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Late anti-apoptotic effect of K_{ATP} channel opening in skeletal muscle

Hossein Farahini,*† Rouhollah Habibey,‡ Marjan Ajami,‡§ Sayed Hossein Davoodi,†††‡‡ Nahid Azad,§§ Mansoureh Soleimani,*** Morteza Tavakkoli-Hosseini††† and Hamidreza Pazoki-Toroudi‡§§**

**Department of Orthopedic Surgery, †Department of Orthopedic Surgery, Rasool-e-Akram General Hospital, ‡Physiology Research Center, Tehran University of Medical Sciences, §Department of Nutrition, School of Public Health, **Faculty of Nutrition Sciences and Food Technology, Shahid Beheshti University of Medical Sciences and Health Services, ††Comprehensive Cancer Control Center, Shahid Beheshti University of Medical Sciences and Health Services, ‡‡Cancer Research Center, Shahid Beheshti University of Medical Sciences and Health Services, §§Nano Vichar Pharmaceutical Ltd, ***Department of Anatomy, Tehran University of Medical Sciences, Tehran, Iran and †††Department of Cardiothoracic Surgery, St George's Hospital, London, UK*

SUMMARY

1. Necrosis and apoptosis caused by ischaemia–reperfusion (IR) result in myocyte death and atrophy. ATP-sensitive K^+ (K_{ATP}) channels activation increases tissue tolerance of IR-injury. Thus, in the present study, we evaluated the effects of K_{ATP} channel activation on skeletal muscle apoptosis after IR.

2. Male Wistar rats were treated with 40 mg/kg, i.p., diazoxide (a K_{ATP} channel opener) or 5 mg/kg, i.p., glibenclamide (a K_{ATP} channel inhibitor) 30 min before the induction of 3 h ischaemia, followed by 6, 24 or 48 h reperfusion. At the end of the reperfusion period, the gastrocnemius muscle was removed for the analysis of tissue malondialdehyde content, superoxide dismutase (SOD) and catalase (CAT) activity, Bax and Bcl-2 protein expression, histological damage and the number of apoptotic nuclei.

3. Ischaemia–reperfusion increased malondialdehyde content ($P < 0.01$) and Bax expression ($P < 0.01$) and induced severe histological damage, in addition to decreasing CAT and SOD activity ($P < 0.01$ and $P < 0.05$, respectively) and Bcl-2 expression ($P < 0.01$). Diazoxide reversed the effects of IR on tissue damage, MDA content, SOD and CAT activity (after 6 and 24 h reperfusion; $P < 0.05$) and Bax and Bcl-2 expression (after 24 and 48 h reperfusion; $P < 0.01$). In contrast, glibenclamide pretreatment had no effect.

4. The number of apoptotic nuclei in the IR and glibenclamide-pretreated groups increased significantly ($P < 0.001$ *vs* Sham). In contrast, diazoxide pretreatment decreased the number of apoptotic nuclei compared with the IR group ($P < 0.01$).

5. The results of the present study suggest that the K_{ATP} channel activator diazoxide attenuates lipid peroxidation during the first hour of reperfusion and modulates apoptotic pathways at later time points.

Key words: apoptosis, diazoxide, ischaemia reperfusion, K_{ATP} channels, oxidative stress, skeletal muscle.

INTRODUCTION

Critical limb ischaemia is considered one of the major factors underlying peripheral arterial disease (PAD), which, in itself, is an important clinical problem that can lead to disability and limb loss.[1,2] In patients with PAD, skeletal muscle ischaemia can induce changes in muscle mass associated with impaired functioning of the limbs.[3] In addition, sustained peripheral arterial insufficiency has detrimental effects on both normal and regenerating muscles by decreasing the force produced, muscle weight and cross-sectional area of muscle fibres.[2] Ischaemic injury is common in clinical conditions such as stroke, myocutaneous tissue transfer, thrombolytic therapy and balloon angioplasty and results in tissue damage because of the restriction of the blood supply followed by its restoration.[4] Consequently, there is intense interest in the development of prophylactic treatments to reverse the effects of critical limb ischaemia.

Ischaemia–reperfusion (IR) injury is a multifactorial process that is induced by the production of reactive oxygen species (ROS), weakening anti-oxidant defence systems, neutrophil infiltration and cellular apoptosis.[2,5] Different studies have shown that one of the main reasons for muscle atrophy in patients with congestive heart failure is apoptosis of the skeletal myocytes.[6] Although Knight *et al.* suggested that necrosis is the single mechanism by which IR induces cell death in skeletal muscle,[7] recent studies demonstrate that IR triggers both necrosis and apoptosis, leading to muscle cell death.[8–10] In addition, factors that were applied to attenuate IR-induced skeletal muscle injury acted by inhibiting the development of the apoptotic process in muscle cells.[11,12]

Previous studies have demonstrated the protective effects of ATP-sensitive K^+ (K_{ATP}) channels, especially mitochondrial

Correspondence: Hamidreza Pazoki-Toroudi, Physiology Research Center, Tehran University of Medical Sciences, PO Box 14515-763, Tehran, Iran. Email: hpazooki@farabi.tums.ac.ir

