

# RENAL HUMORAL MODULATION OF SKELETAL MUSCLE TONE IN MICE; IMPLICATIONS FOR ‘THE PULMONARY – RENAL CASCADE’

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**Abstract :** The pulmonary – renal cascade may regulate the respiration and skeletal muscle contractility. To evaluate this working hypothetical model, we conducted experiments to ascertain the skeletal muscle tone of the Swiss mice (20–35 g). The animals were evaluated for their skeletal muscle tone via several techniques i.e. inclined plane test, grip strength test and swim test.

Groups of mice (n=6) were pre-treated with mefenamic acid (60 mg/kg, i.p), carbenoxolone (100 mg/kg i.p) or vehicle only 15 minutes before the treatment with heparin (500 U/kg, i.v), urokinase (5500 U/kg, i.v) and erythropoietin (150 U/kg, i.v). Heparin potentiated the loss of skeletal muscle tone induced by mefenamic acid and carbenoxolone while urokinase & erythropoietin significantly enhanced the skeletal muscle tone as evaluated by all or one of the tests. Other groups of mice (n=6) were pretreated with mefenamic acid (1 mg i.c.v), carbenoxolone (160 µg i.c.v) or minoxidil (30 µg i.c.v) and the effects of heparin & urokinase and erythropoietin on skeletal muscle tone were evaluated. To study the effects of heparin and urokinase on nerve regeneration, two groups of mice underwent a sham and sciatic nerve crush procedure. The mice treated with urokinase recovered much faster as compared to those treated with heparin or saline.

These experimental results suggest that gap junction blockers and potassium channel openers interact with heparin, urokinase and erythropoietin to control the skeletal muscle tone.

**Key words :** gap junctions                  muscle tone                                  mice  
urokinase                                  erythropoietin                  potassium channels

## INTRODUCTION

Gap junctions play an important role in neurons and glia of brain stem mediating chemoreception and respiratory

rhythmogenesis. Gap junctional intercellular communication (GJIC) and inhibitory synapses also modulate inspiratory motoneuron synchronization (1). In response to the stimulation of the central gap junctions by

changes in inspiratory motoneuron and phrenic nerve impulses and potassium channel modulation results in subsequent renal nerve stimulation. This induces the kidney to release the humoral substances, urokinase, Erythropoietin, heparin binding growth factors and protease nexin-1 which have a prominent effect on the tone and activity of skeletal muscles (2, 3).

Urokinase and erythropoietin have been shown to have effects on myogenesis, skeletal muscle regeneration and regulation of skeletal muscle tone. While heparin mediates a wide variety of complex biological roles by binding to its growth factors and alteration of the tone of skeletal muscles (4).

Gap junctions and potassium channels have the potential to alter the tone and neural impulses to the skeletal muscles (5). This study was therefore undertaken to evaluate the role of gap junctions and potassium channels in the modification of skeletal muscle tone by heparin, urokinase and erythropoietin. The results of our study clearly suggest the importance of these endogenous humoral agents in regulating skeletal muscle tone. The results of this study suggest the significance of these neurohumoral agents in maintaining the 'Pulmonary - Renal cascade'.

## METHODS

### Drugs

The doses of the drugs utilised were selected based on the concentration per kg weight given in standard text books and alongwith the preliminary trials were conducted to evaluate the dosage. The

following drugs were utilised for this study. 0.9% Saline, mefenamic acid (60 mg/kg i.p), minoxidil (30 µg i.c.v), urokinase (5500 U/kg, iv), heparin (500 U/kg i.v), carbenoxolone disodium (160 µg i.c.v), erythropoietin (150 U/kg i.v), ketamine (10 mg/kg i.m).

### Animals

Swiss adult mice of either sex weighing between (20–35 g) were used for this study. The animals were kept under standard laboratory conditions and were provided with chow and drinking water *ad libitum*. A natural 12 hour dark – light cycle i.e 7.00 a.m onwards being the day and 7.00 p.m onwards being the darkness was maintained. Ethical clearance was obtained from the Institutional animal ethics committee to conduct experiments.

### Statistical analysis

Statistical evaluation was done utilising the Mann Whitney U test and Wilcoxon Rank Sum W test for paired experimental data. The values expressed are mean ± SE of n=6 animals. Differences were considered to be significant when P<0.05.

