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Brief Communication

INWARD POTASSIUM TRANSPORT SYSTEMS IN SKELETAL MUSCLE DERIVED CELLS ARE HIGHLY SENSITIVE TO OXIDANT EXPOSURE

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Abstract—Strenuous physical exercise causes a remarkable perturbation of K^+ homeostasis in skeletal muscle tissue. Potassium efflux is crucial for a number of physiological control processes; however, exercise-induced perturbation of K^+ homeostasis in skeletal muscle is suggested to be implicated in the generation of muscle fatigue. Physical exercise is also known to induce oxidative stress; a possible contribution of oxygen free radicals to the development of muscle fatigue has been hypothesized. To reveal the dose-dependent effect of oxidant exposure on inward and outward K^+ ($^{86}RbCl$) transporting systems, skeletal muscle derived L6 cells were treated with different concentrations of *tert*-butylhydroperoxide (TBOOH). We document the responses of (1) the ouabain-sensitive component of K^+ influx (Na^+,K^+ pump), (2) bumetanide-sensitive ouabain-insensitive component of K^+ influx (Na^+,K^+,$2Cl^-$ cotransporter), (3) ouabain- and bumetanide-insensitive component of K^+ influx (passive permeability of the cell membrane to inward K^+), (4) ouabain-insensitive component of K^+ efflux, and (5) passive leakage component of K^+ efflux following exposure of L6 cells to oxidant treatment. Even very low doses of TBOOH (25 μmol) caused powerful activation of the Na^+,K^+ pump. Following TBOOH treatment, activity of the Na^+,K^+,$2Cl^-$ cotransporter was remarkably inhibited. Such a treatment also significantly decreased the permeability of the cell membrane to inward flux of K^+ (passive influx). Thus, we observed that even very low doses of oxidant had remarkable specific effects on the different components of K^+ influx in the skeletal muscle derived cells. However, K^+ efflux mechanisms appeared to be rather insensitive to the extracellular oxidant challenge.

Keywords—Free radicals, Fatigue, Exercise, Ouabain, Bumetanide, Membrane, Oxidative modification

INTRODUCTION

The primary factor triggering muscle contraction is the action potential, an electrical signal based on Na^+ influx followed by K^+ efflux.[1] Human and animal studies have revealed that venous plasma K^+ concentration is markedly elevated following muscular contraction. It has been demonstrated that this K^+ is chiefly effluxed from the contracting muscle.[2] Because of remarkable increase in K^+ efflux and water influx, intense muscular contractions have been shown to result in a 6–20% decrease in intracellular K^+ concentration in the tissue.[3] The membrane potential of the muscle being primarily dependent on the potassium homeostasis of the cell membrane, considerable efflux of K^+ from the cell may impair excitation of sarcolemmal and T-tubular membranes and finally contribute to the onset of fatigue.[2] Increased extracellular K^+ and decreased intracellular K^+ concentrations may independently affect the potassium equilibrium and depolarise the muscle membrane potential.[4,5] Electron paramagnetic resonance signals observed in skeletal muscle homogenates of exhaustively exercised rats revealed that a single bout of exhaustive exercise may result in a two- to three-fold increase in stable radical concentrations in the muscle.[6] Recent studies have revealed that physical exercise is capable of inducing oxidative stress.[7–9] Using pump-perfused mouse soleus muscle and canine gastrocnemius-plantaris muscle preparations, it was shown that xanthine oxidase generated superoxides ($O_2^{\bullet -}$) may attenuate the function and enhance fatigue rate of contracting muscles.[10] Such effects of $O_2^{\bullet -}$ were not observed in the presence of a hydroxyl radical scavenger, a xanthine oxidase activity blocker or a Fe^{2+}-chelator. It was thus assumed that free radicals may be one of the factors that contribute to oxidative

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skeletal muscle fatigue.[10] Very recently, Kuo et al. hypothesized that one of the earliest cellular responses to oxidative stress is the activation of K^+ channels.[11] In this study, we investigated the effect of different doses of oxidant exposure on the primary K^+ transporting systems of skeletal muscle derived L6 cells. We document the responses of (1) the ouabain-sensitive component of K^+ influx (Na^+,K^+ pump), (2) bumetanide-sensitive ouabain-insensitive component of K^+ influx (Na^+,K^+,$2Cl^-$ cotransporter), (3) ouabain- and bumetanide-insensitive component of K^+ influx (passive permeability of the cell membrane to inward K^+), (4) ouabain-insensitive component of K^+ efflux, and (5) passive leakage component of K^+ efflux following exposure of L6 cells to different concentrations of extracellular *tert*-butyl hydroperoxide (TBOOH).

