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**ORIGINAL ARTICLES**

# Eplerenone repolarizes muscle membrane through Na,K-ATPase activation by Tyr10 dephosphorylation

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**Eplerenone, an aldosterone antagonist, repolarizes muscle membrane in-vitro and increases strength in-vivo in channelopathies. In Duchenne dystrophy, it is administered for cardiomyopathy. We studied its mechanism of action on skeletal muscle to test its suitability for increasing strength in Duchenne dystrophy.**

**Using membrane potential measurements, quantitative PCR, ELISA, and Western blots, we examined the effects of eplerenone on skeletal muscle Na,K-ATPase.**

**The repolarizing effect of eplerenone in muscle fibres was counteracted by oubain, an ATPase blocker. In our experiment, ATPA1A mRNA and total ATPase protein were not elevated. Instead, Tyr10 of the α1 subunit was dephosphorylated which would agree with ATPase activation. Dephosporylation of the coupled Akt kinase corroborated our findings.**

**We conclude that eplerenone repolarizes muscle membrane by Na,K-ATPase activation by dephosphorylation at Tyr10. Since ATPase protein is known to be compensatorily increased in Duchenne patients without activity change, eplerenone treatment may be beneficial.**

**Key words:** eplerenone, ATPase, Duchenne muscular dystrophy

## Introduction

In X-linked Duchenne muscular dystrophy, elevated sodium conductance with intramuscular sodium accumulation and subsequent osmotic oedema has been suggested to be pathogenetically relevant (1). Eplerenone, an aldosterone antagonist which could reduce tissue sodium content, has been shown to reduce fibrosis and improve cardiac function in Duchenne boys (2). Since eplerenone is specific to the mineral corticoid receptor, the risk for glucocorticoid myopathy, a limiting factor of the standard therapy, would be negligible. A possible positive effect of eplerenone on the strength of skeletal muscle has been suggested (3). To identify a possible mechanism of rapid, non-genomic action and strengthen a putative suitability of the drug for treatment of weakness in Duchenne dystrophy, we examined its effect on the Na,K-ATPase in skeletal muscle.

## Materials and methods

**Membrane potentials.** Rattus norvegicus animals were sacrificed by CO2-asphyxiation in accordance with German animal protection law (TierSchG) and reported to the Animal Protection Office of Ulm University. Samples of whole rat diaphragm with attached rib fragments and central tendon were kept in carbonate-buffered saline solution of 300 ± 5 mOsmol/L containing 2 mM K+ and 1:500 Dimethyl sulfoxide (DMSO) for up to 4 h during the course of the experiment. To mimic depolarization with sodium overload as observed in Duchenne patients (1), 10 µM of a sodium/potassium ionophore (gramicidin) was added. To prevent a repolarization by ATP depletion during the course of our long-lasting experiments, 4 µM of a blocker of ATP-sensitive potassium channels (glibenclamide) was also added. This combination of drugs has been successfully used in an in-vitro model of Duchenne muscle in the past and enables comparison of our results to previous work (3). For testing of the drug, 20 mg/L eplerenone with and without 10 µM ouabain (Na,K-ATPase blocker) were tested. Incubation time was 30 min. Sharp electrodes with resistance of 5-8 MΩ were used to measure resting membrane potentials of 6-31 fibres per sample. Data are mean of means and standard error.

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**Quantitative polymerase chain reaction (qPCR).** C2C12 cells were cultured in growth medium containing Dulbecco's Modified Eagle Medium (DMEM) with 10% bovine serum at 37°C and 10% CO2. At 95% confluency, medium was replaced by DMEM with 10% horse serum to initiate myotube fusion. On day 6 after beginning of fusion, medium was replaced with serum-free DMEM and 1:500 DMSO and cells kept at 37°C and 10% $CO_2$. 24h later, cells were subjected to fresh solutions with or without 20 mg/L eplerenone for 30 min. To enhance eplerenone effect, 10 nM aldosterone was added. Total ribonucleic acid (RNA) was harvested with RNeasy Mini-Kit and cleaned in a QIAshredder. Using commercial primers from QIAgen for ATP1A1 (Cat.No. PPM04163A) and ACTB (Cat.No. PPM02945B; as control) in the OneStep reverse transciptase(RT)-PCR and SYBR Green Kit, qPCR was performed. The threshold cycle numbers for significant amplification, $C_t$-values, were averaged and the average $C_t$-value of the housekeeping ACTB subtracted ($\Delta C_t$).