### Procedure

Eight groups of mice, each consisting of six animals were used for intraperitoneal injections of mefenamic acid and carbenoxolone disodium with or without other drugs namely urokinase, heparin and erythropoietin. Thirteen groups of mice, each consisting of six animals were used for i.c.v injections of mefenamic acid and carbenoxolone disodium and minoxidil with or without other drugs namely urokinase, heparin and erythropoietin. An i.c.v injection

of saline was also administered to a group which served as control. After administration of injections, the animals were evaluated for the following parameters.

#### **Evaluation of skeletal muscle tone**

##### *a) Inclined Plane test :*

Initially animals were tested for their inherent skeletal muscle tone by their ability to climb up the inclined plane. Test drugs were given and animals tested again for skeletal muscle tone. A small wooden board of 30 × 20 cm was kept inclined to the wall at an angle of 65°. Mice were placed on the rough surface of the inclined plane and the muscle tone was ascertained by their ability to hold on to the surface. Their ability to climb to the top of the inclined plane was evaluated (6).

##### *b) Grip Strength test :*

This test is used to evaluate muscular strength or neuromuscular function in rodents. Initially the animals are tested for their normal reactivity. They were exposed to a horizontal metallic wire 30 cm long suspended in air. Mice are allowed to hang with its fore limb and their ability to catch with their hind limbs within 5 seconds was included for the test. After administration of the drug the animals not able to touch the wire with the hind limbs within 5 seconds or animals which fall off were considered to have decreased tone (7). The animals were injected with test drugs and their muscle tone evaluated by this test.

##### *c) Swim Test :*

The animals were placed in the center

of a water tank (40 × 20 × 10 cm). The temperature of the water was regulated to room temperature (30°C). Normal animals swim perfectly and some times passively, but never sink. The limb movement and the capacity to swim indicated the tone of skeletal muscles. All the animals were tested for their swimming ability with a 3 minute test. Passive swimming was indicative of decreased skeletal muscle tone. Then the animals were treated with the experimental drugs and evaluated. The animals with reduced muscle tone fail to keep afloat, after some time (8).

##### *d) Intracerebroventricular injection of drugs :*

The Intracerebroventricular (i.c.v) technique utilised for this study was based on the method by Haley & McCormick (9). The mice were injected with the anesthetic agent, ketamine (10 mg/kg i.m) 15 minutes before experimental use. The drug solution was injected with a 20 µl Hamilton syringe connected to a plastic cannula with a sleeve around the needle to prevent the later from penetrating more than 3 mm into the skull. All injections were given 1.0–2.0 mm posterior to the bregma.

##### *e) Sciatic Nerve Crush :*

The mice were anesthetized with ketamine (10 mg/kg i.m) injection. The technique utilised was a modification of the technique shown by Siconolfi et al (10). Through a careful incision in the hind limb, the sciatic nerve was located and crushed with the help of a serrated forceps. The twitching of the limb muscle confirmed the effects on the skeletal muscle. After the nerve crush the animals exhibited a distinct

paralytic gait with the affected limb. The animals were treated with the test drugs to evaluate the recovery of sciatic nerve activity.

## RESULTS

### Evaluation of skeletal muscle tone

Different groups of mice (n=6) were evaluated for skeletal muscle tone via the simple tests like the inclined plane test, grip strength test and swim test. The results of our study suggest that pre-treatment with gap junction blocker, mefenamic acid caused mild attenuation of the skeletal muscle tone *per se* as determined by inclined plane test. This mild attenuation in skeletal muscle tone was significantly potentiated by heparin treatment i.e. a decrease of 28.5% while on the other hand the skeletal muscle tone was enhanced by urokinase and showing an increase of 39.2%. The results are shown in Table I. To evaluate the grip strength of the animals, the animals were subjected to pre-treatment with mefenamic acid 15 minutes prior to injections of heparin or urokinase

and we obtained a similar trend i.e. the animals displayed potentiated loss of muscle tone with heparin and enhancement and reversal of skeletal muscle tone with urokinase. On the other hand erythropoietin enhanced the skeletal muscle tone after i.p injections of GJIC blocker, mefenamic acid when evaluated by inclined plane test and grip strength test causing an increase of 19.5 and 60.9% respectively.