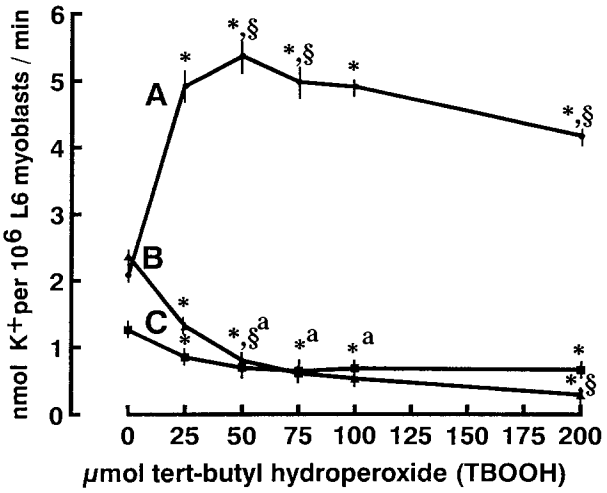

Fig. 1. Influence of different concentrations of *tert*-butylhydroperoxide (TBOOH) on different components of K^+ influx in skeletal muscle derived cells under iso-osmotic conditions. (A) 1 mM ouabain-sensitive component of K^+ influx (Na^+,K^+ pump); (B) 10 μM bumetanide-sensitive ouabain-insensitive component of K^+ influx (Na^+,K^+,$2Cl^-$ cotransporter); (C) ouabain- and bumetanide-insensitive component of K^+ influx (passive inward permeability for potassium). *Significant difference from control values obtained from cells that were prepreincubated in TBOOH free medium, $p < 0.01$. §Significant difference from values obtained from cells exposed to the preceding lower concentration of TBOOH $p < 0.01$. [a]Symbols for statistical significance are for lines B and C. Values are means ± SD of four measurements.

MATERIALS AND METHODS

Materials

L6 cells were from American Type Culture Collection (Rockville, MD). Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS), and other reagents for cell culture were purchased from Gibco laboratories (Grand Island, NY, USA). ^{86}RbCl was obtained from Amersham (Buckinghamshire, England). Ouabain, HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), TBOOH, and Tris [tris-(hydroxymethyl)aminomethane] were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of the highest purity available and were obtained from Merck AG (Darmstadt, FRG) or BDH (Poole, England). Cell culture dishes were purchased from A/S Nunc (Roskilde, Denmark).

Cell culture

Monolayer cultures were grown in DMEM supplemented with 10% FCS, 5 mM glutamine, 0.3% D-glucose, 50 U/ml of penicillin, and 50 μg/ml of streptomycin, in humidified air containing 10% CO_2 at +37°C as described earlier.[12] Cell viability was greater than 95% as estimated by trypan blue exclusion. The cells were split once every 2 days. All experiments were carried out with undifferentiated mononuclear cells (myoblast) of the 3rd to the 7th passage. Prior to 12–14 hours (lag phase of cell multiplication) of ion flux studies, cells were seeded in 24-well ($4–5 \times 10^5$ cells per well) culture dishes.

Determination of ^{86}Rb influx

The rate of ^{86}Rb influx was studied as described previously,[13,14] with minor modifications as described later. L6 cells (seeded in 24-well plates; $4–5 \times 10^5$ cells per well) were washed with 2×2 ml aliquots of medium A (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ in 10 mM Tris-HEPES buffer, pH 7.4) and prepreincubated in 0.5 ml of medium B (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 10 mM D-glucose in 20 mM Tris-HEPES buffer, pH 7.4) for 15 min at +37°C. For some wells, this prepreincubation medium contained TBOOH in the concentration as indicated in the figures. Following such a prepreincubation, the medium from each well was aspirated and cells were washed with 2×2 ml aliquots of medium B. The cells were then preincubated in 0.25 ml of medium B for 10 min at +37°C. In some experiments, this preincubation medium contained the ion transport inhibitors (ouabain and/or bumetanide and/or furosemide) in twice the concentration as indicated in the legends of Figures 1 and 2. Following the 10 min of preincubation as indicated earlier, 0.25 ml of medium B containing 4 μCi ^{86}RbCl per ml was added to each well, and the incubation was continued for another 10 min (influx kinetics is linear up to 35–45 min).[15] The influx of ^{86}Rb was terminated after the appropriate time interval by the addition of 2 ml of ice-cold medium C (100 mM $MgCl_2$

