Effect of magnesium, insulin and insulin-like growth factors on glucose uptake, protein and DNA concentration by skeletal muscle cells in organ culture

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Zusammenfassung

In dieser Studie wurden die Wirkung von Magnesium, Insulin und der insulinartigen Wachstumsfaktoren I und II (IGF-I bzw. IGF-II) auf die Glukoseresorption bei Organkulturen von Skelettmuskeln von Jungratten untersucht. In Abwesenheit von Magnesium waren sowohl die Glukoseaufnahme als auch die DNA-Konzentrationen erhöht, wohingegen hohe Magnesiumspiegel die Glukoseresorption und die DNA-Konzentrationen unterdrückten. Die Gegenwart bzw. die Abwesenheit von Magnesium im Nährmedium beeinflusste nicht die Protein-Konzentrationen. Außerdem waren die Glukoseaufnahme und die DNA-Konzentrationen weiterhin unterdrückt während die Protein-Konzentrationen bei erhöhter Kulturdauer in den Zellen mit hohem Magnesiumgehalt im Nährmedium unbeeinflusst blieb.

Niedrige Konzentrationen von IGF-I, IGF-II und hohe Insulinkonzentrationen stimulierten die Muskelglukoseaufnahme wenn die Kulturen in hohem Magnesiumgehalt im Nährmedium verblieben. Im Vergleich zum Standard förderten IGF-I und II weiterhin die Protein- und DNA-Konzentrationen, aber exogenes Insulin hat keinen großen Einfluss auf die DNA-Konzentrationen und die Protein-Konzentrationen.

Diese Ergebnisse verdeutlichen, dass Magnesiumionen und IGF-I und IGF-II die DNA-Konzentrationen und Protein-Konzentrationen regulieren könnten und die Glukoseaufnahme in Organkulturen von Skelettmuskeln von Jungratten beeinflussen können.

Summary

This study examined the effects of magnesium, insulin and insulin-like growth factor I and II (IGF-I and IGF-II) on glucose uptake in juvenile rat skeletal muscle organ culture. Glucose uptake and DNA concentration were stimulated in the absence of magnesium in this study and high magnesium concentration suppressed glucose uptake and DNA concentration but media magnesium concentration did not affect protein concentration. In addition, glucose uptake and DNA concentration continued to be suppressed whilst protein concentration was unaffected as the time in culture was increased in cells maintained in high magnesium media.

Low concentrations of IGF-I, IGF-II and high concentrations of insulin increased muscle glucose uptake when cultures were maintained in high magnesium media. Simultaneous administration of IGF-I and IGF-II stimulated protein and DNA concentration compared to controls, but exogenous insulin did not significantly affect DNA and protein concentration in cells cultured in high magnesium media.

These results indicate that magnesium ion and IGF-I and IGF-II may regulate DNA and protein concentration and may influence glucose uptake in juvenile rat skeletal muscle cells *in vitro*.

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Introduction

Skeletal muscle is the main tissue responsible for insulin-induced glucose utilisation in humans and in rodents [1-2]. Insulin growth factors (IGF's) are important regulators of muscle growth and development. IGF-I plays a major role in development, growth, differentiation and maintenance of skeletal muscle both in culture [3] and in the intact animal [4] where it increases muscle growth and DNA content. IGF-II increases the cell number, protein levels and [^{3}H] thymidine incorporation of myoblasts. Stimulation of IGF-I initiates proliferation and differentiation of myoblasts [5-6]. The insulin receptor and IGF-I receptors have some homology. IGF-I and insulin can act at either receptor site with varying affinities.

Insulin, IGF-I and IGF-II stimulate many anabolic processes in skeletal muscle cells with the relative potencies: IGF-I > IGF-II > insulin. High levels of insulin stimulate differentiation of muscle cells but the IGF's are more potent stimulators of differentiation [4].

Magnesium is one of the most abundant ions in living cells. Intracellular magnesium is a cofactor for numerous enzymes involved in carbohydrate metabolism. Magnesium accumulation is dependent upon insulin action and correlates with insulin mediated glucose uptake. Thus insulin stimulates magnesium and glucose uptake and thus, influences both glucose metabolism and magnesium homeostasis in humans [7].

It is widely known that hypomagnesaemia is one of the symptoms observed in diabetic patients. Insulin may modulate the shift of magnesium from the extracellular to the intracellular space. A reduction in intracellular magnesium may modulate insulin mediated glucose uptake [8]. Magnesium efflux from skeletal muscle may inhibit insulin stimulated glucose uptake. Skeletal muscle glucose uptake is critical for whole body glucose tolerance, since skeletal muscle accounts for most of the insulin stimulated blood glucose clearance [1].

