Reduced ischemia-reperfusion injury in muscle

Experiments in rats with EPC-K1, a new radical scavenger

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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), a phosphate diester of alpha-tocopherol and ascorbic acid, is a potent antioxidant. We examined the effects of EPC-K1 on ischemia-reperfusion injury in the skeletal muscle of rats, using an ischemic revascularized hind limb model. Warm ischemia (25 °C), produced by vascular pedicle clamping, was sustained for 4 hours. After 24 hours of reperfusion, skeletal muscle injury was evaluated in 2 groups: one group treated by intravenous injection of EPC-K1 (10 mg/kg) prior to ischemia, and a group of controls. The EPC-K1-treated group showed a statistically significant amelioration in the reduction of the isometric muscle contraction, inhibition of the elevation of the muscle wet- to dry-weight ratio, limitation of the muscle level of thiobarbituric acid reactive substances and the serum levels of creatine phosphokinase, lactate dehydrogenase and mitochondrial glutamic oxaloacetic transaminase, and reduction of the extent of muscle injury according to the histological findings. These observations indicate that EPC-K1 acted effectively on ischemia-reperfusion injury in the rat skeletal muscle and thereby improved muscle function.

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The possibility that free radical-mediated reactions may be important in the damage seen following an ischemic episode in several tissues has been discussed for some time (McCord 1985). Recently, a number of authors have examined the role of free radical species in ischemic damage to skeletal muscle (Lindsay et al. 1989, Pang 1990) and, experimentally, the effects of free radical scavengers have been evaluated in skeletal muscle (Korthuis et al. 1985, Walker et al. 1987, Feller et al. 1989, McCutchan et al. 1990, Seyama 1993). However, few radical scavengers are of clinical value for ischemia-reperfusion injury in skeletal muscle.

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., Japan) (Figure 1) is a newly introduced compound representing a phosphate diester linkage of alpha-tocopherol and ascorbic acid. It has been reported to possess potent hydroxyl radical scavenging activity (Mori et al. 1989) and to inhibit phospholipase A2 activity (Kuribayashi et al. 1992). It is amphipathic—that is, soluble in both water and lipid. Therefore, it is used intravenously and may be rapidly absorbed in many organ tissues. Although expected to be of future clinical use, its effect on ischemia-reperfusion injury in skeletal muscle remains unknown. We tried to clarify the effects of EPC-K1 on skeletal muscle during ischemia and the resulting reperfusion.

Animals and methods

Surgical preparation

Male Lewis rats weighing 250–300 g were used 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.

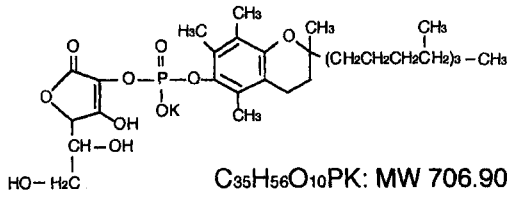


Figure 1. Chemical structure of EPC-K1.

Animals were anesthetized intraperitoneally with sodium pentobarbital (45 mg/kg). EPC-K1 (10 mg/kg) was administered intravenously through the caudal vein (EPC group: n 8). The left thigh was amputated except for the femoral artery and vein and, after 30 minutes of EPC-K1 administration, the vessels were completely occluded with a microvascular clip for 4 hours at room temperature (25 °C). Then the clip was released and patency of the vessels was confirmed by visual inspection. Next, the osteotomy was repaired, using a 18-gauge (1.3 mm in diameter) injection needle as an intramedullary nail, and muscle group and skin were reapproximated. After 24 hours of the reperfusion, the experiments were done.

As the control, other animals received physiological saline instead of EPC-K1 (PS group: n 8) and the same experiments were done.