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K_{ATP} (mitoK_{ATP}) channels, against cardiac ischaemic injury.[13,14] Other studies have demonstrated this effect in other organs that express mitoK_{ATP} channels, including the nervous system and skeletal muscle.[15,16] Other types of K_{ATP} channels, such as sarcolemmal K_{ATP} channels, are found in skeletal muscle tissue and some studies have revealed an important role for these channels in cardiac tissue preconditioning against ischaemic injury.[17] In addition, it has been reported that K_{ATP} channel openers can protect skeletal muscle against IR injury both as a trigger or mediator,[18,19] whereas K_{ATP} blockers decrease muscle tissue tolerance to stress.[19] Furthermore, K_{ATP} channel openers have been shown to inhibit ROS-induced apoptosis in cardiac and nerve cells after IR by downregulating apoptotic proteins, such as Bax and p53, and upregulating the anti-apoptotic protein Bcl-2.[20–23] The effects of the opening or blockade of K_{ATP} channels on the apoptosis of skeletal muscle cells after IR have not been investigated. Thus, we undertook the present study to determine whether K_{ATP} channels can affect apoptotic proteins and muscular tissue injury after IR.

METHODS

All experimental protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation of Tehran University of Medical Sciences (Tehran, Iran) and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.(http://www.nap.edu/openbook.php?record_id=12910, accessed 21 Sept 2009).

One hundred and thirty-five male Wistar rats, weighing between 200 and 244 g, were housed in groups of eight, with food and water available *ad libitum*, under a 12 h light–dark cycle (lights on from 0700 to 1900 hours) and controlled temperature (22 ± 2°C). Of these, 39 rats were used for the evaluation of apoptosis by the terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) technique in a separate series of experiments.

Induction of Ischaemia–Reperfusion (IR)

Rats were anaesthetized using pentobarbitone (45 mg/kg, i.p.; Sigma, St Louis, MO, USA). The inner side of the hind leg was then shaved and an incision made from the inguinal ligament to the insertion of the tendon calcaneus. The femoral artery and vein were exposed and clamped using a single non-traumatic clamp. This area was covered with sterile wet tissue throughout the period of ischaemia to keep the tissue wet and warm. After 3 h, the clamp was removed, blood flow was re-established (reperfusion period) and the incision was closed with three sutures. Rats were placed in separate cages at room temperature, with water and food available, and were allowed to wake up from the anaesthesia. Rats were kept in the same cage until the end of the reperfusion period. At the end of the reperfusion period (6, 24 or 48 h after removal of the clamp) rats were killed, the sutures were released and the skin was removed by T-shaped incisions so that the gastrocnemius muscle could be dissected out. Muscle tissues were cut into two sections, one for histological evaluation and the other for biochemical studies. Muscle tissue for histological evaluation was fixed immediately in 4% paraformaldehyde. The remaining muscle tissues were weighed and homogenized in cold KCl solution (1.5%) to give a 10% homogeneous suspension for biochemical analysis.

Pretreatment groups

Rats were assigned into one of three mean groups depending on the duration of reperfusion (6, 24 or 48 h), each of which was then subdivided into a further four groups, as follows: (i) sham-operated rats that underwent all surgical procedures except that the femoral artery and vein were not clamped; (ii) an IR group; (iii) a diazoxide-pretreated IR group; and (iv) a glibenclamide-pretreated IR group. Diazoxide (40 mg/kg, i.p.; Sigma) and glibenclamide (5 mg/kg, i.p.; Sigma) were administered 30 min before the induction of ischaemia.

Biochemical assays

To determine the level of oxidative stress and the effectiveness of the anti-oxidant system in each group, tissue malondialdehyde (MDA) levels and the activity of the anti-oxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were determined in solutions prepared by centrifuging homogenized gastrocnemius muscle at 15 339 *g*. for 5 min at 4°C.

Muscle tissue MDA content was determined in each group as an index of lipid peroxidation using the thiobarbituric acid (TBA) method described by Ohkawa *et al.*[24] Briefly, as a TBA-reactive substance, MDA reacts with TBA (Sigma) to produce a red complex that has maximal absorbance at 532 nm (Malondialdehyde Assay Kit; Northwest Life Science Specialties, Vancouver, Canada). The MDA content was calculated from the intensity of the red colour of the final product at 532 nm and results are expressed as nmol MDA/g wet weight tissue.[25]

Catalase and SOD activity was determined using commercially available kits (Superoxide Dismutase Activity Colorimetric Assay Kit (ab65354) and Catalase Assay Kit (ab83464); Abcam, Cambridge, UK). Catalase activity was measured spectrophotometrically in an assay based on the formation of a stable complex between hydrogen peroxide and ammonium molybdate. In contrast, the SOD assay is based on the production of a water-soluble formazan dye following reduction of WST-1 by superoxide anion. The rate of reduction by the superoxide anion is linearly related to xanthine oxidase (XO) activity, and the reaction is inhibited by SOD. In the present study, SOD and CAT activity in tissue samples is expressed as U/mg protein.