**Enzyme-linked Immunosorbent Assay (ELISA).** Cultured C2C12 myotubes were lysed with urea buffer and further cleaned with a QIAShredder column. 50 µg of protein was loaded onto a 96-well high-binding plate and stored over night at 4°C. Wells were incubated with 100 µg commercial primary (for α1 subunit of total Na,K-ATPase and of phospho-Na,K-ATPase phosphorylated at Tyr10, ser16, and ser943; all four antibodies form Cell Signalling, Leiden, NL) and HRP-conjugated secondary antibodies for 2h each at 37°C and washed with Tris-buffered saline with 1% Tween 20 (TBST). Bound antibodies were incubated with 150 µl of 3,3',5,5'-Tetramethylbenzidine, the reaction was stopped after 15 min with 50 µl of 2M $H_2SO_4$ and immediately measured. Values for phosphorylation sites were normalized to total protein and their phosphorylated control.

**Western Blot.** Cultured C2C12 myotubes were lysed with Tris-Trition buffer. The supernatant was run on a 9% sodium dodecyl sulphate polyacrylamide gel, blotted to nitrocellulose membrane, and incubated overnight at 4°C with commercial antibodies for Akt, pAkt S473 and pan-Actin (all three from Cell Signalling, Leiden, NL). After washing in TBST, membranes were incubated for 2h with IRDye800CW (Li-Cor Biosciences, Bad Homburg, GER) and washed. Evaluation was performed with Image Studio Lite 5.2 and with normalization to actin.

**Statistics.** Data are average with standard deviation unless stated otherwise. Significance was tested using student's t-test.

## Results

Resting membrane potentials of diaphragm samples were -85.2 ± 4.9 mV (n = 20 diaphragms with altogether 290 myofibres) for the DMSO control, whereby 3.2 ± 1.1% of fibres were more depolarized than -70 mV (P2 fraction). Addition of gramicidin and glibenclamide (to mimic the situation in Duchenne) resulted in depolarization to -70.0 ± 4.2 mV (n = 12 with altogether 204 myofibres, $p < 0.0001$) with 42.9 ± 4.5% ($p < 0.0001$) of fibres in the P2-fraction (Fig. 1A). Incubation with eplerenone repolarized samples by 5.9 mV to -75.9 ± 3.7 mV (n = 9 with altogether 182 myofibres, p = 0.002) and concomitantly decreased P2 by more than half to 19.2 ± 5.1% (p = 0.002, Fig. 1B). To test whether the Na,K-ATPase was involved in the repolarization, we added its blocker, ouabain, which depolarized samples by 3.8 mV to -72.1 ± 7.5 mV (n = 25 with altogether 284 myofibres, p = 0.04) and increased the P2-fraction to 36.3 ± 5.5% (p = 0.11, Fig. 1C). Therefore, oubain eliminated the repolarization effect almost completely suggesting that the Na,K-ATPase may be the effector for repolarization by eplerenone.

To test, whether eplerenone, which has a typical steroid structure, increases transcription of ATPase subunits during the 30 min eplerenone incubation, we performed quantitative RT-PCR of ATP1A1 encoding its α1 subunit. However, even after pre-incubation for 24 h with 10 nM aldosterone expression to maximize effects, eplerenone did not lead to significant changes of ATPA1A expression compared to DMSO control with $\Delta C_t$ values of 12.8 ± 2.8 *vs* 12.4 ± 1.8 (n = 3; p = 0.45).