This study examined the proliferative effects of magnesium, insulin and insulin-like growth factors I and II (IGF-I and IGF-II) singly and in combination in juvenile rat primary skeletal muscle cells in organ cultures.

In addition, the effect of magnesium, insulin and IGF's on glucose uptake was investigated.

Methods and materials

Preparation of primary muscle organ cultures Male

Wistar rats, 9-10 weeks old, (205 g) obtained from Harlan Olac (Oxon, Bicester) were killed by CO_2 suffocation and all skeletal muscle (~ 1 g) was obtained aseptically from the fore and hind limbs. The muscle from each limb was excised, trimmed of fat and connective tissue, washed in Phosphate Buffered Saline (PBS) at 37°C, pooled and then finely diced into 1 mm^3 pieces. The pieces were then mixed to give a representative sample. Primary muscle organ cultures were initiated by inoculating 1 x 10^6 cells (~ 50 mg) onto sterile collagen sponges (Colgen) (~ 1 x 1 x 0.7 cm) in 60 mm diameter tissue culture dishes only if cell viability in preparations was greater than 90 % using the Trypan Blue dye exclusion procedure.

Culture conditions

Organ cultures were serum free and contained 50 unit ml^{-1} penicillin, 50 µg ml^{-1} streptomycin, and Fungizone (0.25 µg ml^{-1}) in 5ml RPMI 1640 media with 11.0 mmol l^{-1} D-glucose. Cultures were maintained in a humidified chamber at 37° C in 95 % air and 5% CO_2 for three days as specified in the experimental methods.

At the end of the incubation period, the culture media was removed separately from each culture, placed into 5 ml sterile Universal tubes and stored at –20° C until glucose analysis. Cell cultures were washed three times with PBS at 37° C, incubated at 37° C for 3 minutes in 200 µl collagenase (0.1 % w/v) to free the cells from the collagen sponge matrix and the collagenase solution was decanted. Cells were then rewashed with PBS, sonicated in lml PBS using an Ultra Sonicator (Microson Heat Systems) to disrupt them and stored at –20° C until utilised for total DNA and protein analysis.

Experimental methods

i. Effect of time in culture

Cultures were initiated as described above, maintained in magnesium complete media (high magnesium media) and supplemented with 100 ng ml^{-1} IGF-I and IGF-II and 10 mU ml^{-1} insulin for three days. A number of cultures were terminated each day for the analysis of cellular protein and DNA concentrations and glucose uptake from the media.

ii. Effect of magnesium concentration in the media

Cultures were supplemented with 100 ng ml^{-1} IGF-I and IGF-II and 10 mU ml^{-1} insulin for three days and maintained in either 5ml magnesium free (without magnesium), low magnesium (containing 0.04 mM total magnesium) or high magnesium (containing 4.06 mM total magnesium) RPMI 1640 culture media. At the end of the culture period, cellular protein and DNA concentrations and glucose uptake from the media were determined.

iii. Effect of varying insulin-like growth factor concentration

Organ cultures were incubated for three days in magnesium complete media (high magnesium media) containing 10 mU ml^{-1} insulin and supplemented with 1, 10 or 100 ng ml^{-1} IGF-I or IGF-II singly and in combination.

iv. Effect of varying insulin concentration

Cultures were maintained for three days in 5 ml magnesium complete media (high magnesium media) with 100 ng ml^{-1} each of IGF-I and IGF-II and supplemented with 0.1, 1.0 or 10 mU ml^{-1} insulin. Cellular protein and DNA concentrations and glucose uptake from the media were determined at the end of the culture period.

Experimental analysis

On completion of experiments, total protein and DNA concentrations were determined on cell homogenates by the methods of *Lowry* [9] and *Sterzel* [10] respectively and glucose concentrations were analysed in the culture media by the Trinder method [11]. Glucose uptake was determined by calculating the reduction of glucose concentration in the incubation media at the end of the experiment. RPMI 1640 media contained 11 mmol l^{-1} D-glucose at the start of each experiment.

Chemicals, culture media and test agents for supplementation were obtained from Sigma Chemical Co. (Poole, Dorset, UK), BDH (Liverpool, UK) or Gibco BRL (Paisley, Scotland).