Cell proteins were extracted from 100 mg tissue in western blot lysis buffer and the samples were boiled for 5 min. The whole tissue extract was stored at –70°C until analysis. Protein concentrations were determined using a bicinchoninic acid protein assay at 560 nm absorbance (Pierce, Rockford, IL, USA). Samples were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis on a 7% gel, incubated at 4°C in 0.1 mol/L sodium phosphate buffer (pH 7.4), and incubated for 2 h with rabbit polyclonal anti-rat Bcl-2 (ab7973; Abcam), anti-rat Bax (ab7977; Abcam) or anti-rat β-actin antibodies (ab8226; Abcam). Finally, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (rabbit IgG secondary antibody; H&L-Pre-Adsorbed; Abcam) and protein bands were visualized with an enhanced chemiluminescence system

(Amersham, Freiburg, Germany).[26] The film was developed and used for the measurement of optical density.

Analysis of DNA fragmentation and apoptosis

In groups that had been subjected to 24 or 48 h reperfusion, DNA fragmentation and apoptotic nuclei were evaluated paraffin-embedded tissue sections using the TUNEL assay (ApopTag1 peroxidase kit; Intergen, Oxford, UK) according to the manufacturer's instructions. Briefly, sequential 8 μm tissue sections adhered onto slides were allowed to dry at room temperature. Sections were deparaffinized and rehydrated, and tissue sections were incubated (30 min at 37°C) in 15 μg/mL proteinase K (catalogue no. 1373196; Boehringer Mannheim, Mannheim, Germany) for protein digestion. Endogenous peroxidase was inactivated with 3% H_2O_2 in methanol for 10 min in the dark at room temperature. Then, biotin-16-dUTP (dUTP; Boehringer Mannheim, Mannheim, Germany) in TdT enzyme buffer was added to sections and samples were incubated at 37°C for 1 h in a humidified chamber. Subsequent to stopping the enzymatic reaction, sections were rinsed with phosphate-buffered saline and incubated in a humidified chamber at 37°C for 30 min. Sections were then incubated in 0.05% diaminobenzidine (DAB) solution in the dark. Finally, sections were washed with distilled water, dehydrated and mounted with Enthelan (Merck, Darmstadt, Germany).

Histological analysis

After tissues samples had been fixed in buffered 4% paraformaldehyde, slices were processed and then embedded in paraffin before being cross-sectioned longitudinally at 5 μm intervals, stained with haematoxylin and eosin and evaluated under an optical microscope. Three sections were prepared from each sample and were evaluated by a pathologist blinded to the experimental group.[27] Paraffin sections were also placed on silane-coated slides [3-(triethoxysilyl)-propylamine] for TUNEL analysis.

Statistical analysis

All data are expressed as the mean ± SEM. Two-way repeated-measures analysis of variance (ANOVA), followed by Tukey's post hoc test, was used to evaluate the significance of differences between different treatment and reperfusion (6, 24 and 48 h) groups. Two-tailed $P < 0.05$ was considered significant.

RESULTS

All animals used in the present study survived until the end of the study.

Biochemical evaluation

In the sham-operated group, MDA content were 0.86 ± 0.32, 0.82 ± 36 and 0.91 ± 41 after 6, 24 and 48 hours reperfusion, respectively. Ischaemia increased lipid peroxidation in muscle tissue, as evidenced by a significant increase in tissue MDA content after 6 and 24 h reperfusion (4.32 ± 0.9 and 2.86 ± 0.73 nmol/mg, respectively; $P < 0.01$; Fig. 1a). After 48 h reperfusion, MDA content had returned to near normal levels (1.78 ± 0.54; $P = 0.09$ *vs* the sham-operated group). Diazoxide significantly decreased IR-induced increases in tissue MDA levels after 6 and 24 h reperfusion (1.99 ± 0.81 and 1.33 ± 0.42 nmol/mg protein; $P < 0.05$ *vs* IR group), but there was no significant difference in the MDA content of the IR and diazoxide + IR groups after 48 h reperfusion ($P = 1.34$). Although, there was a tendency for glibenclamide pretreatment to increase tissue MDA content after IR at 6, 24 and 48 h, the differences failed to reach statistical significance.

After 6 h reperfusion in the IR and glibenclamide + IR groups, MDA content peaked, decreasing significantly by 48 h reperfusion ($P < 0.05$). The highest MDA content in the diazoxide + IR group was seen at 6 h, but did not differ significantly from that after 24 and 48 h reperfusion.

Compared with CAT and SOD activity in the sham-operated group, IR significantly decreased the activity of both enzymes after 6 and 24 h reperfusion ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively; Fig. 1b,c), but there was no significant difference between the two groups after 48 h reperfusion. Minimum values for SOD and CAT activity were obtained after 6 h reperfusion (0.51 ± 0.37 and 53.45 ± 16.07 U/mg protein, respectively), and the activity of both enzymes had increased significantly by 48 h reperfusion (2.07 ± 0.51 and 95.72 ± 13.2 U/mg protein, respectively; $P < 0.05$ *vs* 6 h reperfusion).

