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Benefits of alpha-ketoglutarate versus succinate on rat muscle dysfunction as a result of exposure to a uremic environment

Else M. Bartels[1], Stefan G. Pierzynowski[3], Adrian P. Harrison[2]

[1] *Copenhagen University Library and The Parker Institute, Denmark*
[2] *Institute of Animal and Veterinary Basic Sciences (IBHV), Faculty of Life Sciences, Copenhagen University, Denmark*
[3] *Department of Comparative Physiology, Lund University, Sweden*

Abstract: Muscle weakness is a prominent feature of end-state renal failure. While the cause of this strongly disabling muscle condition is at present unknown, there are suggestions that metabolic factors may play a role in this type of muscle fatigue. *In vitro* measurements of muscle function of the fast-twitch *extensor digitorum longus* (EDL) muscle of adult rats was performed. Isolated muscles were exposed to either a normal ionic environment, a uremic environment – with and without alpha-ketoglutarate (AKG), or with and without succinate, before being field stimulated until they were almost totally fatigued. The addition of AKG to the uremic environment was found to restore muscle performance so that the muscles no longer differed significantly from those incubated in a normal ionic environment. Similar effects to those noted for AKG were observed for succinate. It is concluded that AKG and succinate have a positive and restorative effect on muscle fatigue in uremic fast skeletal muscles *in vitro*. This beneficial form of treatment is proposed to act at the *in vitro* isolated muscle level by means of phosphate-binding, as the literature shows that an elevated plasma P_i concentration with renal failure disrupts normal muscle function.

Key words: uremia, phosphates, succinate, alpha-ketoglutarate

INTRODUCTION

In a recent study [1], we confirmed earlier reports of muscle abnormalities reported in patients with end-state renal disease (ESRD; uremia) [2, 3]. Our studies showed a clear pattern of fatigue in fast muscles in uremic patients with direct measurements using surface EMG and *in vitro* studies of rat muscles incubated in a medium based closely on typical hemodialysis patient values.

Arguably, one of the main differences between the ionic environment of muscle in a uremic condition compared to the normal one is an elevated concentration of inorganic phosphate and urea [1]. The increase in concentration of urea will create a higher osmolarity, which in turn may have an effect on the propagation of action potentials. In metabolic terms, on the other hand, urea may be considered inert. This leaves the inorganic phosphate (P_i) as a more likely candidate, capable of affecting muscle function through changes in cell metabolism. An elevated concentration of plasma phosphate results in an elevated phosphate concentration inside the cell. Furthermore, phosphates are charged and thereby may affect the charge distribution at the cell membrane, apart from any osmotic effect created by an abundance of P_i in general. To date, it is known from studies with genetically modified mice ($CK^{-/-}$), that fast-twitch skeletal muscles display an increased myoplasmic P_i concentration at rest, and show a markedly lower force than that of wild-type mice [4]. Moreover, the work of Millar and Homsher [5] shows that an increase in P_i creates a decreased sensitivity of myofilaments to Ca^{2+}, whilst Fryer et al. [6], reported that an effect of elevated P_i is a decrease in Ca^{2+}-transport leading to a decrease in force over time.

One way of decreasing P_i is to add alpha-ketoglutarate (AKG) to a muscle, since AKG acts as a phosphate binder. AKG, which is part of the Krebs cycle, is normally turned over at a very high rate. Indeed, a clinical crossover open trial study of 10 chronic hemodialysis patients concluded that calcium ketoglutarate may be an effective and safe, yet very costly alternative to treatment with aluminium-containing phosphate binders [7]. Over half a century ago, however, Mudge [8], showed in a study of potassium accumulation by rabbit kidney slices, that oxygen consumption per hour per mg wet weight of tissue increased by 209% *cf.* control levels upon addition of AKG, but increased by some 302% upon addition of succinate. With every completed TCA cycle, succinyl CoA is converted to succinate and a single P_i is converted to a high-energy bond e.g. ATP or GTP. Thus, if addition of TCA intermediates to a tissue causes an increase in metabolism which involves more frequent TCA cycling, one might anticipate a reduction in the level of P_i in a given medium.

The aim of this study, therefore, was to test the hypothesis that the addition of succinate to a uremic environment has an equally beneficial effect on muscle performance to that of alpha-ketoglutarate addition. It is hoped that by testing this hypotheses, a cost effective way of improving muscle function in uremic patients may be achieved.