Statistical analysis

The results of the effect of exogenous supplements on protein and DNA concentration and glucose uptake are representatives of six experimental cultures and each point represents the mean of triplicate analysis. Results are expressed as means ± S.D. Statistical analysis was performed using an ANOVA test and the Bonferroni post hoc test was used to locate the source of significant variance. Differences were taken as statistically significant when $P < 0.05$.

Results

i. Effect of time in culture

Glucose uptake by 1 x 10^6 cultured cells maintained in high magnesium media was measured after 1, 2 and 3 days in culture. Glucose uptake decreased steadily over 3 days in culture with uptake at day 3 in culture significantly less ($P < 0.01$) than that at day 1 (Tab.1).

When considering time in culture, none of the muscle cultures from these rats were significantly different in protein content from the controls. However, cell proliferation declined as

Tab. 1: Effect of time in culture on glucose uptake, protein and DNA concentration by 1 x 10^6 skeletal muscle cells in organ culture.

Time in culture Days	Glucose uptake mmol glucose/ 1 x 10^6 cells	Protein concentration mg protein/ 1 x 10^6 cells	DNA concentration mg DNA/ 1 x 10^6 cells
1 (n = 10)	1.82 (0.80)	1.68 (0.25)	0.017 (0.05)
2 (n = 9)	1.21 (0.24)	1.69 (0.23)	0.012 (0.04)
3 (n = 11)	0.88 * (0.49)	1.53 (0.56)	.0093 ** (0.09)

Results are means (± S.D.).
* compared with day 1, $P < 0.01$. ** compared with day 1, $P < 0.001$.

indicated by a decrease in the DNA content with time in culture, and a significant decrease was obtained at day 3 compared to that at day 1 ($P < 0.001$) (Tab. 1).

ii. Effect of magnesium concentration in the media

The cellular DNA content of cultures decreased with an increase in culture media magnesium ion concentration (Tab 2). The DNA content of cells maintained in low or high magnesium media was significantly less than that for cultures maintained in a magnesium free environment ($P < 0.0001$ respectively). However, the protein concentration in these cultures (Tab. 2) was not affected by media magnesium status.

Glucose uptake by 1 x 10^6 cells was negatively correlated to the dose of magnesium in the media ($r = -0.466$, $P < 0.05$). As such, glucose uptake by 1 x 10^6 cells decreased (but not significantly $P = 0.06$) as the concentration of magnesium in the culture media was increased.

iii. Effect of varying insulin-like growth factor concentration

Figures 1–3 show glucose uptake in cultured cells under insulin-like growth factor stimulation. The effect of a range of concentrations of IGF-I alone, IGF-II alone and IGF-I and II in combination is depicted.

The glucose uptake by 1 x 10^6 cells (Fig. 1) was significantly increased on addition of 1 ng ml^{-1} IGF-I compared to 1 ng ml^{-1} IGF-II ($P < 0.05$). Increasing the dose of and administering either IGF-I or IGF-II alone had no further effects. However, glucose uptake by 1 x 10^6 cells was significantly increased in cultures simultaneously administered with 1 ng ml^{-1} IGF-I and IGF II (over 100 % increase from controls and from cells treated with either 1 ng ml^{-1} IGF-II alone or with 10 and 100 ng ml^{-1} IGF-I and IGF-II combined, $P < 0.0001$ respectively). Furthermore, the addition of 1 ng ml^{-1} IGF-I alone and the simultaneous addition of IGF-I and IGF-II both at 1 ng ml^{-1} significantly increased glucose uptake standardised to protein content in cultures (Fig. 2) (~ 150 % increase from control cells, $P < 0.0001$ respectively). Similar significant results were obtained for comparisons with increasing doses of either IGF-I alone or IGF-I and IGF-II combined ($P < 0.0001$ respectively). Thus, at a dose of 1 ng ml^{-1} the upregulatory effect of IGF-I was not masked by the simultaneous administration of IGF-II. However, increasing the dose of IGF-I and IGF-II did not further enhance glucose uptake standardised to protein.