Functional assessment of gastrocnemius muscle

Both gastrocnemius muscles of each animal were exposed. The animals were fixed to an external frame in a supine position with knee flexion at 90° and ankle flexion at 0°. A 3-0 silk suture was sewn through the distal tendon, which was then sectioned and the suture was attached to a force transducer (Nihon Koden, TB-611T, Japan) to measure isometric contractile force. The exposed muscle was covered with paraffin liquid to prevent drying and muscle temperature was maintained at 32°–35 °C, using an overhead heating lamp. The in situ muscle was stimulated directly (0.1 ms duration,15 volts) through 2 electrodes inserted from a stimulator (Nihon Koden, SEN-1101, Japan). One electrode was inserted in the midmuscle belly and the other in the myotendinous junction. Resting muscle length was adjusted to produce maximal twitch tension.

The isometric twitch contractile properties were determined. These included twitch tension (P_t, N/g), time-to-peak twitch tension (TPT, msec), time to half-relaxation of the twitch ($T_{1/2R}$, msec), and rate of tension increase during a twitch (dPt/dt, N/g/sec). Tetanic tension, which shows the force-frequency relationship, was assessed by recording 1-second trains at frequencies ranging from 5 to 200 Hz. All measurements were separated by 1-minute rest intervals. Twitch and tetanic tensions were reported as newtons (N) per gram of muscle dry weight.

Wet- to dry-weight ratios of gastrocnemius muscle

The gastrocnemius muscles were excised and the wet muscle samples were weighed. Then the muscle dry weight was determined after drying for 24 hours at 60 °C. The wet- to dry-weight ratios were determined as an index of edema formation.

Biochemical assay

1 g of gastrocnemius muscle from the ischemic experimental side was obtained and its thiobarbituric acid reactive substances (TBA-RS) were measured by the method of Ohkawa et al. (1979). Venous blood from the right femoral vein was also obtained after the assessment of gastrocnemius muscle function for analysis of the serum levels of creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and mitochondrial glutamic oxaloacetic transaminase (GOT-m).

Histological study

5 mm transverse sections from the gastrocnemius muscles were mounted on a cork base and frozen in isopentane cooled in liquid nitrogen. The frozen muscles were sectioned at 10 μm, stained with hematoxylin and eosin (HE) and observed with light microscopy.

Data analysis

Differences in experimental populations were evaluated by the Mann-Whitney U-test. Significance was accepted at $p < 0.05$.

Results

Functional assessment of gastrocnemius muscle

The isometric twitch tension (P_t) in the EPC group was significantly greater than in the PS group (Table 1). The time course of twitch contraction, as reflected by the time to peak twitch tension (TPT) and time to half-relaxation of the twitch ($T_{1/2R}$), between the EPC and PS groups was not significantly different. However, the rate of tension increase during a twitch (dPt/dt) was significantly greater in the EPC group. Tetanic tension (force-frequency relationship) in the EPC group was significantly greater than that in the PS group, as also was twitch tension (Table 2). On the other hand, the twitch and tetanic tensions of the nonischemic contralateral gastrocnemius were similar in both groups.

Wet- to dry-weight ratios of gastrocnemius muscle

The muscle wet- to dry-weight ratio was significantly lower in the EPC group than in the PS group (Table 3).

Table 1. Isometric twitch contractile properties. Mean *SD*

	EPC group (n 8)		PS group (n 8)	
	Contralateral limb	Experimental limb	Contralateral limb	Experimental limb
Twitch tension (P_{tt}, N/g)	2.1 *0.2*	1.6 *0.6*[a]	2.3 *0.5*	0.7 *0.4*
Time-to-peak twitch (TPT, msec)	25.8 *3.7*	27.9 *3.4*	27.9 *5.0*	27.4 *6.9*
Half-relaxation ($T_{1/2R}$, msec)	18.5 *2.9*	20.9 *4.1*	18.6 *3.5*	20.9 *6.7*
Speed of contraction (dPt/dt, N/g/sec)	83 *8*	57 *20*	84 *17*	26 *20*

