Protective effect of anisodamine on reperfusion injury of skeletal muscles in rabbit

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Anisodamine is an alkaloid isolated from a Chinese plant, which was subsequently synthesized. Its chemical structure is similar to atropine. It inhibits cholinergic nerve function, improves microcirculation, and was reported to have a protective effect on reperfusion injury in various organs. We used anisodamine in a rabbit model with ischemia and reperfusion injury of hind limb muscles. We evaluated its effect on skeletal muscle cells, using transmission electron microscopy, and analyzed lipid peroxidation by measuring malondialdehyde and lactate dehydrogenase blood concentrations. We found that malondialdehyde and lactate dehydrogenase concentrations after 1 hour of reperfusion were lower in animals treated with anisodamine than in controls. Damage to membrane structures and myofilaments in muscle cells was less severe after anisodamine treatment. Our findings indicate that anisodamine protects skeletal muscles with ischemia and reperfusion injury.

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Reperfusion injury of skeletal muscle is characterized by increased vascular permeability, edema and skeletal muscle necrosis (Korthuis et al. 1985, McCord 1985). It is a common pathologic course after vascular trauma or acute arterial thrombosis. Oxygen-derived free radicals play an important role in ischemia and reperfusion injury of skeletal muscle (Korthuis et al. 1985). Thus, despite technically satisfactory surgery for acute limb ischemia, reperfusion injury may cause failure of limb salvage and the need for amputation. Less seriously, reperfusion injury may lead to muscle fibrosis and contractures.

Anisodamine is an alkaloid originally isolated from the Chinese solancea plant, Ansiodus tanguticus, which was subsequently synthesized and made commercially available. The chemical structure of anisodamine is similar to atropine (Figure 1). Anisodamine shows an inhibiting effect on cholinergic nerve function, but it is less toxic and therefore widely used in Chinese clinics. It has also improved microcirculatory function, especially in treatment of various types of shock (Hock et al. 1983, Su et al. 1984), without major adverse effects. Other studies demonstrated a protective effect on reperfusion injury in lung, skin, eyes, pancreatic beta cells and myocardial muscle (Tan et al. 1989, Zhang et al. 1990, Fu 1991, Gu et al. 1991, Luo et al. 1992, Yao et al. 1995), but the effect of this drug on injured skeletal muscle has not been tested yet. We investigated the effect of anisodamine on skeletal muscle in a rabbit model of hind limb ischemia and reperfusion injury.

Animals and methods

14 New Zealand White adult rabbits were equally divided into two groups. All animals were anesthetized by intravenous injection of 3% pentobarbital (30 mg/kg). After weighing, the right thigh was shaved and washed. The femoral artery and vein were dissected at the groin through a 3 cm incision and occluded with a

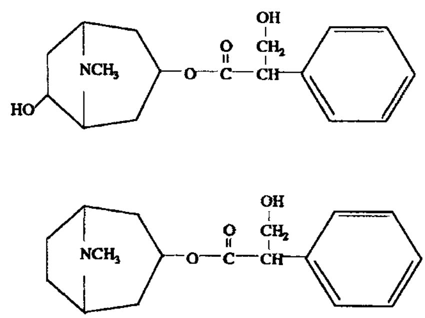

Figure 1. Structure of anisodamine (upper line) and atropine (lower line).

MDA (nmol/mL) and LDH (U/L) concentrations in serum mean (SD)

	Control (n 7)		Anisodamine (n 7)	
	MDA	LDH	MDA	LDH
Preischemia	2.36 (0.66)	139 (60)	2.40 (0.59)	145 (64)
Postischemia	2.86 (0.35)	224 (54) [b]	3.06 (0.40)	192 (74)
Reperfusion	5.41 (0.74) [a]	344 (68) [a]	3.10 (0.94) [c]	242 (44) [b c]

[a] $p < 0.01$, compared with preischemia and postischemia.
[b] $p < 0.05$, compared with preischemia.
[c] $p < 0.01$, compared with the control group.

microsurgery clamp. A rubber band was put around the thigh below the dissected femoral vessels and firmly tightened to occlude the remaining collateral circulation to the limb. After 5 hours, the clamp and rubber band were released and the blood circulation of the limb was restored. Immediately after reperfusion, anisodamine (1 mg/kg, Beijing Pharmacy, Beijing, P.R.China) was injected intravenously in an ear vein in 7 animals (anisodamine group). In the other 7 animals (control group), a similar fluid volume of physiological saline solution was injected. The rabbits were killed 1 hour after limb reperfusion by an overdose of pentobarbital. Blood samples were obtained in all animals from the right femoral vein before ischemia, 5 hours after limb ischemia (just before the injection of anisodamine or saline) and immediately before death (1 hour of reperfusion). At the same times, biopsies were taken from various locations in the gastrocnemius muscle in 3 rabbits in each group. The experiment was approved by the local ethics committee.

