

Coenzyme Q10 protects skeletal muscle from ischemia-reperfusion through the NF-kappa B pathway

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Abstract

Objective: Coenzyme Q10 (CoQ10) has antioxidant and anti-inflammatory activity. The aim of this study was to investigate the effects of CoQ10 on the inhibition of nuclear factor-kappa B (NF-κB) activation during ischemia-reperfusion (I/R) of skeletal muscle.

Methods: For ischemia induction, the animals were anesthetized and the external iliac vessels blocked for three hours. CoQ10 or vehicle was given intraperitoneally during ischemia, just before reperfusion. Four groups received 3, 7, 14 and 28 days' reperfusion, respectively, after the intraperitoneal injection of CoQ10 and four corresponding groups received vehicle only. After reperfusion, the gastrocnemius muscles were removed, fixed and stained for the analysis of edema and mast cell infiltration.

Results: Immuno-histochemistry staining was performed for the detection of tumor necrosis factor alpha (TNF-α) and NF-κB. CoQ10-treated groups showed a significant decrease of mast cell infiltration in the gastrocnemius muscle and edema as compared with the corresponding non-treated groups. Also, CoQ10-treated groups showed a significant TNF-α and NF-κB expression decrease when compared to the corresponding non-treated controls. The results of this study showed CoQ10 administration with ischemia decreased interstitial edema, degeneration of muscle fibers and infiltration of mast cells.

Conclusions: It seems that CoQ10 has inhibitory effects on NF-κB and TNF-α activation.

Keywords

coenzyme Q10; ischemia-reperfusion; edema; TNF-α; NF-κB

Introduction

Lower limb ischemia injury occurs in certain pathological conditions, such as trauma, disease, multiple sclerosis and Guillain-Barre syndrome, surgery and bed sores.^{1–7} Skeletal muscles, as the predominant tissue in the limb, are most vulnerable to ischemia. There are obvious clinical symptoms of skeletal muscle damage, such as localized swelling, muscle dysfunction and necrosis and the degree of the damage is related to the severity and duration of ischemia. Previous studies have shown that skeletal muscle damage occurs after three hours of ischemia and a severe injury occurs when ischemia lasts 4–6 hours, leading to progressive loss (3%) of muscle and limb function.^{8,9}

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Several studies support the concept that reperfusion could, paradoxically, induce a cascade of cellular events, causing further damage. Therefore, muscle ischemia-reperfusion (I/R) injury is classified according to the severity of the edema, infiltration of inflammatory cells and the disruption of the normal structure of muscle cells. Moreover, it has been well established that skeletal muscle (I/R) injury leads to the production of oxygen free radicals and the synthesis/release of proinflammatory cytokines, such as interleukin 6 (IL-6), interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF- α) through nuclear factor-kappa B (NF- κ B) activation. NF- κ B plays an important pathogenic role in skeletal muscle I/R injury. Regulation and inhibition of NF- κ B signaling, which provides physiological protection against severe ischemic stress, has been suggested as a potential therapeutic intervention to treat I/R injury. NF- κ B is activated by oxidative stress and antioxidants can block NF- κ B activation.

Previous studies indicated that coenzyme Q10 (CoQ10), a vitamin-like substance and fat soluble compound which can be found in almost all cell membranes, is capable of decreasing inflammatory cytokine. Like other compounds, CoQ10 has antioxidant, lysosomal function and disease prevention, such as cardiovascular, aging and neurodegenerative diseases.^{10–16}

Although several studies showed that CoQ10 could be useful in the protection of organs against oxidative stress, it is not known whether there is an influence of CoQ10 on NF- κ B activation in skeletal muscle. Therefore, this study was designed to investigate the effects of CoQ10 on the inhibition of NF- κ B activation during I/R of skeletal muscle.

Materials and Methods

Ethical approval

Every effort was made to minimize the number of animals used and their suffering. All experiments were performed in accordance with principles of laboratory animal care of Lorestan University of Medical Sciences.