Although there was a tendency for mean CAT and SOD activity to decrease in glibenclamide-pretreated rats after 6 and 24 h reperfusion, the differences failed to reach statistical significance (Fig. 1b,c). Diazoxide pretreatment significantly increased SOD activity after 6 and 24 h reperfusion (4.59 ± 1.55 and 3.87 ± 1.32 U/mg protein, respectively; $P < 0.05$; Fig. 1b). In addition, CAT activity was significantly increased in diazoxide-pretreated rats after 6 and 24 h reperfusion (122 ± 22 and 142 ± 20 U/mg protein, respectively; $P < 0.05$; Fig. 1c). After 48 h reperfusion, there was no significant difference in CAT and SOD activity between the IR + diazoxide and IR groups.

Although there were no significant changes in CAT and SOD activity after 6, 24 and 48 h reperfusion, peak SOD activity was seen after 6 h reperfusion and peak CAT activity was seen after 24 h reperfusion.

The expression of Bax and Bcl-2 protein in each sample was normalized against that of β-actin. After 6 h reperfusion, there was no significant change in Bax or Bcl-2 expression in the IR compared with the sham-operated group. However, after 24 and 48 h reperfusion, Bax expression increased and Bcl-2 expression decreased in the IR group ($P < 0.05$). Diazoxide pretreatment significantly decreased Bax expression and increased Bcl-2 expression after both 24 h ($P < 0.01$ *vs* both the IR and sham groups; Fig. 2a,b) and 48 h ($P < 0.01$ *vs* both the IR and sham groups) reperfusion, although there was no significant difference in Bax and Bcl-2 expression between the groups after 6 h reperfusion. The expression of Bax and Bcl-2 proteins in diazoxide-pretreated rats after 24 or 48 h reperfusion differed significantly from the expression after 6 h reperfusion ($P < 0.01$; Fig. 2a,b).

Glibenclamide had no effect on Bax or Bcl-2 expression at any time point during reperfusion.

Histological evaluation

In the sham-operated group, at times equivalent to 6, 24 and 48 h reperfusion, there were no marked histological changes

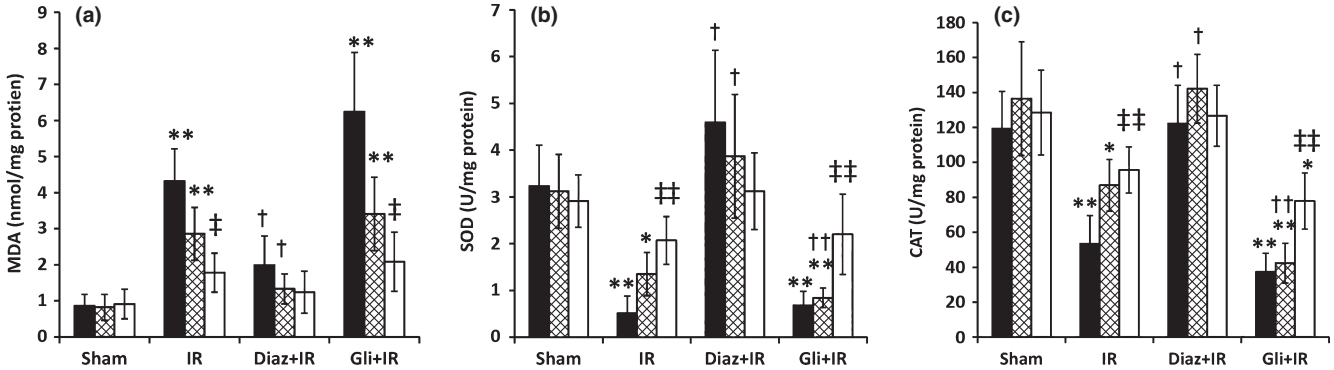

Fig. 1 (a) Tissue malondialdehyde (MDA) content as an indicator of lipid peroxidation and (b) superoxide dismutase (SOD) and (c) catalase (CAT) activity in different groups after 6, 24 and 48 h reperfusion. IR. ischaemia–reperfusion; Diaz, 40 mg/kg, i.p., diazoxide; Gli, 5 mg/kg, i.p., glibenclamide. Data are the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the sham group; $^{\dagger}P < 0.05$ compared with the IR group for the same duration of reperfusion; $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$ compared with 6 h reperfusion within the same group.