Inorder to strengthen our conviction about gap junctions, we utilised another specific GJIC blocker, Carbenoxolone and we obtained almost similar pattern of results as obtained with mefenamic acid (Table I).

To observe the effects on the ability of the animals to swim, and maintain a limb movement during a swim test a separate group of animals were pretreated with mefenamic acid along with heparin, urokinase and erythropoietin and subjected to a 3 minute swim test in a plastic container. Heparin produced a reduction in muscle tone while urokinase and erythropoietin enhanced the ability of the animals to swim. All the

TABLE I: The influence of GJIC Blockers, Mefenamic acid (60 mg/kg, i.p) and Carbenoxolone (100 mg/kg, i.p) on the modification of skeletal muscle tone, by heparin (500 U/kg, i.v), Urokinase (5500 U/kg, i.v) and EPO (150 U/kg, i.v) as evaluated by Inclined plane test, Grip strength and swim test. The results shown are from (n=6) observations and are depicted as % mean  $\pm$  SE change from control.

Drugs	Inclined plane test	Grip strength test	Swim test
Mefenamic acid	7.24 $\pm$ 1.11	7.46 $\pm$ 1.32	8.11 $\pm$ 1.82
Mefenamic + Heparin	-28.5 $\pm$ 1.97 <sup>*a</sup>	-23.3 $\pm$ 3.86 <sup>a</sup>	-18.4 $\pm$ 1.92 <sup>a</sup>
Mefenamic + Urokinase	39.2 $\pm$ 4.43 <sup>*a</sup>	79.6 $\pm$ 7.63 <sup>*a</sup>	81.2 $\pm$ 3.74 <sup>*a</sup>
Mefenamic + Erythropoietin	19.5 $\pm$ 7.36	60.9 $\pm$ 6.28 <sup>*a</sup>	58.1 $\pm$ 6.54 <sup>*a</sup>
Carbenoxolone	-	11.26 $\pm$ 1.71	-
Carbenoxolone + Heparin	-	-38.5 $\pm$ 2.12 <sup>*b</sup>	-
Carbenoxolone + Urokinase	-	62.2 $\pm$ 7.19 <sup>**b</sup>	-
Carbenoxolone + Erythropoietin	-	29.7 $\pm$ 4.82 <sup>*b</sup>	-

Symbol <sup>a</sup>signifies values that are in comparison with the group of animals treated with mefenamic acid alone. Symbol <sup>b</sup>signifies values that are in comparison with the group of animals treated with carbenoxolone acid alone. \*'P' value <0.05, \*\*\*'P' value <0.01.

animals were given scores according to subjective observation. The scores for the tests conducted were given as follows: Severe loss of muscle tone = 1, moderate loss of muscle tone = 2, mild loss of muscle tone = 3 and enhanced muscle tone = 4.

#### Effect of i.c.v injections on skeletal muscle tone

Groups of animals (n=6) were pretreated with mefenamic acid or minoxidil 20 minutes before injections of heparin, Urokinase and erythropoietin. Intracerebroventricular injections of minoxidil *per se* caused some transient loss of skeletal muscle tone although the loss of muscle tone with mefenamic acid was not significant ( $P > 0.05$ ).

In the mefenamic acid treated animals injections of heparin caused decrease in skeletal muscle tone ( $P < 0.05$ ) and urokinase enhanced the tone of skeletal muscles. These changes were observed with the tests conducted on the inclined plane and grip strength. These observations are shown in Table II.

Erythropoietin caused a quick and efficient induction of the skeletal muscle tone *per se*. In animals pretreated with i.c.v mefenamic acid, erythropoietin produced a moderate improvement in skeletal muscle function when evaluated by the inclined plane and grip strength tests as shown in Table II while the improvement was highly significant in the groups of mice treated with i.c.v injection of minoxidil.

In another groups of animals (n=6) pretreated with i.c.v minoxidil, heparin caused marked potentiation in the tone of

skeletal muscles, while urokinase injections caused no enhancement in skeletal muscle tone. Separate groups of mice each were pretreated with carbenoxolone disodium and later given intravenous injections of heparin, urokinase and erythropoietin. The results obtained are shown in Table II.