Animals and study design

CoQ10 was purchased (Sigma-Aldrich, Hamburg, Germany) and a solution with a pH = 6.5 contained 4.0 mg of CoQ, 1.8 mg of phospholipids, 30.0 mg of polyglycols and other solvents and 45.0 mg of d-sorbitol in 1.0 ml prepared. This experimental study was conducted at the Razi Herbal Medicine Research Center of Lorestan University of Medical Sciences. Seventy-two adult male Wistar rats weighing 250–300 g were acclimatized for one week to the conditions. All animals had free access to standard laboratory feed chow and water ad libitum. The

animals were randomly divided into eight groups (experimental and control) with eight rats in each group.

- 1) Q+IR3: Received CoQ10, 20 mg/kg intramuscular (i.m.) (gluteal muscles) and 10 mg/kg intraperitoneally (i.p.)^{17–18} during ischemia followed by three days of reperfusion.
IR3: Received vehicle alone during ischemia followed by three days of reperfusion.
- 2) Q+IR7: Received CoQ10, 20 mg/kg i.m. and 10 mg/kg i.p. during ischemia followed by seven days of reperfusion.
IR7: Received vehicle alone during ischemia followed by seven days of reperfusion.
- 3) Q+IR14: Received CoQ10, 20 mg/kg i.m. and 10 mg/kg i.p. during ischemia followed by fourteen days of reperfusion.
IR14: Received vehicle alone during ischemia followed by fourteen days of reperfusion.
- 4) Q+IR28: Received CoQ10, 20 mg/kg i.m. and 10 mg/kg i.p. during ischemia followed by twenty-eight days of reperfusion.
IR28: Received vehicle alone during ischemia followed by twenty-eight days of reperfusion.

Ischemia induction

The rats were anesthetized intraperitoneally with ketamine HCl (50 mg/kg) and xylazine (5 mg/kg)^{19,20} and placed in the supine position on a heated mat during the operation and recovery. The right external iliac vessels were exposed through an inguinal incision and dissected free of the femoral nerve, under an operating microscope. The gastrocnemius muscle was rendered near-completely ischemic by occluding the external iliac artery and vein with a 6-0 silk suture using a slipknot technique.^{12–15,18,21}

CoQ10, 20 mg/kg i.m. and 10 mg/kg i.p. was administered in all experimental groups with a single dose prior to vascular ligation. In all the groups, the vascular ligation was removed after 3 h of ischemia^{12,21} and the hind limb allowed to re-perfuse for 3, 7, 14 and 24 days in both the control and experimental CoQ10 treatment groups.

The rats were anesthetized with the usual dose of 40–50 mg/kg ketamine and 5 mg/kg xylazine. This dose was efficient for 30–45 min, then repeated. During this time, ischemia induction was performed and then CoQ10, 20 mg/kg i.m. and 10 mg/kg i.p. was administered with a single dose prior to vascular ligation. The vascular ligation was removed after 3 h of ischemia. Then, the inguinal incision was sutured with 2-0 silk. Then hind limb was allowed to re-perfuse for 3, 7, 14 and 24 days in both the control and CoQ10 groups.

After ischemia and release of the ligation, the inguinal incision was sutured with 2-0 silk. The animals were allowed to recover from the anesthesia after the vascular

ligature and incision were sutured. At the end of the experiments, the animals were sacrificed with a high dose of anesthetic and the gastrocnemius muscle was preserved in 10% formalin.

Histopathological studies

After fixation in 10% formalin, the gastrocnemius muscle was embedded in paraffin, cut and stained with hematoxylin-eosin and toluidine blue staining for the analysis of edema and fiber degeneration and mast cell infiltration, respectively. Thirty fields were selected randomly per slide and analyzed with light microscopy (magnification of $\times 40$). Disorganization and degeneration of muscle fibers, infiltration of inflammatory cells and edema of the interstitial fluid on the tissue was performed as follows: 0: normal, 1: mild, 2: moderate, 3: severe, 4: very severe. The sections of gastrocnemius muscle were observed for inflammatory cells and edema under a microscope. The results were analyzed by specialists blinded to the study.