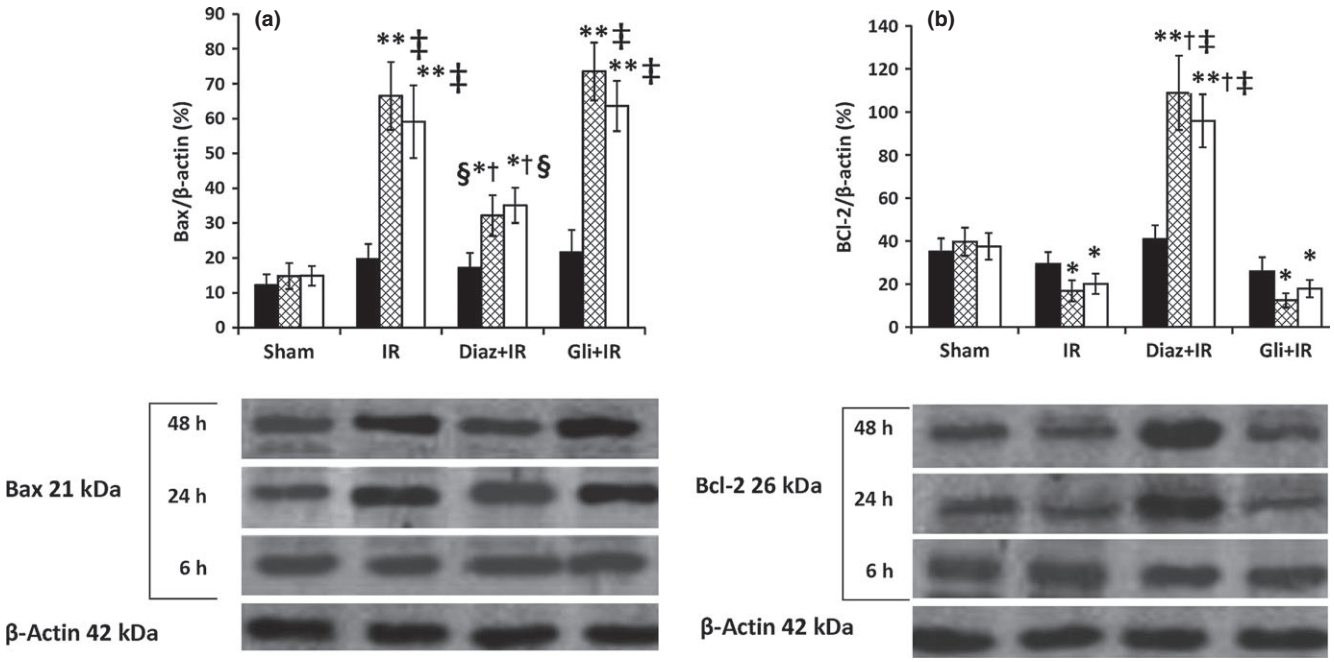

Fig. 2 Expression of (a) pro- and (b) anti-apoptotic proteins after 6, 24 and 48 h reperfusion in skeletal muscle tissue. The upper panels show mean Bax and Bcl-2 expression normalized against that of β-actin. The lower panels show Bax and Bcl-2 expression after different periods of reperfusion (normalized against β-actin). IR. ischaemia–reperfusion; Diaz, 40 mg/kg, i.p., diazoxide; Gli, 5 mg/kg, i.p., glibenclamide. Data are the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the sham group; $^{\dagger}P < 0.05$ compared with the IR group for the same duration of reperfusion; $^{\ddagger}P < 0.01$ compared with 6 h reperfusion within the same group.

evident in tissue samples of the gastrocnemius muscle (Fig. 3a). After 6 h reperfusion, the histological features in muscles from the IR, IR + diazoxide and IR + glibenclamide groups were similar, with no or only very mild changes in noted in the IR and IR + glibenclamide groups (Figs 3a, 3b). However, the histological features were markedly different between the groups after 24 and 48 h reperfusion. For example, after 24 h reperfusion in the IR group, diffuse interstitial oedema, swelling of the muscle fibres and significant neutrophil accumulation were evident (Fig. 3a), which worsened after 48 h reperfusion (Fig. 3a). Diazoxide pretreatment decreased the signs of IR-induced tissue damage. Restricted interstitial swelling and neutrophil infiltration was a common feature of most slices prepared from diazoxide-pretreated rats after 24 and 48 h reperfusion (Fig. 3b). In contrast, in the glibenclamide-pretreated group, similar histological characteristics were evident after 24 and 48 h reperfusion to those seen in the IR group: severe expansion of interstitial tissue and swelling, fragmentation and separation of the muscle fibres and marked infiltration of neutrophils (Fig. 3b).

DNA fragmentation and apoptosis

The results of TUNEL staining in the different groups are shown in Figs 4a and 4b and are summarized in Table 1. Two samples were prepared for TUNEL staining from each muscle tissue and five randomly selected fields (0.05 mm^2) from these samples were evaluated by two pathologists to determine the total number of apoptotic nuclei. The mean number of apoptotic nuclei in the sham-operated