TABLE II: Effect of Intracerebroventricular injection of mefenamic acid (1 mg) minoxidil (30 µg) and carbenoxolone (160 µg) on the modification of skeletal muscle tone by heparin (500 U/kg i.v), urokinase (5500 U/kg, i.v) and erythropoietin (Epo) (150 U/kg i.v) as evaluated by inclined plane and grip strength tests. Pre-treatment with mefenamic acid, carbenoxolone and minoxidil was conducted 20 minutes before intravenous injections. The data are shown as mean  $\pm$  SE for six observations. \*denotes values significant from control ( $P < 0.05$ ).

Drugs	Inclined plane	Grip strength
Saline	2.83 $\pm$ 0.17	2.83 $\pm$ 0.17
Mefenamic acid	2.08 $\pm$ 0.19	1.83 $\pm$ 0.16
Mefenamic + Heparin	1.83 $\pm$ 0.31*	1.66 $\pm$ 0.33
Mefenamic + Urokinase	2.5 $\pm$ 0.34*	2.33 $\pm$ 0.45*
Mefenamic + EPO	2.83 $\pm$ 0.17*	2.17 $\pm$ 0.17
Minoxidil	1.41 $\pm$ 0.19	1.33 $\pm$ 0.14
Minoxidil + Heparin	2.83 $\pm$ 0.35*	2.33 $\pm$ 0.45*
Minoxidil + Urokinase	1.66 $\pm$ 0.17	1.33 $\pm$ 0.33
Minoxidil + EPO	2.66 $\pm$ 0.22*	2.33 $\pm$ 0.17*
Carbenoxolone (CBX)	1.33 $\pm$ 0.21	1.33 $\pm$ 0.11
CBX + Heparin	0.83 $\pm$ 0.31*	1.17 $\pm$ 0.17
CBX + Urokinase	2.0 $\pm$ 0.37*	2.17 $\pm$ 0.17*
CBX + EPO	1.83 $\pm$ 0.31*	1.67 $\pm$ 0.21*

\*denotes 'P' value  $< 0.05$ .

#### Effect of drugs on Sciatic nerve crush recovery

Groups of mice (n=6) undergoing a sham operation or a sciatic nerve crush were treated with intravenous heparin or urokinase. The animals undergoing a sciatic nerve crush displayed a distinct paralytic gait. The animals receiving urokinase

showed faster recovery from nerve injury and became normal as compared with control (Saline treated) animals. The recovery observed after day 3 and day 6 was evaluated and subjective scores allotted on the basis of improvement in gait, limb movement and ability to climb the inclined plane. Our results clearly demonstrate that the urokinase treatment significantly enhanced the recovery of the hind limb following sciatic nerve crush displaying an improvement of 69% & 84% on day 3 and day 6 respectively, in comparison, heparin was less effective and saline was least effective in reversing the nerve function following sciatic nerve crush showing an improvement of only 46% and 63% respectively. The results of our study are shown in Figure 1. The scores for the

recovery from sciatic nerve crush were given as following :

1: Severe loss of muscle tone, 2 = moderate loss of muscle tone, 3 = mild loss of muscle tone, 4 = enhanced skeletal muscle tone.

## DISCUSSION

In the background of the increasing evidence on the physiological role of humoral mediators released from the kidney e.g urokinase, erythropoietin, protease nexin-1 and heparin binding growth factors, we visualized a working neurohumoral circuitry functioning in human body serving multiple physiological functions in the body including skeletal muscle control (Fig. 1). We conducted experiments to prove this hypothesis by using simple techniques for the evaluation of skeletal muscle tone (11).

To elucidate the possible role of central gap junctions and potassium channels different groups of the animals received either i.c.v. injections of mefenamic acid, carbenoxolone or minoxidil.

Mefenamic acid when injected intraperitoneally *per se* caused a mild attenuation of the skeletal muscle tone. Intravenous injections of heparin after GJIC blockade produced a significant reduction in the skeletal muscle tone, as evaluated by all the tests i.e inclined plane, grip strength and swim tests. Heparin has been shown to have direct effects on the skeletal muscle through its actions on the excitation – contraction coupling (12). Heparin has also been shown to block  $IP_3$  receptors and following gap junctional blockade it seems that peripheral actions of heparin get