The malondialdehyde (MDA, nmol/mL) concentration in serum, used as an index of lipid peroxidation, was measured with the fluorometric method using thiobarbituric acid (Yagi 1976). The lactate dehydrogenase (LDH, U/L) concentration in serum was analyzed with an automatic biochemical analyzer (ENCORE System II, Baker Instruments, USA), based on the method described by Gay et al. (1968).

The muscle biopsies were fixed in 2% glutaraldehyde for 2 hours immediately after sampling and then postfixed in 1% osmic acid after a rinse with sodium cacodylote buffer solution. The samples were dehydrated with acetone and embedded with epoxy resin. Ultrathin sections were cut and examined with a JEM-100CX transmission electron microscope (JEOL Ltd., Tokyo, Japan).

For statistical analysis, two-way ANOVA and post-hoc tests were used. $P < 0.05$ was required for a significant difference.

Results

In the control group, MDA concentrations were found to be increased after reperfusion compared to pre- and postischemic values ($p < 0.0001$) (Table). In animals treated with anisodamine, MDA concentrations were similar at the three time intervals. Thus, anisodamine-treated and control animals had similar MDA concentrations before and after ischemia, but after reperfusion the values were lower in the treated group ($p = 0.0003$).

In the control group, LDH concentrations were increased after ischemia and further increased after reperfusion ($p = 0.006$). In the anisodamine group, although the LDH concentrations after reperfusion were found to be higher than the preischemic values ($p = 0.03$), the increase between postischemia and reperfusion was not significant, and the concentration was lower after reperfusion than in the controls ($p = 0.006$) (Table). Pre- and postischemic LDH values were similar in both groups.

Ultrastructural changes

The muscle biopsies looked normal before ischemia. After 5 hours of ischemia, muscle cells in both groups showed a decrease in glycogen granules, slight dilation of the sarcoplasmic reticulum and swollen mitochondria, with somewhat disordered and obscure cristae arrays, but intact inner and outer membranes. The myofibrils had a normal appearance (Figure 2). After reperfusion, the muscle cells in the controls showed severe swelling, with large spaces between myofibrils, and apparent dilation of the sarcoplasmic reticulum and mitochondria. The myolemma was not continuous in some places. Mitochondria cristae and inner and outer membranes were found damaged in this group. Also many myofilaments were found dissolved and the Z line was streaming and disrupted, so that myofibrils were destroyed (Figure 3). In the anisodamine group, the muscle cells also showed edema, with swollen mitochondria and sarcoplasmic reticulum and disordered mitochondria cristae array, but the

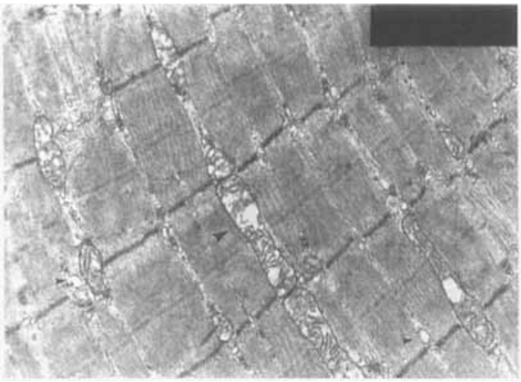

Figure 2. Electron micrograph of a muscle sample after 5 hours of ischemia. The sarcoplasmic reticulum is slightly dilated (small arrow heads). The mitochondria are somewhat swollen and its cristae array disordered, but the inner and outer membranes are intact (big arrow head). Normal myofilaments (×10,000).

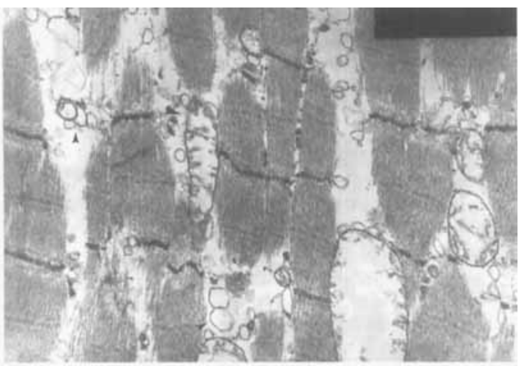

Figure 3. Electron micrograph of a muscle sample in the control group after 1 hour of reperfusion. The sarcoplasmic reticula are markedly swollen (small arrow heads). The mitochondria are dilated dramatically, its cristae have disappeared, and the inner and outer membranes are disrupted (big arrow head). Obvious edema is seen between myofilaments. Many myofilaments have dissolved and disappeared (×10,000).

damage seemed less than in the controls. Destruction of mitochondria membranes and dissolution of myofilaments were only rarely seen in this group, and the arrangement of the Z lines was kept in order, preserving the organization of the basic structural and functional unit of the myofibrils (Figure 4).