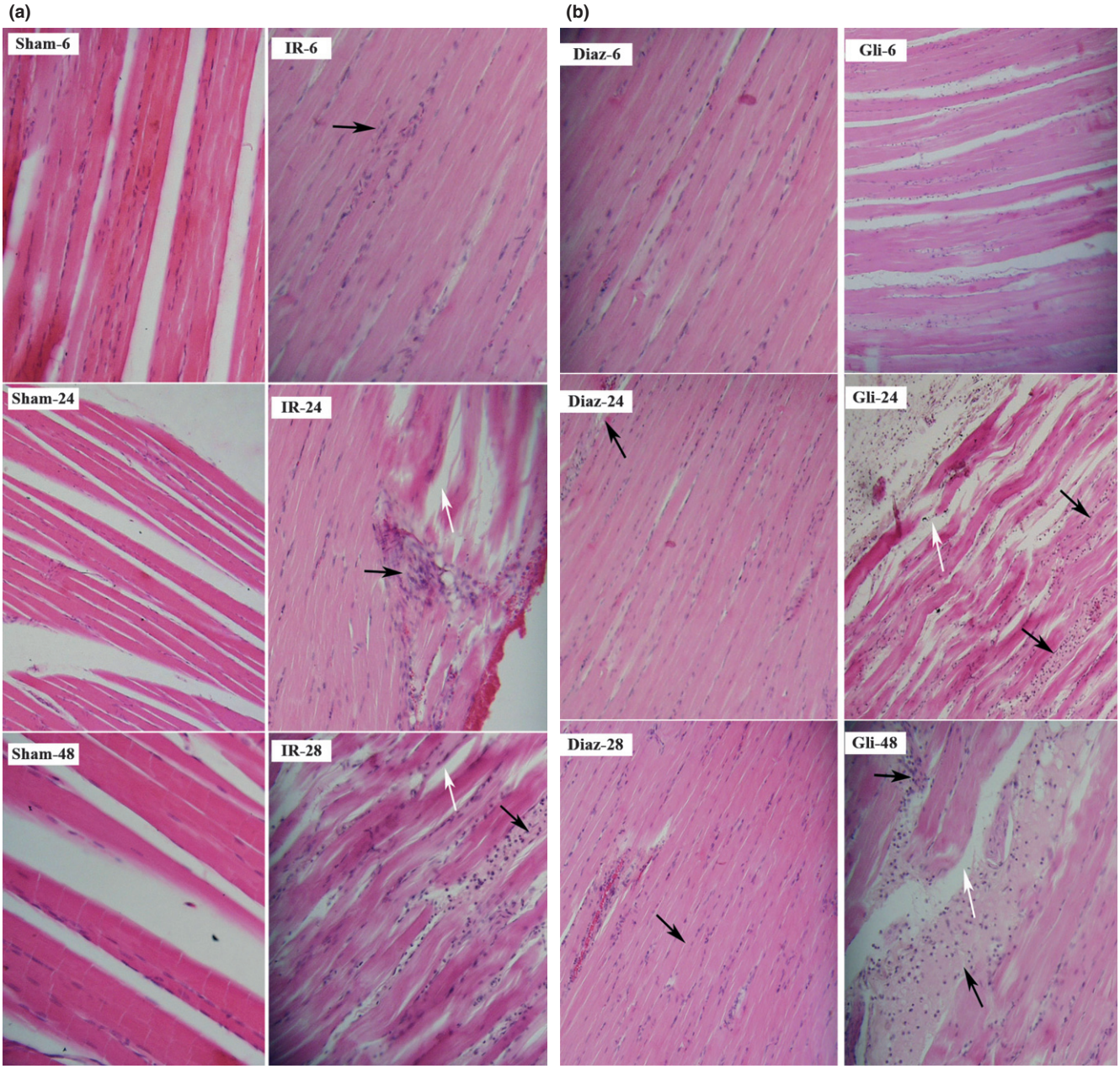

Fig. 3 Representative photomicrographs showing cross-sections of skeletal muscle samples from the sham-operated, ischaemia–reperfusion (IR), 40 mg/kg, i.p., diazoxide-pretreated (Diaz) and 5 mg/kg, i.p., glibenclamide-pretreated (Glib) groups. (a) In the sham group, all fibres are of normal appearance with several dark-stained peripheral nuclei. There are no signs of interstitial or fibre swelling or leucocyte infiltration at any time point. After 6 h reperfusion, there are no signes of oedema or neutrophil infiltration in tissues from the IR group. In contrast, after 24 and 48 h reperfusion, interstitial oedema (white arrows) is visible and fibre swelling, with disappearing nuclei, is a common feature of most samples in the IR group, concomitant with severe neutrophil infiltration (black arrows). (b) Diazoxide pretreatment decreased muscle damage after 24 and 48 h reperfusion compared with the IR group. Signs of interstitial oedema and neutrophil infiltration were seen after 24 and 48 h reperfusion, but were restricted to a few places throughout the tissue (black arrows). Fibre swelling was rarely seen in samples from the IR + Diaz group. In the glibenclamide-treated group, there were very mild signs of tissue damage after 6 h reperfusion, including dilation of the interstitial space. After 24 and 48 h reperfusion interstitial oedema was evident at different sites; indeed, swollen and distended fibres had separated from each other because of severe interstitial oedema. Visible nuclei were rare throughout muscle fibres from the IR + Glib group. (Haematoxylin and eosin stain).

group after 24 and 48 h reperfusion was 3.1 ± 0.5 and 2.7 ± 0.4, respectively. In the IR group, there was a significant increase in the number of apoptotic nuclei after 24 and 48 h reperfusion (42.9 ± 7.1 and 47.2 ± 6.5, respectively; $P < 0.001$ *vs* the sham-operated group; Table 1; Fig. 4a). Although glibenclamide pretreatment had no significant effect on the number of apoptotic nuclei after 24 and 48 h reperfusion (Table 1; Fig. 4b), diazoxide pretreatment significantly decreased the number of apoptotic nuclei per slice after 24 and 48 h reperfusion (12.2 ± 2.8 and 14.0 ± 2.6, respectively; $P < 0.01$ *vs* the IR group; Table 1; Fig. 4b). There were no significant differences in TUNEL findings within groups after 24 and 48 h reperfusion.