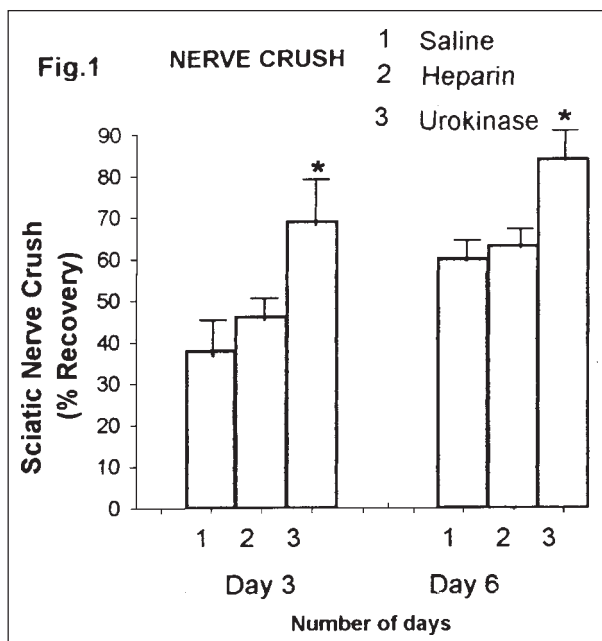


Fig. 1: The effect of heparin (500 U/kg i.v), urokinase (5500 U/kg, i.v) and saline treatment on the recovery of sciatic nerve crush in mice. The results shown are representative of n=6 observation. The data are given as mean  $\pm$  SE of the percentage recovery as observed on day 3 and day 6. \*shows 'P' values significant from control (P<0.05).

potentiated. The results are depicted in Table I. On the other hand, urokinase has been shown to improve skeletal muscle viability. In our study, we observed a significant improvement in skeletal muscle tone following intravenous injections of urokinase. It appears that this enhancement in skeletal muscle tone is because of the direct effects of uPA on slow and fast acting muscle fibres.

In another groups of animals, pretreated with gap junction blocker and later with erythropoietin, we observed an improvement in muscle functioning, however it was less pronounced compared to urokinase. Erythropoietin has been found to be beneficial in improving skeletal muscle function in renal failure patients, normal patients and experimental animals. Erythropoietin, a 165 amino acid glycoprotein stimulates the PI-3 kinase enzyme and improves the oxygenation state of the skeletal muscles (13). The results of our study are shown in Table I.

Similar pattern of results were obtained when we utilised a more specific central GJIC blocker carbenoxolone in combination with heparin, urokinase and erythropoietin.

In another group of animals injected with i.c.v minoxidil (30 µg), the improvement in muscle function was potentiated and they displayed improved muscle function after erythropoietin injection. These results are shown in Table I. Erythropoietin may thus prove to be an important drug for improving skeletal muscle function, erythropoietin treatment results in a greater force and generates a longer duration of contraction.

It also causes faster relaxation of the type II muscle fibers. This study thus elaborates the recent emphasis on gene transfer of erythropoietin for erythropoiesis and muscle degenerative states. The observation that it overcomes central polysynaptic mediated skeletal muscle inhibition strengthens its role as an important mediator of the 'Pulmonary – renal cascade' (14).

Heparin administered after GJIC blockade produced a significant reduction in skeletal muscle performance, while enhancing the tone in mice treated with minoxidil. These results suggested that there is an interaction of potassium channels and inositol phosphate mediated pathway at the cellular level, unmasking the effects of  $IP_3$  on skeletal muscles (15). Urokinase caused an increase in muscle tone after GJIC and potassium channel modification suggesting that phospholipase mediated signalling was predominant and intensified muscle tone was because of multiple signalling mechanisms (16).

In order to ascertain the neurotrophic role of heparin and urokinase, we conducted studies with the sciatic nerve crush model. The results of our experiments with sciatic nerve crush are demonstrated in Table I. Heparin treatment although less effective than urokinase enhances the extent of muscle reinnervation, the recovery of nerve evoked muscle twitch tension and motor neurons reinnervation are greatly enhanced compared to saline treated rats. Urokinase has been shown to cause peripheral nerve regeneration by digestion of adhesive cell contacts activating proteases e.g matrix metalloproteases (17, 18).

In conclusion, the results of our study demonstrate that gap junctional activity and potassium channels may significantly influence the release of endogenous humoral agents, viz. heparin and its binding growth factors, urokinase, erythropoietin and protease nexin-1 to regulate the skeletal muscle tone. The modulation of the release and expression of these humoral mediators

might have implications in the treatment of musculo-skeletal disorders.

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