Discussion

Since free radicals are highly reactive and short-lived, it is hard to detect them directly. Thus, detection of lipid peroxidation relies on analyses of secondary or end products derived from hydroperoxide transformation, metabolism or decomposition. The fluorometric

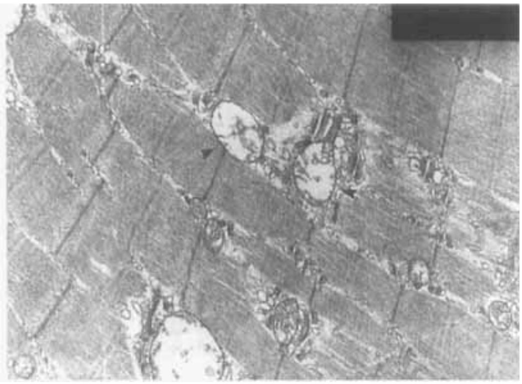

Figure 4. Electron micrograph of a muscle sample in the anisodamine group after 1 hour of reperfusion. The muscle cell shows some swelling. The mitochondria are swollen and their cristae array disordered, but the membranes of the mitochondriae are intact (arrow heads). Some of the myofilaments have dissolved, but to a lesser extent than in the control group (×10,000).

method using thiobarbituric acid that we applied quantifies lipid peroxidation by measuring the concentration of MDA, which is an end product of oxidative lipid degradation. The MDA concentration has been used previously as an indicator of lipid peroxidation (Janero 1990).

MDA concentrations in our study did not change after ischemia, but were found much increased in the controls after 1 hour of reperfusion, indicating obvious lipid peroxidation after reperfusion. Substantial formation of free radicals had also been found in previous experiments with reperfusion injury (Korthuis et al 1985, McCord 1985). Lipid peroxidation of the membrane structures of muscle cell may damage the cell organelles. The increased blood levels of LDH after ischemia, and especially after reperfusion, may reflect increased release of intracellular LDH from injured muscle cells. The destruction of mitochondria and myofilaments which we found on structural analysis further confirmed the severe cell damage. The high production of free radicals may be caused by the relatively high oxygen levels at the onset of reperfusion after ischemia. In the normal skeletal muscle, the occasional free radical is detoxified by a system of intracellular enzymes. During ischemia, calcium-activated proteases convert xanthine dehydrogenase to xanthine oxidase. Hypoxanthine, a byproduct of adenosine triphosphate degradation, is the natural substrate for xanthine oxidase. In the absence of oxygen during ischemia, this reaction is blocked; but at the moment of reoxygenation, the reaction between xanthine oxidase and hypoxanthine forms uric acid and free radicals (Weisfeldt 1987). Oxygen free radicals can initiate chain reactions in the lipid bilayers of organelle membranes by removing hydrogen atoms from the polyunsaturated fatty acids, thus forming lipid free radicals that are also highly reactive and capable of propagating cell damage (Freeman and Crapo 1982).

Gu et al. (1991) showed that anisodamine is able to to reduce oxygen free radical release thereby improving the condition after granulocyte-mediated lung injury in dogs. Likewise, when anisodamine was used in rat abdominal skin flap reperfusion injury, the survival of the graft was enhanced, and MDA and water contents were significantly lower in the anisodamine group than in the placebo group (Fu 1991). After ligation of the main coronary artery in rats, anisodamine can reduce infarct size and improve myocardial contractility (Tan et al. 1989). The failure of the MDA concentration to increase after reperfusion of skeletal muscle that we found suggests that anisodamine can inhibit lipid peroxidation of muscle cell membranes and therefore prevent or reduce reperfusion injury. The comparably lower levels of LDH and better preserved cell structures after reperfusion in the anisodamine-treated animals compared to the controls confirm the protective effect of this drug. This is in agreement with a recent study of the effect of anisodamine on myocardial reperfusion injury, which showed that MDA and creatine kinase were lower in the anisodamine-treated group than in the saline-treated group (Yao et al. 1995). However, the mechanism for this protective antioxidant effect is not completely understood. Luo et al. (1992) showed that anisodamine cannot scavenge exogenous superoxide as superoxide dismutase did. The result threw doubt on the direct antioxidant action of anisodamine. It was found in other studies that anisodamine can act as a calcium antagonist (Tang et al. 1985, Li et al.1989, Chen et al. 1992). Calcium is thought to convert xanthine dehydrogenase to xanthine oxidase, which is a key enzyme for the production of oxygen free radical (Lee et al. 1987). An indirect cellular membrane-stabilizing effect of anisodamine was demonstrated by Wang et al. (1993). They found that anisodamine can interdigitate with the phospholipid bilayer, thus influencing the fluidity of membranes.

The content of oxygen-derived free radicals increased as early as in the first 10 seconds of reperfusion (Weisfeldt 1987). According to this time course, to be effective, a free radical scavenger should be administered at the moment of reperfusion. Previous experiments also indicated that the first 30 to 60 minutes of reperfusion are vital for the recovery of function (Long et al. 1989, Wei et al. 1995). Therefore, we on purposely administered anisodamine immediately after reperfusion, which is feasible in clinical work, to ameliorate the reperfusion injury.

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