# Effects of a hydroxyl radical scavenger, EPC-K1, and neutrophil depletion on reperfusion injury in rat skeletal muscle

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**ABSTRACT – Oxygen free radicals (OFR) and neutrophils are potent sources of reperfusion injury. We compared the effect of EPC-K1, a new OFR scavenger, and neutrophil depletion on the reperfusion injury in skeletal muscle, using an ischemic revascularized hindlimb model in rats. Warm ischemia, produced by vascular pedicle clamping, was sustained for 4 h. After 24 h of reperfusion, muscle function and damage were evaluated in 4 groups: a sham operation group, a control study group, a group treated by EPC-K1 (EPC group), and a group that received nitrogen mustard to induce neutropenia (NM group). Both the EPC and NM groups had limited muscle damage compared to the control group. The EPC group preserved muscle function significantly better than the control group and the mean isometric tetanic tension in the EPC group appeared to be higher than that in the NM group. Furthermore, levels of lipid peroxides in muscle and serum, and muscle edema in the EPC group, were significantly lower than in the NM group. Histological examinations supported these results. These findings suggest that limiting OFR generation by EPC-K1 in the early phase of reoxygenation is more potent than depletion of neutrophils in reducing reperfusion injury.**

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Postischemic reperfusion injury has many causes. With the reintroduction of molecular oxygen to tissue subjected to an ischemic insult, oxygen free radicals (OFR) are formed, proinflammatory agents are produced, and circulating neutrophils accumulate (Rubin et al. 1996, Homer-Vanniasinkam et al. 1997, Gute et al. 1998). These mediators then combine through an intricate series of interactions, resulting in endothelial cell dysfunction and parenchymal cell damage.

A growing body of evidence has recently implicated neutrophils in the pathogenesis of this reperfusion injury. The finding that neutrophils are activated and accumulate in the postischemic tissue in proportion to the severity of the reperfusion injury also suggests their involvement. Various potential mechanisms for neutrophil-induced injury have been proposed, including OFR generation, capillary plugging, and the release of proteolytic enzymes (Schmid-Schönbein 1987, Weiss 1989). Neutrophil adhesion to the vascular endothelium significantly increases after 30 min of reperfusion (Ferrante et al. 1996), with maximum infiltration into muscle tissue after 4 h (Seekamp et al. 1993).

By contrast, an earlier burst of OFR formation occurs in the 2–5 min immediately after reperfusion (Kurose and Granger 1994). These OFR initiate lipid peroxidation of cell membranes, which causes cellular dysfunction and further tissue necrosis (Girotti and Thomas 1984). The possible sources of OFR include xanthine oxidase, the mitochondrial electron transport chain, arachidonic acid metabolism, and activated neutrophils (Weiss 1989, Rubin et al. 1996, Homer-Vanniasinkam et al. 1997, Gute et al. 1998). Neutrophils may therefore contribute little to OFR generation immediately after reoxygenation, since their adhesion to the vascular endothelium and their infiltration into skeletal muscle are relatively slower than the immediate burst of OFR production. In the present study, we sought to test whether suppressing OFR production immedi-

ately after reoxygenation would be more effective than depletion of neutrophils to reduce the ischemia-reperfusion injury.

We used a new compound, L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2H-1-benzopyran-6-yl hydrogen phosphate] potassium salt (EPC-K1, Senju Pharmaceutical Co., Ltd., Osaka, Japan), as an OFR scavenger. This has been said to have potent hydroxyl radical scavenging activity (Mori et al. 1989) and to inhibit phospholipase A2 activity (Kuribayashi et al. 1992). The effects of EPC-K1 on reperfusion injury have been reported in brain (Block et al. 1995), heart (Tanemoto et al. 1993), liver (Yagi et al. 1997), and skeletal muscle (Hirose et al. 1997, 1999).

## Animals and methods

### *Animal preparation*

Male Lewis rats, weighing 250–300 g, were used in these studies according to the Guide for Care and Use of Laboratory Animals (DHEW Publication No. (NIH) 78–23, revised 1978) and local guidelines for humane use of animals in research.

They were anesthetized intraperitoneally with sodium pentobarbital (45 mg/kg), and a surgical plane of anesthesia was maintained, with additional doses of intraperitoneal pentobarbital (10 mg/kg). All procedures were performed with the animals breathing spontaneously. The left thigh was amputated with transection of the muscles and femur at the midthigh level but not the femoral artery and vein. The vessels were completely occluded with a microvascular clip for 4 h at room temperature (25 °C). The clip was then released and vessel patency was confirmed by visual inspection. Next, the osteotomy was repaired, using an 18-gauge (1.27 mm diameter) injection needle as an intramedullary nail, and muscle group and skin were reapproximated. After 24 h of reperfusion, the rats were reanesthetized and skeletal muscle function and damage were evaluated in the following groups.