Glucose uptake standardised to DNA content of cultures (Fig. 3) showed that on addition of 1 ng ml^{-1} IGF-I, glucose uptake was significantly increased by up to 150 % over control values ($P < 0.0001$) and values observed with 10 ng ml^{-1} and 100 ng ml^{-1} IGF-I ($P < 0.0001$ and $P < 0.001$ respectively). Treatment with 1 ng ml^{-1} IGF-II also significantly increased glucose uptake standardised to DNA by approximately 100 % over control values ($P < 0.0001$) and further increases were observed with 10 ng ml^{-1} IGF-II (~ 200 % over control) ($P < 0.0001$). Thus, a dose response relationship between glucose uptake standardised to DNA and IGF-II concentration was apparent up to the 10 ng ml^{-1} dose. However, on simultaneous addition of 1 ng ml^{-1} IGF-I and II, glucose uptake standardised to DNA was reduced to almost control levels (Fig. 3). Thus, the separate actions of IGF-I and IGF-II were abolished in the presence of each other. In contrast, increasing the concentration of and simultaneously administering both growth

Tab. 2: Effect of exogenous magnesium concentration on glucose uptake, protein and DNA concentration by 1 x 10^6 skeletal muscle cells in organ culture.

Magnesium concentration (mmol l^{-1})	Glucose uptake mmol glucose/ 1 x 10^6 cells	Protein concentration mg protein/ 1 x 10^6 cells	DNA concentration mg DNA/ 1 x 10^6 cells
Mg free (0.00) (n = 8)	1.078 (0.57) (0.80)	2.47 (0.428) (0.25)	0.020 (0.038) (0.05)
Low Mg (0.04) (n = 8)	0.92 (0.25)	3.00 (0.688)	0.0093* (0.039)
High Mg (4.06) (n = 8)	0.54 (0.42)	2.54 (0.16)	0.0103* (0.012)

Results are means (± S.D.).
* compared with Mg free, $P < 0.0001$.

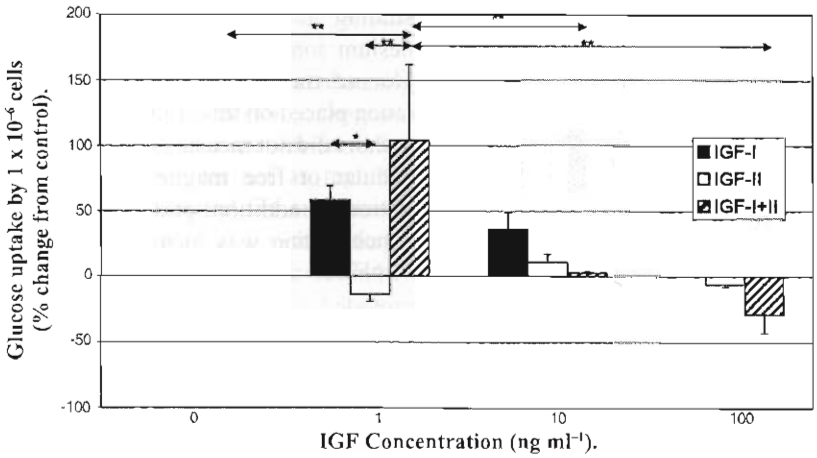

Fig. 1: Effect of insulin-like growth factors on glucose uptake by 1x 10^6 rat skeletal muscle cells in organ culture.
Results are mean (± SD) glucose uptake by 1x 10^6 cells (% change from control). * $P < 0.05$; ** $P < 0.0001$.

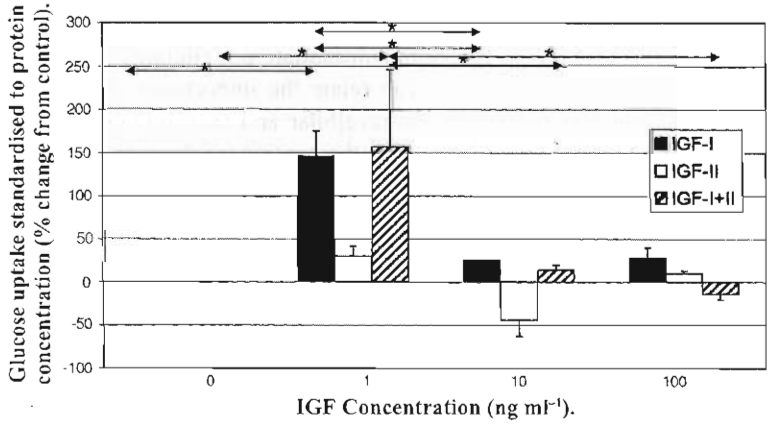

Fig. 2: Effect of insulin-like growth factors on glucose uptake standardised to protein concentration by 1x 10^6 rat skeletal muscle cells in organ culture.
Results are mean (± SD) glucose uptake standardised to protein concentration. (% change from control). * $P < 0.0001$.