MATERIALS AND METHODS

Preparation of animals and muscle. Wistar Hannover rats housed at the animal facilities of The Faculty of Life Sciences, Copenhagen University were used. The animals

Corresponding author: Else M. Bartels, MD, Copenhagen University Library, Noerre Allé 49, 2200 Copenhagen N, Denmark. E-mail: emb@dnlb.dk

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were raised under identical conditions with a 12/12 light cycle, and had a body weight of 280-350 g. The rats were fed rodent pellets *ad libitum* (Altromin No.1314 Spezialfutterwerke, Lage, Germany) and given free access to drinking water. The rats were killed by exposure to 95% CO_2 and cervical dislocation, in accordance with local and national guidelines.

Extensor digitorum longus (EDL) muscles were dissected intact with both tendons attached. This lower hind-limb muscle has its origin in the distal portion of the femur and inserts upon the distal phalanx of digits 2-5. Muscles were securely attached at one end to the force transducer, and at the other to 2 metal pins on the mounting/stimulating block. As described in part [9, 10], isolated rat muscles were mounted vertically in thermostatically-controlled chambers, stimulated directly with supra-maximal pulses, and force development was recorded. No neuronal blockers were used in this study to avoid the risk of any side-effects of such compounds on muscle performance. The thermostatically-controlled chambers had an internal depth and diameter of 5.5 and 3.2 cm, respectively, holding 44 ml of incubation buffer. The mounting/stimulation block, made of perspex, was 8 cm long, 1.5 cm wide, and 1 cm thick. Into this perspex block were inserted 2 steel pins to hold the isolated muscle, and 2 silver stimulating electrodes (0.88 mm diameter fashioned out of jewellery-grade silver (Dansk Hollandsk Ædelmetal A/S, Copenhagen, Denmark).

The stimulator used was a DS3 Isolated Stimulator (Digitimer Ltd.) linked to an 8S MacLab A/D Converter (AD-Instruments, UK). Twitch and tetanic contractions were measured using a FTO3 force displacement transducer (Grass Instrument, West Warwick, RI) connected to a home-built bridge amplifier and interfaced with a 8S MacLab A/D Converter (ADInstruments, Chalgrove, Oxfordshire, UK).

Incubation solutions. Isometric force was measured under the following conditions: normal Ringer (Ringer), Human Uremic Ringer (HU) and Human Uremic Ringer with AKG replacing glucose (HUA). Another series of similar experiments were performed using the HU Ringer, but with succinate as the key metabolic compound rather than AKG, denoted as HUS. The composition of the various Ringers used is shown in Table 1. The HU Ringer was based on typical haemodialysis patient values [1]. Suspended isolated muscles were placed into the thermostatically-controlled chambers at 38°C, filled with one of the above Ringer solutions and continuously oxygenated with a mixture of 95% O_2 and 5% CO_2 (pH 7.4). All Ringers were adjusted to approximately the same osmolarity.

Force measurements. The muscles were suspended vertically in a force-displacement FT03 Grass Force transducer (Grass Instruments, Quincy, MA) setup. The recorded signal was adjusted to zero for muscle slack with the aid of an offset dial mounted on the pre-amplifier unit. Each muscle was exposed to 5 single stimulations where the length of the muscle was adjusted to a length that gave the maximal twitch tension (duration 2 ms, 2 Hz, maximal voltage 90 V, current 32 mA) with the aid of a PowerLab /8S A/D converter (AD-Instruments, Chalgrove, Oxfordshire, UK). The transducer scale was set at 200 mV, and a recording speed of 1,000 data samples per second was used. Muscles were left in either normal Ringer or a uremic Ringer, to equilibrate for 30 minutes. Following the equilibration period, the muscle was stimulated continuously to fatigue.