### *Experimental groups*

32 rats were randomized into each of the following 4 groups:

1. Sham group, in which rats underwent only the surgical amputation/repair and were not subjected to ischemia (n 8),

2. Control group, in which rats underwent surgical amputation, ischemia and repair (n 8),

3. EPC group, the OFR scavenger treatment group, in which rats were given 10 mg/kg of EPC-K1, a potent hydroxyl radical scavenger, intravenously 30 min before ischemia (Hirose et al. 1997) (n 8),

4. NM group, the leukocyte-depleted group, in which rats were given 1.75 mg/kg of nitrogen mustard (Sigma Chemical Co., St. Louis, MO, U.S.A.) intravenously 48 h before ischemia (Müller-Berghaus and Eckhardt 1975, Taoka et al. 1997) (n 8).

EPC-K1 and nitrogen mustard were administered intravenously through the caudal vein under anesthesia.

### *Hematological evaluation*

In a preliminary study, the number of leukocytes was counted in a hemocytometer. Differential cell analysis was performed, using peripheral blood smears with Wright-Giemsa staining.

### *Functional assessment of gastrocnemius muscle*

The gastrocnemius muscle of each animal was exposed. The animals were fixed to an external frame in a prone position, with knee flexion at 90° and ankle flexion at 0°. A 3-0 silk suture was sewn through the distal tendon, and the tendon was sectioned. Then the suture was attached to a force transducer (Nihon Koden, TB-611T, Tokyo, Japan), to measure isometric contractile force. The exposed muscle was covered with liquid paraffin to prevent drying. Temperature in the muscle, continuously monitored by a temperature probe (Unique Medical Co., LTD, KU-96098, Tokyo, Japan), was maintained at 32–35 °C using an overhead heating lamp. The in situ muscle was stimulated directly (0.1 msec duration, 15 volts) via two electrodes connected to a stimulator (Nihon Koden, SEN-3301, Tokyo, Japan). One electrode was placed in the midmuscle belly and the other in the myotendinous junction. The length of resting muscle was adjusted to produce maximal twitch tension. Then tetanic tension, which shows the force-frequency

relationship, was assessed by recording 1-sec trains at frequencies ranging from 5 to 200 Hz. All measurements were made at 1-min rest intervals. The tetanic tensions were measured in newtons (N) per gram of muscle dry weight.

### *Water content in gastrocnemius muscle*

After measuring muscle function, gastrocnemius muscles were excised and weighed (wet weight). The muscle was then dried at 60 °C in a convection oven for 72 h and reweighed (dry weight). Wet-to dry-weight ratios (W/D ratios) were used as an index of edema formation.

### *Biochemical study*

An additional 32 rats were randomized into the 4 experimental groups above. After 4 h of ischemia and 24 h of subsequent reperfusion, venous blood from the right femoral vein was taken for assay of thiobarbituric acid reactive substances (TBA-RS) as an index of lipid peroxidation. The experimental gastrocnemius muscle was then obtained. A 5 mm transverse section was prepared for histological examination. The remaining segment was immediately frozen in liquid nitrogen for assays of TBA-RS and muscle neutrophil content.

TBA-RS in serum and gastrocnemius muscle were measured by Yagi's (1976) and Ohkawa et al.'s (1979) methods, respectively. The gastrocnemius muscle neutrophil content was assessed by measuring the neutrophil-specific enzyme myeloperoxidase (MPO) as an index of neutrophil infiltration. Muscle MPO activities were determined by a modified method of Allan et al. (1985).

### *Histological examination*

5 mm transverse muscle sections were mounted on a cork base and frozen in isopentane cooled in liquid nitrogen. The frozen muscles were sectioned at 10 μm and stained with hematoxylin and eosin (HE). Histological sections were examined under light microscopy. Neutrophil counts (number per high-power field) and edema were evaluated by 2 pathologists with no prior knowledge of the experimental groups. The following grading system of 0 to 3 was utilized for evaluating edema in each tissue: grade 0 = normal appearance, grade 1 = mild edema (partial inter-perimysium), grade 2 = moderate edema (whole inter-perimysium), and grade 3 = severe edema (inter- and intra-perimysium).

### *Data analysis*

Differences in experimental populations were evaluated by a one-way analysis of variance (ANOVA) followed by Fisher's PLSD for multiple comparisons. Histological examination of edema in muscle tissue was compared with the nonparametric Kruskal-Wallis test with Fisher's PLSD. The force-frequency relationship was analyzed using repeated-measures ANOVA, and the differences between experimental groups at each frequency were compared with Scheffe's method. $P < 0.05$ was regarded as significant.