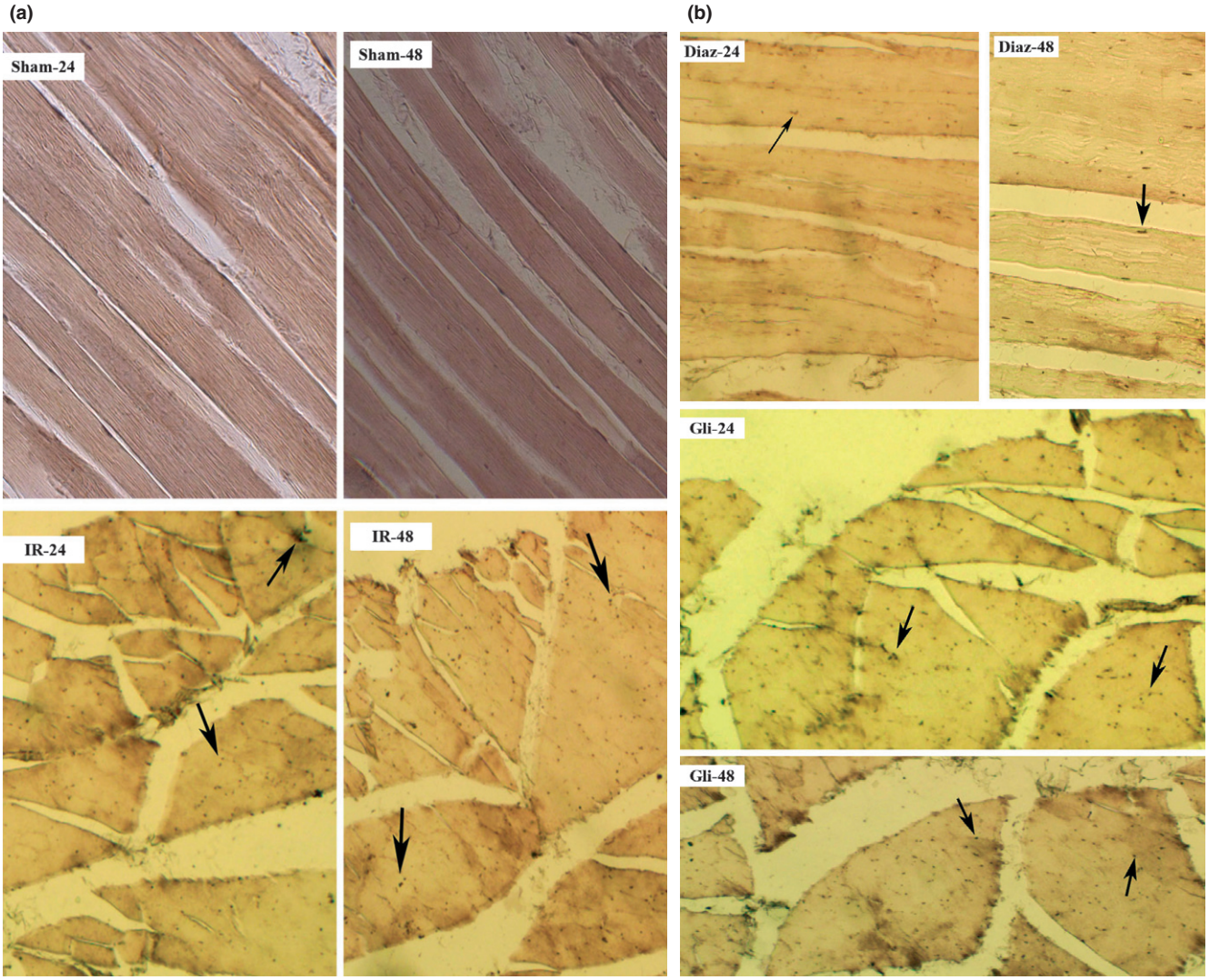

Fig. 4 Terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) staining of a cross-section of gastrocnemius muscle after 24 and 48 h reperfusion. (a) Sham operated group (original magnification × 10) and ischaemia–reperfusion group (original magnification × 40). Black arrows indicate one of many apoptotic nuclei in a selected field in a sample from the IR group. (b) Tissues from diazoxide-pretreated (original magnification × 40) and glibenclamide-pretreated rats (original magnification × 40) after 24 and 48 h reperfusion. Black arrows indicate one of many apoptotic nuclei in a selected field in samples from each group.

DISCUSSION

The main aim of the present study was to determine the effect of K_{ATP} channel opening and blockade on skeletal muscle cell apoptosis over a period of 48 h reperfusion. Diazoxide, a putative opener of mitoK_{ATP} channels, augmented the anti-oxidant defence system (during the first 24 h of reperfusion), downregulated Bax expression, upregulated Bcl-2 expression (during the last 24 h of reperfusion) and decreased the number of apoptotic nuclei after ischaemia. The peak anti-apoptotic effect of diazoxide was seen after 24 h reperfusion.