[a] p = 0.01 compared with experimental limb in PS group

Table 2. Tetanic tension measurement as N/g dry muscle. Mean *SD*

	EPC group (n 8)		PS group (n 8)	
Stimulation frequency	Contralateral limb	Experimental limb	Contralateral limb	Experimental limb
5Hz	2.3 *0.3*	1.7 *0.6*[a]	2.4 *0.5*	0.7 *0.5*
10Hz	2.4 *0.4*	1.8 *0.6*[a]	2.5 *0.6*	0.8 *0.5*
20Hz	2.6 *0.5*	2.2 *0.9*[a]	2.8 *0.6*	1.0 *0.7*
30Hz	5.5 *3.5*	3.3 *1.4*[a]	5.5 *2.4*	1.6 *1.1*
40Hz	9.1 *3.7*	6.6 *3.8*[a]	10.0 *2.4*	2.6 *2.0*
50Hz	13.2 *3.0*	9.2 *3.7*[a]	12.2 *2.1*	3.8 *2.8*
75Hz	17.3 *3.7*	11.0 *3.5*[a]	15.3 *3.4*	5.0 *4.1*
100Hz	17.9 *4.0*	11.3 *3.2*[a]	16.4 *5.2*	5.3 *4.4*
150Hz	16.7 *4.8*	11.2 *2.5*[a]	16.5 *5.4*	4.8 *3.8*
200Hz	15.2 *5.4*	10.0 *2.3*[a]	14.9 *6.3*	4.1 *3.1*

[a] p ≤ 0.02 compared with experimental limb in PS group

Table 3. Gastrocnemius wet- to dry-weight ratio. Mean *SD*

EPC group (n 8)		PS group (n 8)	
Contralateral limb	Experimental limb	Contralateral limb	Experimental limb
4.04 *0.11*	4.21 *0.14*[a]	4.13 *0.08*	4.70 *0.52*

[a] p = 0.01 compared with experimental limb in PS group

Table 4. Muscle level of thiobarbituric acid reactive substances (TBA-RS) in experimental limb and serum levels of CPK, LDH and GOT-m. Mean *SD*

	EPC group (n 8)		PS group (n 8)	
TBA-RS, nmol/g	46	*22*[a]	77	*24*
CPK, IU/IL	2242	*665*[b]	3444	*857*
LDH, IU/L	2414	*1517*[b]	5431	*2660*
GOT-m, IU/L	29	*7*[b]	44	*14*

[a] p = 0.01 compared with PS group
[b] p = 0.02 compared with PS group

Biochemical assay

The muscle level of TBA-RS and the serum levels of CPK, LDH and GOT-m activities in the EPC group were significantly lower than in the PS group (Table 4).

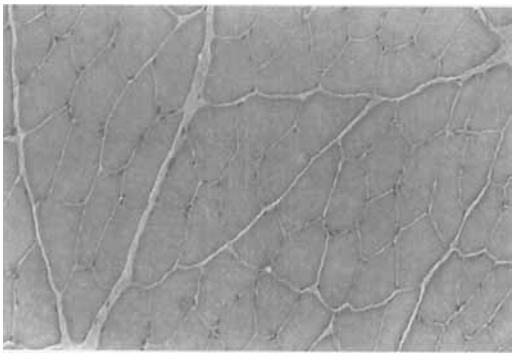
Histological study

The PS group showed marked muscle fiber destruction and intense cellular inflammatory reaction, but these changes were slight in the EPC group (Figure 2).