## Results

### *Hematological evaluation*

Circulating leukocyte counts, including the number of neutrophils in the peripheral blood from NM-treated rats, were dramatically reduced compared to the sham group (Table 1). In contrast, leukocyte and neutrophil counts in the EPC group were similar to those of controls, which were significantly higher than in the sham group.

### *Functional assessment of gastrocnemius muscle*

There was a significant difference in the isometric tetanic tension (force-frequency relationship) analyzed by repeated-measured ANOVA ($p < 0.001$) (Figure 1). The tetanic tension in the sham group at frequencies 40–200 Hz and that in the EPC group at 50–200 Hz were significantly higher than in the control group. The tetanic tension in the EPC group averaged 83% of that in the sham group, while the NM group averaged 70% of that in the sham group.

### *Water content in gastrocnemius muscle*

The muscle W/D ratios in the EPC group were significantly lower than those in the NM and control groups (Table 2). The W/D ratios in the NM group were similar to those of the control group.

### *Biochemical study*

The TBA-RS levels in the serum and muscle tissue in the sham, EPC and NM groups were signifi-

**Table 1. Hematological study. Mean (SD)**

| | Sham group (n 8) | Control group (n 8) | EPC group (n 8) | NM group (n 8) |
|---|---|---|---|---|
| Leukocyte counts | 5800 (912) [b] | 8563 (2644) | 8575 (1865) [f g] | 337 (185) [a d] |
| Neutrophil counts | 3565 (560) [c] | 5074 (1567) | 5277 (1147) [e g] | 203 (111) [a d] |

[a] $p < 0.001$ compared to control group
[b] $p = 0.003$ compared to control group
[c] $p = 0.006$ compared to control group
[d] $p < 0.001$ compared to sham group
[e] $p = 0.002$ compared to sham group
[f] $p = 0.003$ compared to sham group
[g] $p < 0.001$ compared to NM group

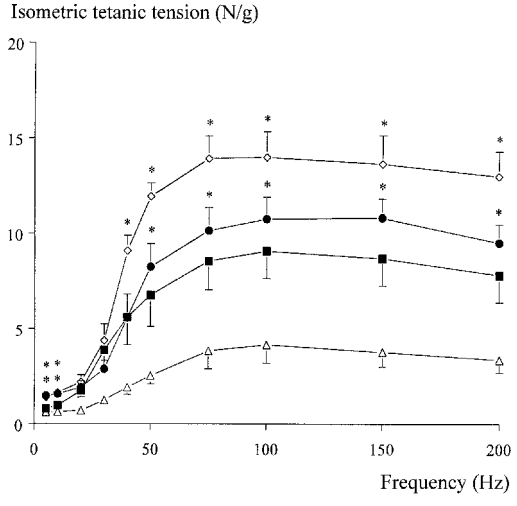


Figure 1. Force-frequency relationship of the gastrocnemius subjected to 4 h of ischemia and 24 h of reperfusion. Values are means ± SE for 8 rats in each group. Isometric tetanic tension in the sham and EPC groups was significantly better preserved than in the control group (* $p < 0.02$). There were no statistically significant differences between the EPC and sham groups at all frequencies tested. Tetanic tension in the NM group was not significantly higher than in the control group. Sham group (♦), EPC group (●), NM group (■), and Control group (▲).

cantly lower than those in the control group (Table 3). In the EPC group, the serum levels of TBA-RS were significantly higher than in the sham group, but TBA-RS levels in the EPC-treated muscle tissue were similar to those in the sham group. Both serum and muscle TBA-RS levels were significantly lower in the EPC group than in the NM group.

Similarly, muscle MPO activities in the sham, EPC and NM groups were significantly lower than those of the control group (Table 3). The EPC-treated muscle MPO activities, however, were significantly higher than those of the sham and NM groups.

### *Histological examination*

The control group showed marked edema, swelling and destruction of muscle fibers, and an intense cellular inflammatory reaction (Figure 2). These changes were rarely observed in the EPC and NM groups. The sham, EPC and NM groups had significantly lower neutrophil counts in the muscle tissue than those in the control group (Table 4). Histological evaluation of muscle edema showed a significant difference among the experimental groups. The NM group showed more edematous changes than the EPC group.

