for muscle fiber cross sectional areas and capillary basement membrane thickness after varying periods (min) of ischemia (I) and 60 min reperfusion (R). Significant differences: § vs. contralateral control, # vs. 60 I / 60 R, + vs. 90 I / 60 R.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Fiber Area</th>
<th>Basement Membrane Thickness</th>
</tr>
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<tbody>
<tr>
<td>60 I / 60 R</td>
<td>85.1 ± 1.1 §</td>
<td>152.2 ± 9.3 §</td>
</tr>
<tr>
<td>90 I / 60 R</td>
<td>79.5 ± 2.3 #</td>
<td>162.6 ± 9.6 §</td>
</tr>
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</table>
Muscle morphology after ischemia/reperfusion

Interstitial edema seemed to become more pronounced with longer periods of ischemia. The muscle fibers of the 60 I / 60 R group showed a light vacuolization mostly in their peripheral parts that originated from destructed mitochondria and contained some lysosomes. Moreover, residual bodies were frequently found with prolonged periods of ischemia (Fig. 3). Some fibers appeared necrotic with a complete disorganization of their internal structures or showed peripherally condensed contractile material while the fiber center was filled with structureless precipitates (Fig. 4). In the 90 I / 60 R group, such was encountered frequently with various patterns of complete internal destruction of the fibers (Fig. 5). With longer periods of ischemia (120 I / 60 R), even more necrotic fibers were found (Fig. 6) contain-

Figure 1. Electron micrograph (90 I / 60 R) of a capillary with intraendothelial edematous blebs; bar represents 2 µm.

Figure 2. Electron micrograph (60 I / 60 R) of a narrowed capillary with fenestrated endothelium (insert) surrounded by a thickened basement membrane; bars represent 2 µm and 0.5 µm in the insert.

Figure 3. Electron micrograph from a 60 I / 60 R muscle with wide interstitial space and a lysosome in the muscle fiber, the insert shows residual bodies from a 120 I / 60 R muscle; bars represent 2 µm.

Figure 4. Light micrograph (60 I / 60 R, above) with two severely altered muscle fibers and interstitial edema; an electron micrograph of the marked portion is shown below; bars represent 50 µm (LM) and 5 µm (EM).
Muscle morphology after ischemia/reperfusion

Discussion

In accordance with current metabolic concepts on ischemia/reperfusion [8, 12, 22, 30] the blood capillaries are among the first structures to suffer from oxidative stress during reperfusion which at least partly should be derived from xanthine oxidase. Already during short periods (15 min.) of ischemia (without reperfusion), capillary obstruction and endothelial swelling has been described [2]. This observation may be attributed to alterations in ionic exchanges across the endothelial membrane [20], which is assumed to be a major cause for microcirculatory failure during postischemic reperfusion [23]. Endothelial pathology is further manifested by an impaired structural integrity of the microvascular bed. Especially the occurrence of fenestrated or gap capillaries is most uncommon in skeletal muscle [14]. Such results in an escape of proteins and other macromolecules from the bloodstream into the interstitial space. The enlargement of the endothelial basement membrane might be interpreted as a (yet obsolete) defense mechanism of the capillaries against increased extravascular pressure [14], but should rather be looked at as the morphological correlate of functionally puffed constituents of the basement membrane due to extravascular water accumulation. Macromolecular leakage has mainly been held responsible for the I/R tissue injury.
Muscle morphology after ischemia/reperfusion

[16] and results in an increased osmotic pressure in the interstitial space that is being aggravated during reperfusion with an edematous water accumulation. Especially for muscles contained in a tight fascial compartment, such will result in a compartment syndrome [9]. Such, together with the “no reflow” phenomenon, may lead to secondary ischemia during reperfusion [23].

These considerations receive support from earlier studies using the same experimental protocol as present [8] where the muscle wet weight increased by up to 90% and the protein content of muscle increased by up to 40%. This is morphologically reflected by an edematous enlargement of the interstitial space and by the morphometric muscle fiber data. It could have been expected that the muscle fibers, like endothelial cells, increased in volume because of homeostatic disturbances leading to intracellular edema. This mechanism cannot be ruled out, but is most probably masked by the higher osmotic pressure in the interstitial space forcing water to escape from the muscle fibers. Consequently, the cross sectional areas of the muscle fibers were reduced, and this effect was increasing with the period of ischemia. This finding correlates with earlier observations on the intensity of oxidative stress and muscle protein content as a temporal function of the ischemic period [8].

Since the formation of reactive oxygen species during ischemia and reperfusion is assumed to play a major role, several attempts have been made to reduce I/R damage by administration of antioxidants or inhibitors of xanthine oxidase (XO). The efficacy of allopurinol as an XO inhibitor has been controversially reported in the literature. While some experiments showed a protective effect against oxidative stress and against muscle damage [1, 10], another study did not show major protective effects [18]. Vitamin E as a radical scavenger, irrespective of the origin of reactive oxygen species, has been successfully used in various I/R experiments [1, 11, 24]. In a series of experiments with the same experimental protocol, administration of Vitamin E did not only prevent the muscles from oxidative stress, but especially from endothelial damage, intramuscular edema, and major muscle fiber damage [1].

Although the present pathological alterations finally entering into fiber necrosis have not been quantified for methodological reasons, it appears that the longer the ischemic period had lasted the more the muscle fibers were affected. Mitochondrial destruction, condensation of the contractile material, and occurrence of lysosomes probably represent a pathophysiological cascade. The formation of reactive oxygen species, increasing with time of ischemia [8] also affects the muscle fibers and especially their sarclemmal and mitochondrial membrane systems [24]. Consequently, an impaired sarclemmal integrity facilitates an influx of calcium ions into the muscle fibers leading to a loss of calcium homeostasis; this promotes the condensation of contractile material and mitochondrial disturbances and also activates the lysosomal system entering into an autolytic process [3, 21].

It seems, however, surprising that under the experimental conditions not all muscle fibers underwent the described signs of pathological alterations and necrosis to the same or a comparable extent. Similar phenomena hold true for other types of muscle damage induced by aggressive experimental protocols. In accordance with recent concepts [5], the observed cellular damage may have occurred only in an especially susceptible fiber population. In this sense, the homeostatic disturbances cannot sufficiently be tolerated by those fibers, while other fibers obviously are more resistant. It remains unclear whether fiber necrosis represents a direct effect of the above described mechanisms induced by ischemia and reperfusion or whether some fibers enter into a programmed cell death (apoptosis) under such circumstances.

Address correspondence to:

Prof. Dr. Hans-Joachim Appell, Institute of Sport Orthopedics, German Sport University, D-50927 Cologne, Germany, phone +49 221 4982543, fax +49 221 4912001, Email appell@hrz.dshs-koeln.de.

References


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