Table 1 Comparison of chemical composition of incubation buffers used in connection with isolated EDL muscles of adult rats. HU – human uremic conditions; HUA – HU buffer where glucose has been exchanged for alpha-ketoglutarate (AKG); HUS – HU buffer where glucose has been exchanged for succinate. Values are presented as mmol/l

	Ringer	HU	HUA	HUS
Na^+	145	140	134.8	134.8
HCO_3^-	25	19	14	14
Ca^{2+}	1.27	1	1	1
Cl^-	127	109.5	109.5	109.5
Mg^{2+}	1.2	0.75	0.75	0.75
PO_4^{3-}	1.2	4	4	4
K^+	5.9	3.6	3.6	3.6
Urea	-	30	30	30
Glucose	5	5	-	-
AKG	-	-	5	-
Succinate	-	-	-	5
SO_4^{2-}	1.2	0.75	0.75	0.75
Mosmol/l	313	303	303	303
Na^+ / K^+ ratio	24.5 : 1	38.8 : 1	37.4 : 1	37.4 : 1
Na^+ / Cl^- ratio	1.14 : 1	1.27: 1	1.23 : 1	1.23 : 1
Final pH	7.4	7.4	7.4	7.4

Incubation buffers were prepared using the following fine chemicals supplied by Merck: $NaHCO_3$, $CaCl_2$, $MgSO_4$, KH_2PO_4, Na_2HPO_4, NaCl, KCl, H_2NCONH_2 D-Glucose; the latter 2 two chemicals were added last. pH was adjusted by the addition of HCl or NaOH where necessary; however, we found that the buffers had a final pH very close to 7.4. Buffers did not fall out of solution; pH was checked routinely at the start and finish of each experiment.

In each experiment, EDL muscles from contra-lateral legs were measured simultaneously in normal Ringer and in a uremic Ringer in order to carry out a valid comparison between the control and uremic environments. In order to facilitate comparison of muscles from different rats, the relative decline in force was calculated as a percentage of the maximum isometric force – reached approx. 1 sec after the onset of stimulation, as described previously [9].

Statistical analysis. Data are presented as means ± SE. Differences between means were tested for statistical significance with the use of a student's paired *t*-test, with an additional test for Gaussian Normal Distribution using the Kolmogorov-Smirnov KS distance. Data were found to be normally distributed and to have equal variance. Differences showing a *P* value >0.05 were considered non-significant.

RESULTS

In a previous study involving the slow-twitch soleus muscle of the rat, the rate of decline in force during a period of sustained field stimulation was not significantly different for muscles incubated in a uremic solution *cf.* those incubated in Ringer [1].

Force measurements. In the current study, the isometric force for a continuously stimulated EDL muscle under normal and uremic conditions are shown in Figures 1 and 2. EDL muscles, which are fast-twitch, showed a significantly faster rate of fatigue as a result of field stimulation when incubated in the human uremic (HU) Ringer *cf.* those incubated in normal Ringer. The rate of decline in force measured over the first 8 seconds after maximum force production was 8.0%/sec

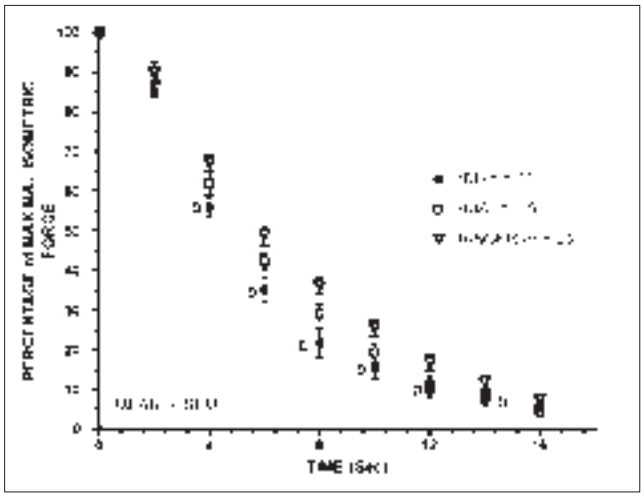

Figure 1 The relation between percentage maximal isometric force vs. time for stimulated *extensor digitorum longus* (EDL) muscles of rats incubated in normal Krebs Ringer (▽) or, either a human uremic (HU) solution (■) or a human uremic + alpha-ketoglutarate (HUA) solution (❑), respectively. For details of bathing solutions used, see Table 1. Isolated muscles were continuously stimulated at 90 Hz for a period of 16 s, with 32-mA pulses of 1 ms duration; this represents supramaximal, constant-current field stimulation. Force recordings made on a force transducer attached to an A/D converter, sampling rate 1,000 samples/s. Each point represents mean ± SE. Significant differences between muscles incubated in uremic solutions and muscles incubated in normal Krebs Ringer: a = p<0.05 and b = p<0.01.