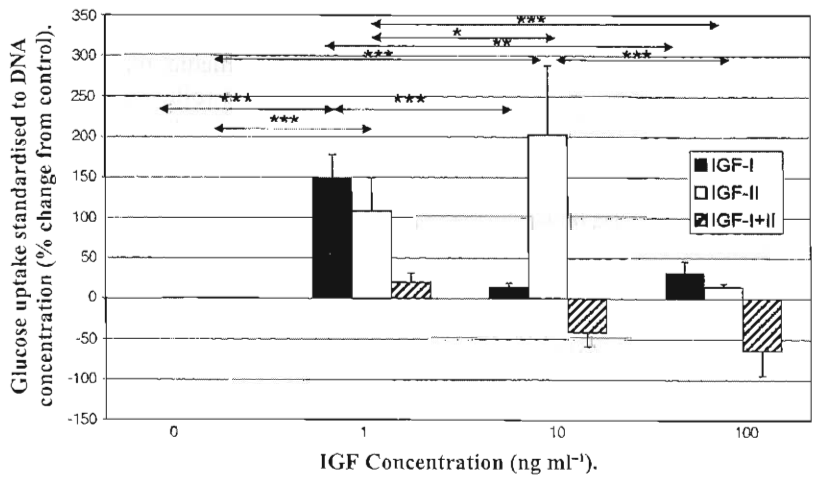

Fig. 3: Effect of insulin-like growth factors on glucose uptake standardised to DNA concentration by 1x 10^6 rat skeletal muscle cells in organ culture.
Results are mean (± SD) glucose uptake standardised to DNA concentration. (% change from control). * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$.

factors together, correlated with a decrease in glucose uptake referenced to DNA content of cells ($r = -0.532$, $P < 0.0001$) in a dose dependent manner.
When IGF-I and IGF-II were administered singly, protein and DNA concentration were reduced in cells ($P < 0.0001$ respectively for protein; $P < 0.05$ and $P < 0.0001$ respectively for DNA) compared to controls, in the presence of insulin (Fig. 4). However, at 10 ng ml^{-1} IGF-II, DNA concentration was significantly reduced compared to controls ($P < 0.0001$) whilst protein concentration was stimulated but not significantly. IGF-I and IGF-II administered in combination at the concentrations employed in this study stimulated both protein and DNA concentration above controls with significant values obtained on administration of 10 ng ml^{-1} IGF-I and IGF-II for protein and 100 ng ml^{-1} IGF-I and IGF-II for DNA ($P < 0.05$ respectively).

iv. Effect of varying insulin concentration

In contrast, glucose uptake was positively correlated to increasing insulin concentration ($r = 0.458$; $P < 0.01$) and the addition of 10 mU ml^{-1} insulin to cultures significantly increased the glucose uptake by 1 x 10^6 cells compared to controls ($P < 0.05$) (Tab. 3). However, on addition of insulin, irrespective of concentration, there were no significant differences in total protein or DNA concentrations in these cultures.

Discussion

The uptake of glucose in juvenile muscle cells in organ culture was studied in order to gain more insight into the importance of magnesium, insulin and insulin like growthfactors I and II (IGF-I and II) and their interplay in regulating glucose uptake and to determine magnesium and IGF requirements for growth and differentiation in these cells.
DNA concentration but not protein concentration was increased with