In the ELISA, incubation with eplerenone did not change total ATPase protein amount which was in agreement with unchanged transcription. Therefore, we postulated a non-genomic effect such as secondary modification of this ATPase subunit. Three phosphorylation sites of different metabolic pathways were considered: i) Ser16 which affects ATPase trafficking (4) and is phosphorylated by the aldosterone-inducible protein kinase C (PKC) (5), ii) Ser943 which affects ATPase activity (6) and is phosphorylated by aldosterone-inducible protein kinase A (PKA) (7), and iii) Tyr10 which affects ATPase activity (8) and is phosphorylated by Lyn kinase which also phosphorylates Akt (9), a kinase that is dephosphorylated by aldosterone (10). Na,K-ATPase α1 subunit phosphorylation at Ser16 or Ser943 was unchanged (Fig. 1D). In contrast, protein phosphorylated at Tyr10 was significantly reduced (by 56.1 ± 29.5% compared with DMSO control; n = 4, p = 0.038, Fig. 1D). To support this finding, we tested the phosphorylation of a coupled protein, Akt kinase, in Western blot. Total Akt protein was unchanged, but its phosphorylation at Ser473 was decreased significantly to 46 ± 57% of the DMSO control (n = 14; p = 0.01, Fig. 1E).

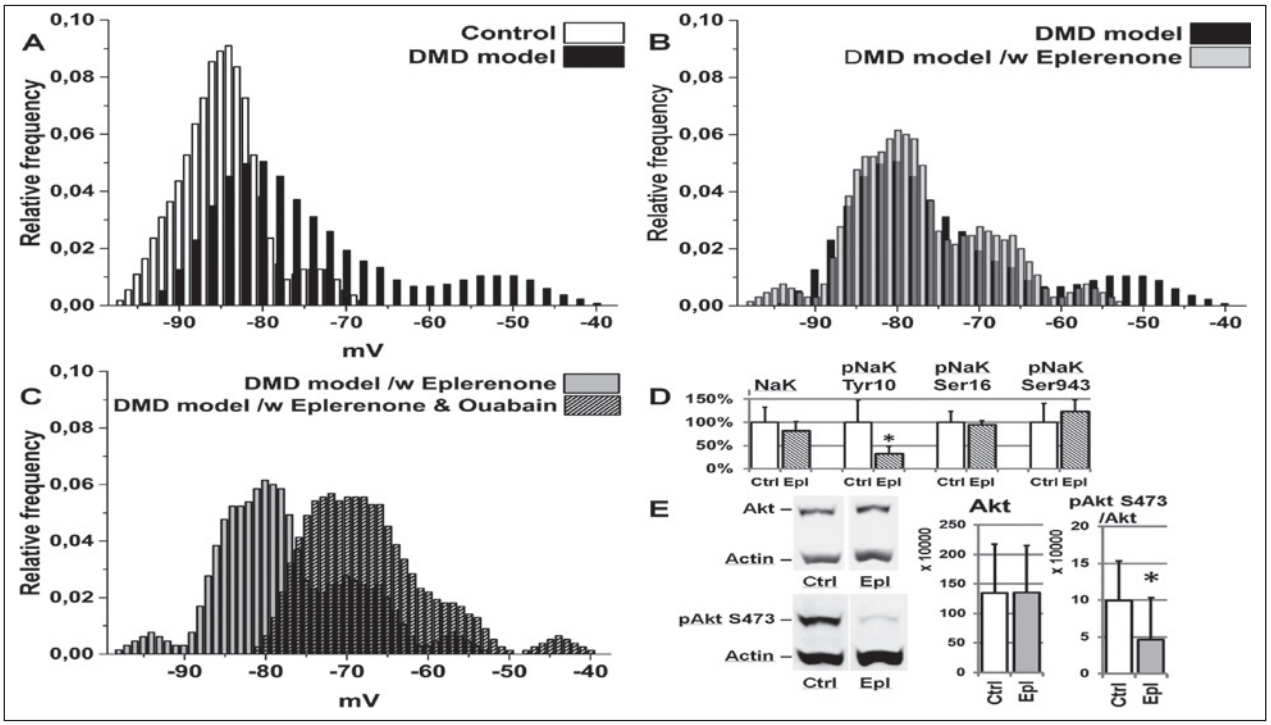


**Figure 1.** (A-C) Smoothed histograms of relative frequency of resting membrane potentials of all myofibres of all rat diaphragm samples. For smoothing, three data histograms shifted by 1 mV each (class width of 3 mV) were added together (n = 182-290). (A) Depolarization in DMD model caused marked depolarization compared to control; (B) Eplerenone repolarised large portion of fibres; (C) Ouabain counteracted the eplerenone effect. (D) ELISA. Original blots for Extinction normalized to total protein and phosphorylated control respectively. Data are mean and standard deviation. Total Na,K-ATPase (82.2 ± 20.0%, n = 4) and phosphorylation of Ser16 (95.2 ± 24.9%; n = 4) and Ser943 (123.4 ± 24.9%; n = 4) remained unchanged, while phosphorylation of Tyr10 was significantly reduced (56.1 ± 29.5%; n = 4; p = 0.038) after eplerenone incubtation. (E) Western blot. Original blots of Akt (1:1.000) and phosphorylated Akt (1:500) with actin (1:10.000) as standard (left). Intensities normalized to actin and phosphorylated protein relative to total amount (right). Data are mean and standard deviation. Akt remained unchanged (100 ± 59%; n = 18), while phosphorylation at Ser473 was significantly reduced to 46 ± 57% (n = 14; p = 0.01) after 30 min eplerenone incubation.

## Discussion

The degree of depolarization in our gramicidin/glibenclamide model and the degree of repolarization by eplerenone in this study are consistent with an earlier report (3). Also, the oubain blockage of the eplerenone-induced repolarization in skeletal muscle strongly suggests Na,K-ATPase activation which is in agreement with a study showing that eplerenone counteracts oubain-induced decrease of Na,K-ATPase current density in heart muscle (11).