**Table 2. Muscle wet- to dry-weight ratios. Mean (SD)**

| | Sham group (n 8) | Control group (n 8) | EPC group (n 8) | NM group (n 8) |
|---|---|---|---|---|
| W/D ratio | 4.13 (0.10) [a] | 4.70 (0.53) | 4.21 (0.14) [b d] | 4.57 (0.25) [c] |

[a] $p < 0.001$ compared to control group
[b] $p = 0.003$ compared to control group
[c] $p = 0.007$ compared to sham group
[d] $p = 0.02$ compared to NM group

**Table 3. Biochemical analysis. Mean (SD)**

| | Sham group (n 8) | Control group (n 8) | EPC group (n 8) | NM group (n 8) |
|---|---|---|---|---|
| Serum TBA-RS (nmol/mL) | 39 (10) [a] | 192 (44) | 99 (24) [a e h] | 144 (47) [b c] |
| Muscle TBA-RS (nmol/g) | 35 (13) [a] | 126 (22) | 46 (22) [a f] | 92 (17) [a c] |
| Muscle MPO (U/min/g) | 104 (27) [a] | 1699 (693) | 776 (216) [a d g] | 259 (116) [a] |

[a] $p < 0.001$ compared to control group
[b] $p = 0.009$ compared to control group
[c] $p < 0.001$ compared to sham group
[d] $p = 0.001$ compared to sham group
[e] $p = 0.002$ compared to sham group
[f] $p < 0.001$ compared to NM group
[g] $p = 0.009$ compared to NM group
[h] $p = 0.02$ compared to NM group

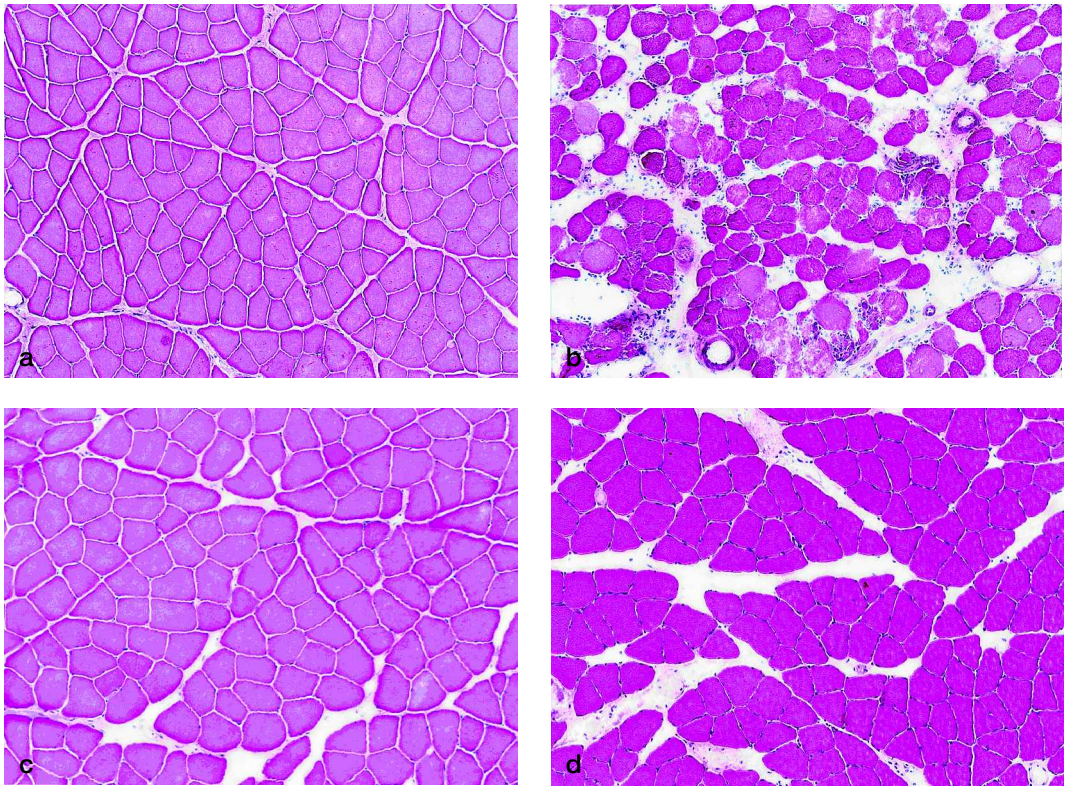


Figure 2. Histological transverse sections of the gastrocnemius (HE, ×100).
a. Sham group showing no abnormalities.
b. Control group showing marked change with edema, muscle fiber swelling and destruction, and cellular inflammatory reaction.
c. EPC group showing slight change with edema and muscle fiber swelling.
d. NM group showing almost the same change as the EPC group, but a more marked edematous change than the EPC group.