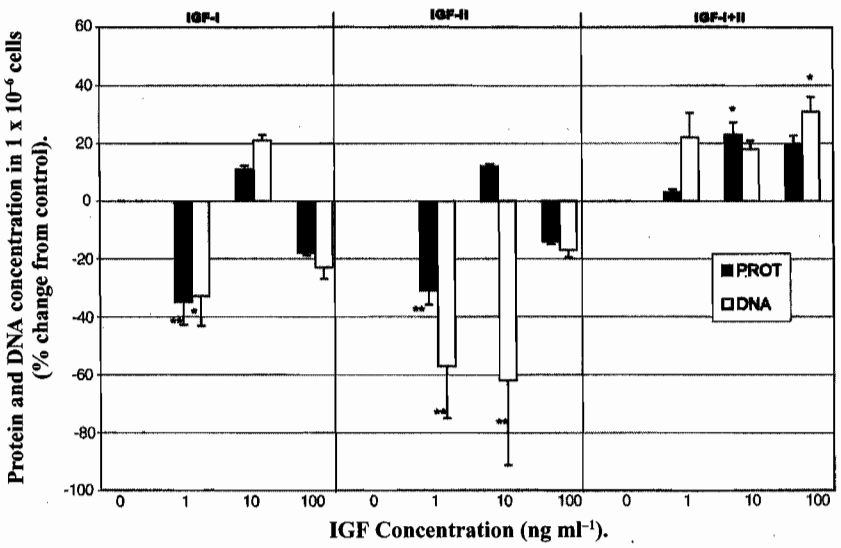

Fig. 4: Effect of insulin-like growth factors on protein and DNA concentration by $1x10^6$ rat skeletal muscle cells in organ culture.
Results are mean (± SD) protein and DNA concentration in 1 x 10^6 cells (% change from control).
* Compared with control, $P < 0.05$; ** compared with control, $P < 0.0001$.

magnesium ion under-nutrition. The dramatic reduction in muscle DNA content resulting from an increase in magnesium ion status (Tab. 2) and number of days in culture (Tab. 1) paralleled changes in glucose uptake without affecting protein concentration. Thus, magnesium may inhibit DNA synthesis by affecting the proportion of cells in the S-Period of the cell cycle [12] or perhaps by promoting DNA destruction.
In otherwords, these results suggest that culture media magnesium ion restriction or under-nutrition as indicated by culture in magnesium free media in this study enhanced viability as indicated by increased DNA concentration whilst stimulating glucose uptake but did not affect protein concentration. Nutrition has been previously shown to profoundly affect growth and maturation of developing tissue [13] and muscle is a tissue, which responds rapidly to changes in hormonal and dietary status.
At high magnesium ion levels, glucose uptake was suppressed, further demonstrating the effectiveness of magnesium ion status in modulating the glucose transport process. One limitation placed on this study was that the authors did not measure cellular, intracellular or free magnesium concentration. In addition, protein and DNA concentration was measured and not synthesis. Clearly, this present study provided information about the influence of exogenous media magnesium concentration on glucose uptake, protein and DNA concentration and provided essential insight into the possible importance of magnesium concentration.
Our results are consistent with, but do not prove a role for magnesium in regulating glucose uptake in rat skeletal muscle. Further studies are required to substantiate our findings before we can relate the importance of cellular, intracellular and free magnesium and their the precise mechanism of action on glucose uptake in skeletal muscle.
In this study, the glucose uptake was more responsive to either IGF-I and/or IGF-II (at low concentration) than to insulin, such that the glucose uptake was similarly stimulated by IGF-I and IGF-II and less by insulin. Insulin did not significantly affect DNA and protein concentration by skeletal muscle cells at the concentrations employed in this study. However, these results indicate that high levels of insulin stimulate glucose uptake by skeletal muscle cells maintained in high magnesium media, but the IGF's were more potent (effective at lower concentrations) stimulators of differentiation and glucose uptake as previously shown [4]. Magnesium has previously been shown to maintain insulin-sensitivity of isolated rat diaphragm and small amounts of magnesium ions was critically important for insulin responsiveness [14].
On administration of either IGF-I or IGF-II, the apparent decrease in the skeletal muscle protein and DNA content was largely accompanied by an increase in glucose uptake. However, muscle protein and DNA concentration was improved by the

Tab. 3: Effect of insulin concentration on glucose uptake, protein and DNA concentration by 1 x 10^6 skeletal muscle cells in organ culture.

Insulin concentration mU l^{-1}	Glucose uptake mmol glucose/ 1 x 10^6 cells	Protein concentration mg protein/ 1 x 10^6 cells	DNA concentration mg DNA/ 1 x 10^6 cells
0.0 (n = 11)	**0.940** (0.324)	**2.01** (0.41)	**0.020** (0.071)
0.1 (n = 12)	**1.033** (0.510)	**2.45** (0.64)	**0.0234** (0.035)
1 (n = 10)	**0.990** (0.593)	**2.40** (0.28)	**0.0234** (0.0421)
10 (n = 12)	**1.568*** (0.596)	**2.18** (0.16)	**0.0222** (0.042)

Results are means (± S.D.).
* compared with control, $P < 0.05$.

simultaneous administration of IGF-I and II.

In conclusion, at high media magnesium concentrations, exogenous IGF-I, IGF-II and insulin (at a high concentration) can act as regulators of glucose metabolism and increase glucose uptake in juvenile skeletal muscle in vitro. In addition, IGF-I and IGF-II in combination can increase DNA and protein concentration in a high magnesium environment.

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