TNF- α and NF- κ B expression

TNF- α and NF- κ B expression were analyzed by immune-histochemistry according to AbD Serotec (Bio-Rad, Kidlington, Oxfordshire, UK) and cell signal procedure, with slight modifications.²²

TNF- α

After fixation, the specimens were embedded in paraffin, cut, cleared by xylene and dehydrated in ethanol. The samples were incubated with 0.3% (w/v) H_2O_2 to block endogenous peroxidase activity and incubated overnight with primary antibody (rat anti-mouse TNF- α , AbD Serotec, # MCA 1488). Then, samples were incubated for 30 min with one drop of secondary antibody (Rabbit F (ab') 2 anti-rat IgG: HRP, AbD Serotec, # STAR21B), washed and incubated with one drop of SignalStain[®] DAB substrate Kit (Cell signaling, #8059, New England Biolabs (UK) Ltd., Hitchin, Hertfordshire, UK). The results were analyzed by specialists blinded to the study and the expression level was determined by multiplying the average density of the image by the percentage of the stained areas.

NF- κ B

After fixation, the specimens were embedded in paraffin, cut, cleared by xylene and dehydrated in ethanol. The samples were incubated with 0.3% (w/v) H_2O_2 to block endogenous peroxidase activity and incubated overnight with primary antibody (Rabbit anti NF-Kb p65 (D14E12) XP[®] Rabbit mAb, Cell signaling, #8242, New England

Biolabs). Then, the samples were incubated for 30 min with one drop of secondary antibody (SignalStain[®] Boost IHC Detection, Cell signaling, #9997, New England Biolabs) washed and incubated with one drop of SignalStain[®] DAB Substrate Kit (Cell signaling, #8059, New England Biolabs).

The results were analyzed by specialists blinded to the study and the expression level was determined by multiplying the average density of the image by the percentage of the stained areas.

Statistics

Statistical analysis was made with SPSS V.22 (IBM Corp., Armonk, NY, USA) and the variables were tested using the Kruskal–Wallis test and the Mann–Whitney U Test. The Kruskal–Wallis test was used to analyze the differences between the groups and the Mann–Whitney U test was used for pairwise comparisons. All data were expressed as mean \pm SD in each group and significance was set at $p \leq 0.05$.

Results

Histopathological studies

The CoQ10-treated groups of Q+IR7, Q+IR14, and Q+IR28 showed a significant mast cell infiltration decrease in the gastrocnemius muscle ($p=0.001$) compared with the corresponding non-treated groups (Table 1, Figure 1).

The CoQ10-treated groups of Q+IR7, Q+IR14 and Q+IR28 showed a significant decrease in gastrocnemius muscle edema compared with the corresponding non-treated groups (Table 1).

TNF- α and NF- κ B expression

The CoQ10-treated groups of Q+IR3, Q+IR7, Q+IR14 and Q+IR28 showed a significant TNF- α expression decrease ($p=0.001$) when compared to the corresponding non-treated controls (Table 2).

The CoQ10-treated groups of Q+IR3, Q+IR7, Q+IR14 and Q+IR28 showed a significant NF- κ B expression decrease ($p=0.001$) compared with the corresponding non-treated group (Table 2).

Discussion

The results of this study showed that CoQ10 administration with ischemia decreased interstitial edema, degeneration of muscle fibers and infiltration of mast cells. Moreover, this compound decreased TNF- α expression and inactivated NF- κ B. I/R induced the generation of reactive oxygen species (ROS) and apoptosis

Table 1. Data of edema are expressed as median and mast cell infiltration data are expressed as mean \pm SD.