## Discussion

Our results indicate that OFR and neutrophils induce muscle dysfunction caused by reperfusion injury, and that damage was more reduced by scavenging OFR than by depleting neutrophils. It is known that OFR-induced cytotoxicity depends largely on the subsequent production of highly reactive species of free radical. One of these, the hydroxyl radical, has been reported to be an extremely toxic compound (Willson 1984), and was effectively scavenged by EPC-K1 (Mori et al.

**Table 4. Histological evaluation. Mean (SD)**

| | Sham group (n 8) | Control group (n 8) | EPC group (n 8) | NM group (n 8) |
|---|---|---|---|---|
| Neutrophil counts | 0 (0) [a] | 43.0 (27.1) | 3.1 (2.9) [a] | 1.1 (1.6) [a] |
| Edema | 0 (0) [a] | 2.3 (0.9) | 0.5 (0.5) [a c] | 1.1 (0.6) [a b] |

[a] $p < 0.001$ compared to control group
[b] $p < 0.001$ compared to sham group
[c] $p = 0.04$ compared to NM group

1989). We found that the muscle TBA-RS levels in the EPC group were similar to those in the sham group, and were significantly lower than those in the NM group. This implies that lipid peroxidation of muscle cellular membranes was almost entirely inhibited by EPC-K1 treatment.

Muscle edema, measured by W/D ratios in the NM group, was significantly greater than in the EPC group. Edema formation in the skeletal muscle occurred due to an increase in microvascular permeability initiated by the cytolytic effect on endothelial cell membranes induced by free radicals through lipid peroxidation (Korthuis et al. 1985). In both the EPC and NM groups, TBA-RS levels in the serum were lower than those of controls, however, EPC-K1 injection more effectively reduced serum TBA-RS levels than NM treatment. Inhibition of the lipid peroxidation of endothelial cell membranes was therefore more marked in the EPC group than in the NM group. These findings suggest that microvascular permeability was greater in the NM group than in the EPC group. Histological findings further supported this view, since the edematous change in the skeletal muscle was seen in the NM group more often than in the EPC group. Taken together, OFR scavenging appears more effective than neutrophil depletion in preventing muscle edema formation from reperfusion injury.

Muscle MPO levels and leukocyte counts in the peripheral blood and muscle indicated that neutrophils were sufficiently depleted in the NM group. In contrast, muscle MPO levels in the EPC group were significantly higher than in the NM and sham groups. This shows that neutrophil infiltration into the skeletal muscle was not entirely prevented by EPC-K1. Nevertheless, muscle TBA-RS levels in the EPC group were very low, demonstrating that OFR generation was reduced despite the presence of neutrophils. These experiments suggest that OFR produced by neutrophils may be effectively scavenged by EPC-K1.

Moreover, neutrophil infiltration into the skeletal muscle in the EPC group was significantly prevented, compared to the control group, despite the similar neutrophil counts in peripheral blood in both groups. This indicates that scavenging OFR with an EPC-K1 injection reduced vascular endothelial damage and subsequent activation and recruitment of inflammatory cells, since neutrophil-endothelium interactions significantly increase after skeletal muscle injury and then neutrophils accumulate in the damaged tissue (Menth-Chiari et al. 1998). Leukocyte-endothelium interactions after neutrophil activation are considered a central pathologic event in the evolution of tissue damage. There is increasing evidence that these processes are initiated at the level of the microcirculation and that microcirculatory damage may affect tissue injury (Korthuis et al. 1985, Homer-Vanniasinkam et al. 1997). On the basis of this information, our results suggest that limitation of the OFR-induced tissue injury in the microvascular and the skeletal muscle at an early stage of reperfusion by EPC-K1 prevents further neutrophil accumulation in the post-ischemic muscle tissue.

These findings support our hypothesis that suppressing OFR production immediately after reoxygenation would be more effective than depletion of neutrophils to reduce the reperfusion injury in skeletal muscle. Furthermore, limiting OFR generation reduces secondary neutrophil infiltration indirectly, and muscular contractile function is well preserved as a result. Induction of neutropenia is impractical in clinical application for acute ischemia, although it is important to understand the role of neutrophils in the mechanism of reperfusion injury. On

the other hand, EPC-K1 is amphipathic and easily administered in intravenous form for use in human trials. Other investigators are conducting phase II trials to examine EPC-K1's efficiency in acute brain infarction. EPC-K1 is therefore expected to be of clinical use in acute ischemia and resulting reperfusion. In conclusion, both OFR and neutrophils influenced muscular contractile dysfunction; however, EPC-K1 effectively limits generation of OFR in the early phase of reoxygenation and is more potent than neutrophil depletion in preventing reperfusion injury.

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