To avoid transcription-mediated effects by eplerenone, we set our maximal eplerenone incubation time to 30 minutes. Our RT-PCR findings confirmed that during this incubation time, no changes of transcription of the Na,K-ATPase genes took place. In agreement with this, the ELISA did not show any change in ATPase protein abundance, i.e. no translation changes either. Therefore, a modulation of ATPase activity may be the source of the repolarizing effect of eplerenone. WE considered a representative phosphorylation site of the three kinases PKA, PKB, and PKC.

Ser16 is phosphorylated by PKC and regulates membrane trafficking of the Na,K-ATPase (4, 5). Previously, aldosterone has been shown to activate PKC-signalling and this pathway was inhibited in cardiomyocytes by eplerenone after 24 h (12). We found no change in ser16 phosphorylation after an incubation time of 30 minutes and deduced that inhibition of this pathway may not be a rapid effect of the drug. However, in long-term therapy, this effect could contribute to fibrosis inhibition and improvement of cardiac function found in the aforementioned clinical trial (2).

Ser943 is phosphorylated by the aldosterone-inducible PKA which regulates Na,K-ATPase (6, 7). However,

we show that eplerenone does not change phosphorylation at this site. This could be due to either short incubation time with drug, or the lack of pre-incubation with aldosterone. Either way since pre-incubation with aldosterone was not required for repolarization in the membrane potential measurements of native rat myofibres, modification of Ser943 phosphorylation does not explain the rapid repolarization we found in the in-vitro Duchenne model.

In contrast, Tyr10 phosphorylation of Na,K-ATPase $\alpha$1 subunit was reduced by eplerenone. Since Tyr10 phosphorylation reduces ATPase activity (14), we assume that dephosphorylation will increase its activity. Further, since Duchenne muscle has increased Na,K-ATPase protein quantity without relevant single-protein activity change (15), the rapid effect of eplerenone on Tyr10 which increases ATPase activity could ameliorate muscle weakness in Duchenne dystrophy by repolarizing muscle membrane.

To our knowledge, this is the first report of regulation of Tyr10 phosphorylation by eplerenone. Since Tyr10 is phosphorylated by Lyn kinase (8) and dephosphorylated by protein tyrosine phosphatase 1B (14), our study additionally implicates, for the first time, that the Lyn kinase and/or the protein-tyrosine phosphatase 1B may be regulated by eplerenone.

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