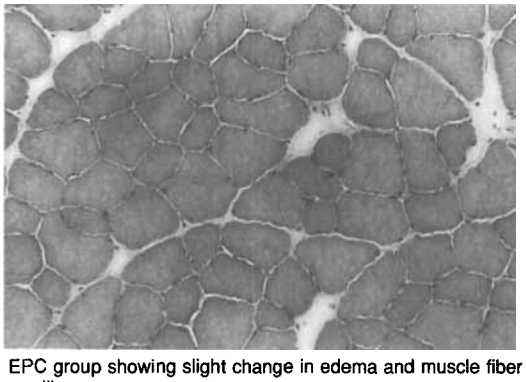
Discussion

The main finding of our study is that administration of EPC-K1 improved the reduction in muscle contractile function induced by ischemia and reperfusion. Although contractile function is a clinically relevant assessment of muscle viability, only a few studies have reported the effects of antioxidant therapy on postischemic muscle contractile function (Feller et al. 1989, McCutchan et al. 1990). The twitch and tetanic tensions of experimental ischemic limbs in the PS group averaged 31% and 33% of nonischemic contralateral

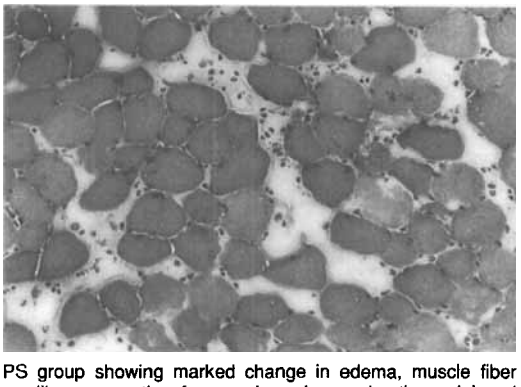
Figure 2 Histologic transverse section of the gastrocnemius (HE, ×100).



Nonischemic contralateral gastrocnemius in PS group showing no abnormalities.



EPC group showing slight change in edema and muscle fiber swelling.



PS group showing marked change in edema, muscle fiber swelling, separation from endomysium, pyknotic nuclei and cellular inflammatory reaction.

values, similarly to previous studies (Fish et al. 1989, McCutchan et al. 1990, Stompro et al. 1994). By contrast, in the EPC group the twitch and tetanic tensions averaged 75% and 73%. Since ischemic damage predominates in complete severe ischemia-reperfusion injury (Seyama 1993), the loss of contractile function in the EPC group may have been caused by ischemic damage in our complete ischemic model.

It has been reported that oxygen free radicals contribute to skeletal muscle injury associated with ischemia and reperfusion (Lindsay et al. 1989, Pang 1990). We measured the muscle TBA-RS level, which is a secondary product of lipid peroxidation and therefore an indicator of free radicals (Ohkawa et al. 1979). EPC-K1 limited the increase in that level—i.e., it inhibited the production of free radicals in skeletal muscle. Oxygen free radical-induced cytotoxicity depends largely on the subsequent production of a highly reactive species of oxygen free radical, the hydroxyl radical, reported to be an extremely toxic compound (Willson 1984). These radicals have a direct lytic effect on cellular membranes through lipid peroxidation (Bulkley 1983). We suggest that EPC-K1 inhibited lipid peroxidation of cellular membranes by virtue of its hydroxyl radical scavenging properties.

EPC-K1 limited muscle edema formation, elevation of the serum CPK, LDH and GOT-m levels and muscle damage in the histological study. Based on muscle wet- to dry-weight ratios, fluid accumulation in skeletal muscle occurred because of an increase in microvascular permeability initiated by the free radical's direct lytic effect on endothelial cell membranes through lipid peroxidation (Korthuis et al. 1985). The increase in the serum CPK, LDH and GOT-m levels and the histological findings also showed the destruction of skeletal muscle structure induced by the same pathological change in the muscle cell membranes. These changes cause muscle contractile dysfunction.

Our study shows that EPC-K1 reduces the severity of injury and loss of contractile function in skeletal muscle during ischemia and the subsequent reperfusion condition. Many radical scavengers have been reported to reduce skeletal muscle damage in numerous experimental models of ischemia-reperfusion injury. From the clinical point of view, however, only a few are effective. Clinical administration of EPC-K1 may play an important role in preventing ischemia-reperfusion injury in replantation and transplantation of digits or limbs and in free vascularized graft salvage.

Acknowledgments

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