	Edema	Mast Cell Infiltration
Q+IR3	3.25 \pm 0.463*	3.25 \pm 0.886*
IR3	3.75 \pm 0.463	5.25 \pm 1.669
Q+IR7	2.13 \pm 0.641*	3.25 \pm 0.886*
IR7	3.75 \pm 0.463	5.13 \pm 1.553
Q+IR14	1.38 \pm 0.518*	1.63 \pm 0.744*
IR14	3.38 \pm 0.916	4.13 \pm 1.808
Q+IR28	0.50 \pm 0.535*	0.75 \pm 0.886*
IR28	3.88 \pm 0.354	4.25 \pm 0.707

*Test and results considered significant at $p \leq 0.05$.

Table 2. TNF- α (an inflammatory protein) and NF- κ B (controls transcription of DNA). Data are expressed as mean \pm SD.

	TNF- α	NF- κ B
Q+IR3	9.13 \pm 3.75	2.63 \pm 0.51*
IR3	10.63 \pm 3.58	8.13 \pm 3.48
Q+IR7	1.63 \pm 0.74*	2.75 \pm 0.88*
IR7	10.13 \pm 2.16	10.13 \pm 2.16
Q+IR14	2.25 \pm 2.18*	3.88 \pm 0.64*
IR14	10.5 \pm 2.72	11.5 \pm 1.77
Q+IR28	0	0
IR28	6.63 \pm 2.32	6.63 \pm 2.32

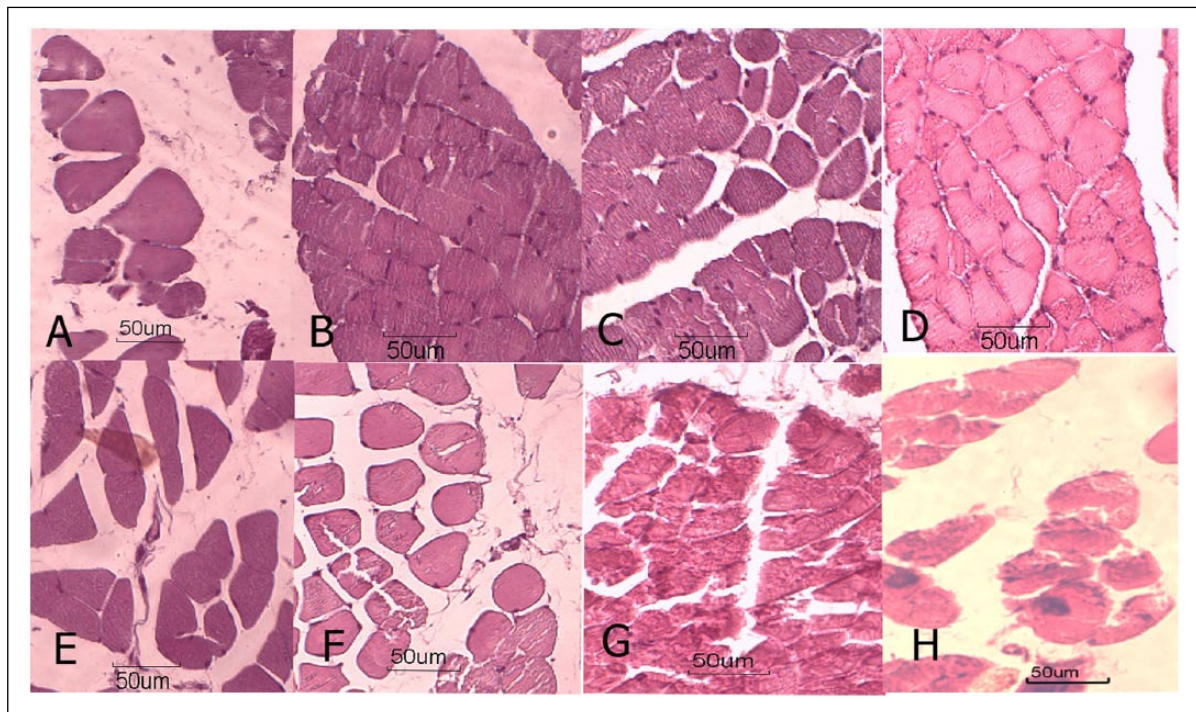
*Test and results considered significant at $p \leq 0.05$.

in muscle cells. However, the cellular source of these ROS during I/R is poorly defined. This situation could lead to the activation of NF- κ B which is activated in affected muscle cell.