Metabolic changes in cardiac disease, such as activation of catabolic pathways and decreased anabolic capacity, are often accompanied by significant loss of lean body mass; that is, a decrease in muscle mass and atrophy. The K_{ATP} channels, which are expressed in different muscle cells, including cardiac muscle,[28] smooth muscle[29] and skeletal muscle,[30] are directly affected by the metabolic conditions within the cell and provide a feedback system between muscle cell metabolism and electrical activity.[31] Calcium overload occurs during ischaemic injury and K_{ATP} channels, by controlling muscle contractility, especially under conditions of reduced energy availability (e.g. ischaemia), can protect against calcium overload and fibre damage.[31]

Most studies have demonstrated that the opening of K_{ATP} channels contributes to the resistance of tissues against ischaemic insult.[32] In accordance with previous studies,[33,34] we have confirmed in the present study that activation of K_{ATP} channels protects the skeletal muscle against IR injury, whereas blocking K_{ATP} channels results in a worsening of IR-induced stress. In the present study, diazoxide pretreatment deceased MDA content in muscle tissue, an effect that persisted for 24 h after ischaemia and is suggestive of decreased oxidative damage. Increased activation of the antioxidant enzymes SOD and CAT is another component of diazoxide-induced protection. It has been reported

Table 1 Number of apoptotic nuclei as determined by terminal deoxyribonucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling

Group	*Z*	No. stained nuclei
24 h reperfusion		
Sham	3	3.1 ± 0.5
IR	5	42.9 ± 7.1***
IR + Diaz	5	12.2 ± 2.8*††
Glib + IR	5	52.1 ± 6.3***
48 h reperfusion		
Sham	3	2.7 ± 0.4
IR	6	47.2 ± 6.5***
IR + Diaz	6	14.0 ± 2.6**††
Glib + IR	6	60.2 ± 7.5***

Unless indicated otherwise, data are given as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the sham group

††$P < 0.01$ compared with the ischaemia–reperfusion (IR) group for the same duration of reperfusion.

IR, ischaemia–reperfusion; Diaz, 40 mg/kg, i.p., diazoxide; Glib, 5 mg/kg, i.p., glibenclamide.

that diazoxide reduces the number of leucocytes infiltrating ischaemic muscle tissue[28] and prevents ROS overproduction by mitochondrial sources, which protects cells against DNA fragmentation and apoptosis.[32]

Although it has been reported that K_{ATP} channels on sarcolemmal membranes of muscle cells have a role in diazoxide-induced protection against IR injury of the heart,[17] recent findings suggest that inhibition of sarcolemmal K_{ATP} channels could be as protective as the opening of mitoK_{ATP} channels.[35,36] Such apparent discrepancies may be due to differences in the experimental set-up and the doses of pharmacological agents used. In addition, it seems that the opening of sarcolemmal ATP channels prevents cardiomyocyte apoptosis and mitochondrial damage via an interaction between Ca^{2+} and the mitochondria.[37]

Muscle cells are multinucleated and their metabolism is compartmentalized. When apoptosis occurs in one nucleus of a muscle fibre it will not lead to cell death; rather, each muscle fibre will lose only a part of itself, resulting in atrophy.[6] In fact, it has been demonstrated that the number of apoptotic nuclei is correlated with the degree of muscle atrophy[38] and, interestingly, DNA breaks in myocytes nuclei are associated with greater caspase activity, decreased Bcl2 expression and increased Bax expression.[39] In the present study, diazoxide pretreatment significantly reversed the increased Bax expression and decreased Bcl-2 expression seen in the IR group over the 24–48 h period of reperfusion period. The relationship between oxidative stress and apoptotic status in myocytes is an indirect consequence of mitochondrial damage that triggers ROS production and apoptosis signalling.[6,40,41]

The protective effects of diazoxide pretreatment were observed after 6 h reperfusion and persisted for 48 h, which suggests the involvement of K_{ATP} channels in both the acute and chronic phases of protection against IR injury in skeletal muscle. There are often two distinct phases of protection against IR injury: the first occurs approximately 2 h after initiation of reperfusion and the second occurs after 24 h and may last for several days.[42,43] In cardiac models of IR injury, K_{ATP} channels have a role in both phases of protection.[44] In the present study, K_{ATP} channel activation prevented ROS overproduction by activation of the anti-oxidant defence system. One possible explanation for the results is that the K_{ATP} channel opener induced mild ROS production and oxidative stress prior to the ischaemic insult that activated protective mechanisms to cope with the subsequent severe oxidative stress induced by ischaemia.[45] Although high levels of ROS are detrimental to tissues, mild oxidative conditions after a brief period of ischaemia or specific types of treatments can induce the second phase of protection against IR injury.[46,47]

There was a difference in the expression of apoptotic and anti-apoptotic proteins in diazoxide-pretreated after 24 and 48 h reperfusion. This seems to be subsequent to the mild oxidative conditions in first hours of the reperfusion period. An *in vitro* study by Ichinose *et al.*[20] revealed that diazoxide-induced opening of mitoK_{ATP} channels acts as a trigger to reduce apoptotic cell death in myocytes. In the present study, there were no significant differences in skeletal muscle antioxidant enzyme activity after 48 h reperfusion between the between groups, although the ratio of Bax:Bcl-2 expression and the number of apoptotic nuclei were significantly lower in diazoxide-pretreated rats compared with the IR group.

In conclusion, the findings of the present study suggest that, in skeletal muscle, changes in oxidative conditions are responsible for the acute phase of diazoxide-induced protection, whereas the subsequent expression of protective proteins plays an important role in protecting tissue against cellular apoptosis that happens during the later phases of the reperfusion period.

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