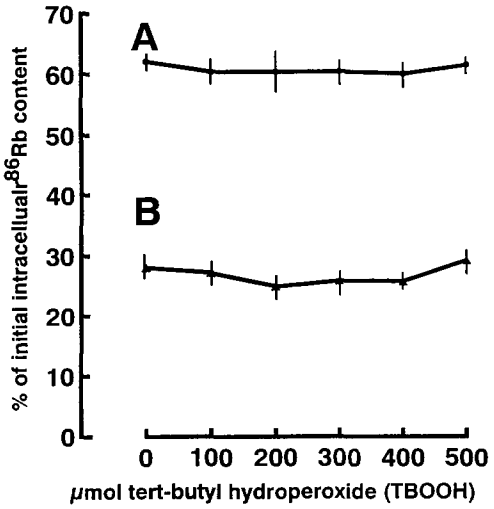

Fig. 2. Influence of different concentrations of *tert*-butylhydroperoxide (TBOOH) on different components of K^+ efflux from skeletal muscle derived cells under isoosmotic conditions. Cells were prelabelled by 4 h incubation in ^{86}RbCl at +37°C. The amount of ^{86}Rb thus loaded into the cell is referred to as initial intracellular ^{86}Rb content. Results express the percentage of initial intracellular ^{86}Rb extruded. (A) control, incubation medium contained 1 mM ouabain (represents net K^+ efflux); (B) incubation medium contained 1 mM ouabain + 10 μM bumetanide + 1 mM furosemide (represents "passive leakage" of K^+). No significant effect of oxidant exposure was observed. In addition to the doses indicated in the figure, the following doses of extracellular TBOOH were also investigated: 25, 50, and 75 μmol. Data are mean ± SD of four measurements.

in 5 mM Tris-HEPES, pH 7.4). Dishes were immediately placed on ice and the cells were washed with 3 × 2 ml of ice-cold medium C. Following the aspiration of medium C, 1 ml of 5% sodium dodecyl sulfate (SDS) solution containing 2 mM Na_2-EDTA was added to each well to obtain the cell lysate. The cell lysate followed by 4 ml of OptiPhase 'HiSafe'3 (LKB Wallac, Finland) was then added to scintillation vials. Radioactivity in the incubation medium and in the cell lysate was determined by a 1214 RACKBETA liquid scintillation counter (LKB Wallac, Finland). The rate of ^{86}Rb influx (nmol per 10^6 cells per min) was calculated as $(A)\cdot(a\cdot n\cdot t)^{-1}$, where A is the radioactivity (cpm) in cell lysate, a is the specific radioactivity (cpm · $nmol^{-1}$) of the incubation medium, n is the number of 10^6 cells per well, and t is the time interval (in minutes) of incubating the cells with the isotope.

Determination of ^{86}Rb *efflux*

L6 cells (seeded in 24-well plates; 4–5 × 10^5 cells per well) were prelabelled with ^{86}Rb by incubating the cells with 0.5 ml per well of medium B containing 4 μCi of ^{86}RbCl per ml. The incubation was carried out at +37°C and lasted for 4 h. Following the time interval of incubation, the incubation medium was aspirated and replaced by the same medium with or without TBOOH. Such an incubation with the oxidant supplemented medium was continued for 15 mins at +37°C. The 24-well culture plates were then placed on ice, and the radioactive incubation medium was aspirated. Subsequently, the cells were washed with 4 × 2 ml aliquots of medium A. To initiate ^{86}Rb efflux, 1 ml of prewarmed (+37°C) medium B containing 1 mM ouabain (and other ion transport inhibitors as indicated in the legend of Fig. 2) was added to each well. Cells were incubated at +37°C for 5 min (efflux kinetics is linear up to 15 min; our unpublished observation). Following appropriate time interval of incubation, 1.5 ml of an ice-cold medium A was added to each well, and 1 ml of the medium (overlaying buffer) from each well was transferred to scintillation vials. To determine the amount of ^{86}Rb still remaining within the cells, cell lysates were obtained as described earlier and transferred to scintillation vials. Values of ^{86}Rb efflux were expressed as the percent of initial ^{86}Rb content (as a result of prelabelling) in the cells and was calculated as $(100\cdot A_1)\cdot(A_1+A_2)^{-1}$, where A_1 is the radioactivity in the withdrawn medium (overlaying buffer), and A_2 is the residual radioactivity in the cell lysate.

Results are expressed as mean (± SD) of four measurements. The difference between means was tested by Student's t-test for unpaired data.