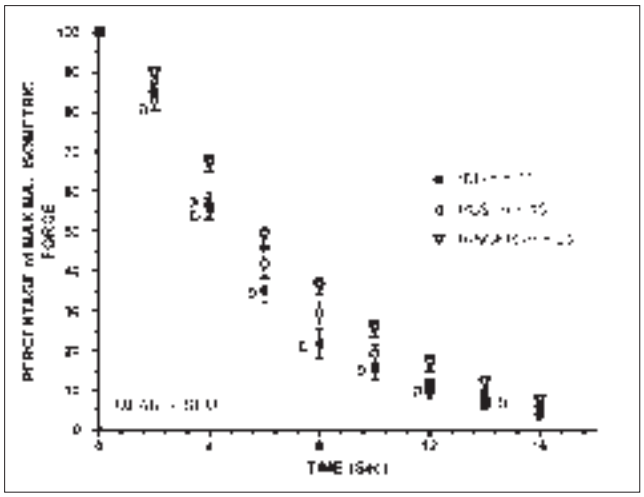

Figure 2 The relationship between percentage maximal isometric force vs. time for stimulated *extensor digitorum longus* (EDL) muscles of rats incubated in normal Krebs Ringer (▽) or, either a human uremic (HU) solution (■) or a human uremic + succinate (HUS) solution (○), respectively. For details of bathing solutions used, see Table 1. Isolated muscles were continuously stimulated at 90 Hz for a period of 16 s, with 32-mA pulses of 1 ms duration; this represents supramaximal, constant-current field stimulation. Force recordings made on a force transducer attached to an A/D converter at sampling rate of 1,000 samples/s. Each point represents mean ± SE. Significant differences between muscles incubated in uremic solutions and muscles incubated in normal Krebs Ringer: a = p<0.05 and b = p<0.01.

for muscles incubated in Ringer, a value that was significantly different from those values measured for muscles incubated in the HU solution: 9.8 %/sec, (22% faster $P<0.01$).

The substitution of AKG for glucose in the HU Ringer, producing the Ringer denoted as HUA, resulted in an improved fatigue profile for stimulated EDL muscles. Indeed, the improvement in the fatigue pattern for muscles incubated in HUA, as opposed to the HU Ringer, meant that these muscles were not found to be significantly different from those muscles incubated in normal Ringer. Substitution of succinate for glucose in the HU Ringer, producing the Ringer denoted as HUS, also resulted in an improved fatigue profile for stimulated EDL muscles. As with muscles incubated in HUA, the rate of decline in force measured over the first 8 seconds after maximum force production was 8.8 %/sec, a value that did not differ significantly from that of muscles incubated in Ringer. However, unlike the HUA Ringer, EDL muscles incubated in HUS were initially significantly weaker in terms of percentage of maximal isometric force for the first 4 seconds of force production, compared with those muscles incubated in Ringer. After 2 seconds of force production, muscles incubated in HUS were 7.5 % weaker, and after 4 seconds they were 15.5 % weaker than muscles incubated in Ringer (both $P<0.05$); thereafter there was no significant difference between the HUS and Ringer muscles.

EDL muscles measured in the current study produced an isometric force of 0.90 ± 0.06 N, which is very comparable with previously published values [1].

DISCUSSION

The results of this study not only confirm previous work showing that the effect of a uremic environment on fast-twitch skeletal muscle is an impaired contractile performance, but also show that such effects can be alleviated to a great extent by the addition of the TCA intermediates alpha-ketoglutarate or succinate.

Uremic patients – apart from renal failure – have a serious problem with muscle fatigue which diminishes their quality of life. If this fatigue could be treated, or even partly treated, the patients' lives would therefore improve significantly. However, at present, loss of fast muscle function remains symptomatic of these patients, with some studies showing atrophy of the type II fibres, and unpreventable loss of fine-motor skills.

In the present study, we have examined normal rat muscles exposed to uremic conditions. In this situation, the muscles have not had time to develop long-term effects of uremia; rather, we have measured the immediate consequences for muscle performance of exposure to the uremic environment. The osmolality in these *in vitro* experiments was deliberately kept constant as a hypertonic environment is known to diminish twitch tension alone [11], and although this must play a role *in vivo,* the present study has attempted to focus on other underlying effects.