IR-induced injury to muscle leads to the inflammatory response. Several studies have demonstrated that CoQ10 enhances antioxidant enzyme activities and lowers inflammation. This compound exerts anti-inflammation effects via the reduction of NF- κ B-dependent gene expression. Recent studies have suggested that one of the several possible molecular mechanisms of I/R-induced muscle cell injury could be through the induction of NF- κ B. Previous studies indicated that NF- κ B acts as an immuno-modulating factor and CoQ10 inhibits NF- κ B activation.^{12-15,19,21}

The decrease in TNF- α activation by muscle cell following CoQ10 administration observed in the present work is in accordance with the findings reported by another study which has shown that "CoQ10 prevents over-expression of TNF- α after exercise, together with an increase in TNF-RII that limits the pro-inflammatory actions of TNF".²³ Moreover, one study indicated that NF- κ B suppress apoptosis.²⁴

NF- κ B can be activated by TNF- α or the oxidant hydrogen peroxide (H₂O₂). TNF- α is a factor involved in systemic inflammation that is mainly produced by activated macrophages and activates NF- κ B as an indicator of apoptosis. NF- κ B is a transcription factor that is

**Figure 1.** Transverse section of hematoxylin and eosin stained gastrocnemius muscles.

A: Q+IR3, B: Q+IR7, C: Q+IR14, D: Q+IR28, E: IR3, F: IR7, G: IR14, H: IR28.

Scale bar: 50 μ m magnification: $\times 400$.

transported to the nucleus and initiates the transcription of many factors involved in cell survival and proliferation in response to inflammatory and anti-apoptotic factors. NF- κ B normally exists in the cytoplasm of cells in an inactive form which binds with an inhibitor protein called NF- κ B inhibitor (I κ B). During NF- κ B activation, the α subunit of I κ B (inhibitor of NF- κ B) is phosphorylated and dissociated from the inactive cytoplasmic complex. In response to TNF- α , I κ B is phosphorylated and, subsequently, degraded by the proteasome. As a result of this process, NF- κ B is activated and translocates into the nucleus and binds with promoter sites located in areas of immune-regulatory genes and activates them. The primary mechanism for regulating NF- κ B is through inhibitory I κ B proteins. This process includes translocation of the active dimers of p50 and p65 into the nucleus. Therefore, specific target genes of pro-inflammatory mediators and cytokines become immediately up-regulated. Several studies revealed that the NF- κ B-activating cascade was inhibited by antioxidants and hypothesized that oxygen radicals are key players in the activation of NF- κ B through a redox-dependent mechanism.^{23,24} The results of our study showed that CoQ10 may play an important role against muscle ischemia-reperfusion injury by the reduction of TNF- α expression. The inhibition of TNF- α activation may contribute to the myoprotective effects.

Several studies revealed that the NF- κ B-activating cascade is inhibited by antioxidants and hypothesized that oxygen radicals are key players in the activation of NF- κ B through a redox-dependent mechanism. In previous studies, CoQ10 supplementation reduced oxidative stress and prevented over-expression of TNF- α after exercise and muscle damage.²³ Moreover, CoQ10-revealed neuroprotection effects via the inhibition of mitochondrial complex-1 down-regulation and NF- κ B activation.²⁴

In general, it seems that the CoQ10 has inhibitory effects on NF- κ B and TNF- α activation.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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