RESULTS AND DISCUSSION

Oxidative stress to tissues, for example, skeletal muscle, may be induced by reactive oxygen species (i) generated within the cell, and/or (ii) originating from an extracellular source either close to the tissue or having a half-life long enough to allow distant transport and damage. Physical exercise is known to increase plasma level of lipid peroxides.[8] High levels of lipid peroxides in the plasma reflect free radical damage to tissues. Lipid peroxyl radicals (ROO·), a lipid peroxidation byproduct, is also a member of the reactive oxygen species family that has sufficient energy and stability (half-life 7 s)[16] to initiate further lipid peroxidation reaction at structures distant from their point of origin. Plasma lipid peroxyl radicals are, therefore, a potent inducer of extracellular oxidative challenge. Michna[17] showed that skeletal muscle tissue, damaged by physical exercise, releases chemotactic signals that invite the mass invasion of leukocytes. Myeloperoxidase and NADPH oxidase dependent superoxide production in neutrophils is a physiological process having microbicidal activity. Neutrophils rep-

resent 50–60% of the total circulating leukocytes, and Smith et al.[18] have shown that a single bout of exercise may remarkably increase the production of reactive oxygen species by the neutrophils. Thus, marginated neutrophils may serve as a potent inducer of extracellular oxidative challenge to the muscle tissue. Another major possible source of extracellular reactive oxygen species that may threaten the muscle tissue is the endothelial cell. Localized in close proximity of the muscle tissue, the capillary endothelial cells host the production of xanthine oxidase dependent superoxides and nitric oxide synthase dependent nitric oxides. Superoxides and nitric oxides may react to form the peroxynitrite radical. Similar to the hydroxyl radicals ($^{\bullet}OH$), peroxynitrites ($ONOO^{\bullet-}$) are highly deletorious. A relatively long half-life (0.05^{-1} s of $ONOO^{\bullet-}$ vs. 10^{-9} s of $^{\bullet}OH$)[16] of peroxynitrites allow them to incite oxidative damage to structures distant from their origin. TBOOH treatment does not only induce extracellular oxidative challenge. Chance et al.[19] have discussed biological membranes are highly permeable to hydroperoxides and that addition of TBOOH or cumene hydroperoxide to tissues or cells is especially effective in inducing intracellular oxidative challenge. Previously we have reported that treatment of L6 cells with 150 μM of TBOOH resulted in a rapid decrease in intracellular glutathione content.[12]

In humans, oxygen accounts for 65% of the total body weight. On average, a rat cell processes 10^{12} O_2 molecules daily.[20] The leakage of partially reduced forms of oxygen at rest has been estimated to be about 2%,[20] yielding about 33 mmol of $O_2^{\bullet-}$ and H_2O_2 per 10^{12} cells per day. In the normal anesthetized rat, the liver produces 380 nmol H_2O_2 per min per gram tissue.[19] During physical exercise, oxygen flux through the active skeletal muscle tissue increases by $\sim 10^2$ folds with a $\sim$30 fold increase in the rate of blood flow and a $\sim$3 fold increase in arteriovenous O_2 difference.