Human studies provide clear scientific evidence that the intramuscular concentration of P_i increases linearly with an increase in plasma P_i concentration, since the plasma membranes of skeletal myocytes are sufficiently permeable to P_i to allow detectable bulk movement of P_i across the membrane within a time-scale of minutes [12]. The precise effect of increased P_i on intact muscle-cell function has been difficult to prove without also affecting other metabolic changes. However, with the use of a genetically modified mouse ($CK^{-/-}$), it is now possible to address this very issue [4]. Westerblad and colleagues (2002), have shown that fast-twitch skeletal muscles from such ($CK^{-/-}$) mice display an increased myoplasmic P_i concentration at rest, and show a markedly lower force than that of wild-type mice. Additional support for a coupling between myoplasmic P_i concentration and force production in intact muscle cells comes from experiments in which reduced myoplasmic P_i has been associated with increased force production [13]. The exact mechanism of P_i on force production remains to be explained, but it may be speculated that the increased myoplasmic P_i may decrease force production by direct action on cross-bridge function; alternatively, increased P_i may inhibit the ATP-

driven sarcoplasmic Ca^{2+} uptake, so that less Ca^{2+} would be available for release, leading to a decline in force [14].

Whatever the mode of action of phosphate, keto-acid supplements, which are: 1) phosphate free, and so help to reduce phosphate intake, and 2) are often provided with calcium salts which promote phosphate binding in the intestine, thereby increasing phosphate excretion, and have been shown to reduce the progression of renal insufficiency in chronic renal failure [15,16].

While the current results show that both the TCA cycle intermediates investigated have a beneficial effect on muscle performance for muscles exposed to a uremic condition *in vitro*, the precise means of action still needs to be determined. However, the short time-frame adopted in this study means that any TCA effects on transcription/translation within the isolated muscles can effectively be ignored. It was reported recently that a decrease in inorganic phosphorus levels occurred in rats after 7 days of treatment with AKG (p.o.) [17]. We therefore suggest that the data of Mudge [8], which provides clear evidence of a significant increase in cellular metabolism after exposure to both AKG and succinate, combined with the results of Bhattacharya and co-workers [17], and the observations of the effects of a high level of P_i on muscle performance [4, 13], lend considerable weight to the role of elevated plasma and cellular P_i in the muscle weakness seen with exposure to a uremic environment, and the alleviating role of TCA cycle intermediates. Furthermore, we hypothesise that the addition of TCA cycle intermediates to muscle incubated in a uremic environment results in incorporation of P_i into high energy bonds as the succinyl CoA is converted to succinate. In the present study, it appears that AKG is slightly more efficient than succinate in alleviating or restoring the effects imposed on muscle performance by exposure to a uremic environment *in vitro*. In an *in vivo* situation, however, one might anticipate an even greater beneficial affect of AKG *cf.* succinate, since it is known that AKG can and does complex with ammonia in muscle to form glutamine. In so doing, AKG would not only spare the organism the 'cost' of 3 molecules of ATP with the release of 3 x P_i as part of urea synthesis, it would additionally provide the release of glutamine which acts as an obligatory substrate for the kidney in ammonia production.

Clearly, this study shows that AKG, and for that matter succinate as well, have an effect on the fast muscles exposed to uremic conditions. Therefore, a supplementary intake, perhaps in the form of AKG tablets, could be a possible means of improving the fast muscle function affected by the uremic condition. Indeed, in terms of dosage it has been ascertained that AKG can be administered in doses of 2.0 g kg^{-1} (*per os*) without any change in haematology, biochemistry, or histology of the vital organs in rats [17]. However, administering AKG orally is a simple matter in the case of healthy individuals, but to uremic patients this could be a problem. Perhaps a means of overcoming this problem may be found in the dialysis solutions used for the treatment of these patients, or the use of succinate rather than AKG.

CONCLUSION

The results of this study indicate that the internal environment bathing nerve and muscle cells plays an important role in the occurrence of the muscle weakness and fatigue, which is symptomatic of chronic renal failure. The measurements of muscle fatigue during normal and uremic conditions clearly show that a uremic environment, in itself, will rapidly induce muscle weakness, and that this weakness can be alleviated to a great extent by the presence of the TCA intermediates alpha-ketoglutarate or succinate. Further studies are necessary to elucidate the precise means by which these protective/restorative effects are achieved, and the potential for such treatment in a clinical situation.

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