In erythrocytes and Ehrlich ascites cells, the ouabain-insensitive bumetanide sensitive pathway of K^+ influx was identified as an electroneutral transport system for $Na^+,K^+,2Cl^-$ cotransport.[21,22] Using human erythrocytes it was shown that bumetanide and DIOA ([dihydroindenyl]oxy alkanoic acid) are selective inhibitors of $Na^+,K^+,2Cl^-$ and K^+,Cl^- cotransport, respectively, whereas furosemide at concentrations 0.5–1.0 mM inhibits both of the above-mentioned ion transport pathways.[23] Our results suggest that the $Na^+,K^+,2Cl^-$ cotransporter is highly susceptible to oxidant challenge. At very low doses (25 μmol) of the oxidant, the capacity of the cotransporting system to transport K^+ into the cell was remarkably reduced. Increase in the concentration of TBOOH to 50 μmol caused further inhibition of the cotransporter. However, when the concentration of TBOOH was further increased to 75 μmol or 100 μmol, such a dose-dependent response was not evident. When present at a concentration of 200 μmol, TBOOH dependent inhibition of $Na^+,K^+,2Cl^-$ cotransport was significantly higher compared to that caused following 100 μmol of oxidant treatment. No effect of DIOA on ^{86}Rb influx in L6 myoblasts was observed in the presence of bumetanide in the incubation medium (our unpublished observation). Such an observation indicates negligible activity of K^+,Cl^- cotransport in the L6 myoblasts studied. Therefore, the ouabain + bumetanide insensitive component of K^+ influx was interpreted as the passive permeability of the cell membrane toward the inward flow of K^+ (Fig. 1, line C). Passive inward flow of K^+ was also affected by the oxidant exposure. A significant decrease in such flow of the cation was observed following exposure of the cells to 25 μmol of TBOOH. Oxidant-dependent decrease in passive inflow of K^+ and decreased inflow of K^+ via the $Na^+,K^+,2Cl^-$ cotransporter did not appear to have any remarkable net impact on the inward flow of K^+ in the L6 cells. This is primarily because of the fact that exposure to the oxidant (25 μmol) resulted in a strong activation of the Na^+,K^+ pump (Fig. 1, line A). Such a response of the pump may be compensatory in nature, the activation may have been triggered by the sharp decline in the activity of $Na^+,K^+,2Cl^-$ cotransporter- and passive inflow-dependent inward transport of K^+. Activation of the pump was further increased when the concentration of extracellular TBOOH was increased to 50 μmol. However, with further increase in the concentration of the oxidant, a slight decrease in the activity of the pump was observed (Fig. 1, line A). Previously it has been observed that following exercise, activity of the pump increases considerably. The primary factors underlying the activation of the pump were described to be as follows: (i) rise in intracellular Na^+, and (ii) action of several hormones (adrenaline, noradrenaline, etc.) mediated via β_2-adrenergic mechanisms.[24,25] Our study indicates that reactive oxygen species (ROS), generated in excess in the muscle during exercise, are capable of activating the Na^+,K^+ pump as well. However, as described previously large increases in extracellular K^+ and intracellular Na^+ concentrations, as are observed following strenuous muscular exercise, are because perturbation of ionic fluxes during exercise exceed the capacity of the Na^+,K^+ pump to maintain the ionic homeostasis.[24,26] Although ROS may directly interact with the cation transport systems to cause the inhibition and activation as described earlier, such effects may be second messenger dependent as well.

Membrane changes caused by ROS may activate second messengers like protein kinases, cAMP or NO. ROS themselves, either as H_2O_2 or generated by the immunomodulatory cytokine tumor necrosis factor α, may serve as second messengers as well and are known to be capable of activating the cytosolic multisubunit transcription factor NF-$_K$B.[27] Previously we have shown that Na^+/H^+ exchanger and Ca^{++} uptake pathway (s) in L6 cells are activated by guanosine triphosphate binding proteins, a second messenger-dependent mechanism. However, the activities of Na^+,K^+ pump, $Na^+,K^+,2Cl^-$ cotransporter or inward K^+ leakage in the muscle derived cell were not modulated by the guanine nucleotide binding proteins.[15] Phosphorylation of K^+ channel proteins by protein kinases is also known to modulate K^+ current in tracheal smooth muscle cells.[28]

In experiments related to the study of ^{86}Rb efflux from prelabelled L6 myoblasts, 1 mM ouabain was added to the incubation medium to block Na^+,K^+ pump mediated efflux of K^+ by way of K^+/K^+ exchange.[29] Unlike the inward K^+ transporting systems, K^+ efflux was indifferent to the oxidant exposure. Doses of TBOOH, as high as 500 μmol, did not cause any change in the rate of K^+ efflux from the L6 cells (Fig. 2). No change in passive leakage of K^+, a concentration gradient favored movement of the cation, from cells exposed to the oxidant suggests that the exposure protocol perhaps did not cause any nonspecific membrane damage. Because exposure to the oxidant did not result in any remarkable alteration of net K^+ influx and efflux it appears that ROS dependent enhanced fatigue rate[10] may not be mediated by the perturbation of intracellular K^+ homeostasis.

This study presents unique evidence suggesting that brief exposure to even very low levels of ROS may act as a powerful activator and inhibitor of the Na^+,K^+ pump and $Na^+,K^+,2Cl^-$ cotransporter in muscle derived cell, respectively. Also, low dose oxidant treatment reduced passive permeability of the L6 cell membrane to inward K^+. Understanding of the actual mechanism of such specific and sensitive modulation of K^+ influx systems by oxidants demands further studies.

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ABBREVIATIONS

DIOA—[dihydroindenyl]oxy alkanoic acid
DMEM—Dulbecco's Modified Eagle Medium
EDTA—[Ethylenedinitrilo]tetraacetic acid
FCS—Fetal calf serum
HEPES—(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)
SD—standard deviation
SDS—sodium dodecyl sulfate
Tris—[tris-(hydroxymethyl)aminomethane]
TBOOH—*tert*-butylhydroperoxide