Effect of insulin on intracellular pH and phosphate metabolism in human skeletal muscle \textit{in vivo}

D. J. TAYLOR* , S. W. COPPACK†, T. A. D. CADOUX-HUDSON*, G. J. KEMP*,
G. K. RADDA*, K. N. FRAYN† AND L. L. NG†

*MRC Biochemical and Clinical Magnetic Resonance Unit, John Radcliffe Hospital, Oxford, U.K., and †The Sheikh Rashid Diabetes Unit, Radcliffe Infirmary, Oxford, U.K.

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SUMMARY

1. $^{31}$P nuclear magnetic resonance spectroscopy and the hyperinsulinaemic–euglycaemic clamp were used simultaneously to assess the effect of insulin on intracellular pH and the major phosphorus-containing metabolites of normal human skeletal muscle \textit{in vivo} in four normal subjects.

2. Insulin and glucose were infused for 120 min. Plasma insulin increased approximately 10-fold over pre-clamp levels (5.6 ± 0.9 m-units/l pre-clamp and 54 ± 5 m-units/l over the last hour of infusion; mean ± SEM, n = 4). Plasma glucose concentration did not change significantly (5.4 ± 0.2 mmol/l pre-clamp and 5.5 ± 0.1 mmol/l over the last hour of infusion).

3. Insulin and glucose infusion resulted in a decline in the intracellular pH of forearm muscle of 0.027 ± 0.007 unit/h (P < 0.01), whereas in control studies of the same subjects, pH rose by 0.046 ± 0.005 unit/h (P < 0.001).

4. In the clamp studies, intracellular inorganic phosphate concentration rose by 18%/h, whereas ATP, phosphocreatine and phosphomonoester concentrations did not change. In plasma, inorganic phosphate concentration was 1.16 ± 0.05 mmol/l before infusion, and this decreased by a mean rate of 0.14 mmol h$^{-1}$ l$^{-1}$. No change was observed in any of these intracellular metabolites in the control studies.

5. The results show that, under physiological conditions, insulin does not raise intracellular pH in human muscle, and thus cannot influence muscle metabolism by this mechanism. The results also suggest that insulin causes a primary increase in the next flux of inorganic phosphate across the muscle cell membrane.

**Key words:** insulin, \textit{in vivo}, muscle, $^{31}$P nuclear magnetic resonance spectroscopy, pH, phosphate.

INTRODUCTION

Skeletal muscle is one of the major sites of insulin action, and the potential for insulin to influence its metabolism is substantial. Administration of this hormone leads to an increase in the K$^+$ content of muscle, whereas Na$^+$ efflux is increased due to changes in both Na$^+$/K$^+$-ATPase activity and Na$^+$/H$^+$ exchange [1]. P$_i$ flux across the muscle cell membrane may also be increased, as P$_i$ uptake by muscle is thought to be important in the hypophosphatemia associated with insulin infusion [1, 2].

It has been argued that insulin-induced changes in ion fluxes, particularly in Na$^+$/H$^+$ exchange, stimulate phosphofructokinase activity by increasing intracellular pH (pH$_i$), thereby accounting for the stimulation of glycolysis [3]. Accurate measurement of pH$_i$ is technically difficult, but can be achieved in cells and isolated tissues by using pH-sensitive dyes or in almost any biological system by using $^{31}$P n.m.r. Moore and co-workers [3, 4] used both methods to show that, in isolated frog muscle, insulin can raise pH$_i$ within 30–60 min. An increase of up to 0.12 pH unit was paralleled by an increase in glycolytic activity [3].

An increase of similar magnitude in the pH$_i$ of cultured L$_929$ rat muscle cells was found to occur within 3 min of addition of the hormone. This change lasted for up to 60 min and preceded increased glucose uptake [5]. Other investigators have failed to detect any effect of insulin on pH$_i$ in perfused cat muscle [6]. The significance of the various findings remains uncertain. This is partly because the insulin concentrations used in these studies were several orders of magnitude higher than physiological concentrations, and also because the pH response of cells to insulin depends on other experimental conditions. For example, insulin induced a change in the pH$_i$ of cultured human fibroblasts in the presence of epidermal growth
factor, but not in its absence [7]. Also the presence of HCO$_3$\(^{-}\) in the medium has been shown to affect pH$_i$ regulation through effects on HCO$_3$\(^{-}\)-dependent exchange systems in the cell membrane [8]. Thus, it is not possible to extrapolate straightforwardly from studies of other systems to human skeletal muscle in vivo.

We have used $^{31}$P n.m.r. and the hyperinsulinaemic-euglycaemic clamp techniques to investigate the effects of physiological concentrations of insulin on pH$_i$ and P, in the skeletal muscle of normal human subjects in order to clarify the relationship between insulin action and pH in vivo.

**METHODS**

**Subjects**

Four normal male subjects were studied after an overnight fast. They were non-smokers, were aged 29–41 years and had a body mass index (weight/height$^2$) of 19–24 kg/m$^2$.

The studies were approved by the Central Oxford Research Ethics Committee, and all subjects gave informed consent.

**Hyperinsulinaemic-euglycaemic clamp**

Cannulae (‘Exacta’ 20-gauge arterial cannulae; Viggo Products, Swindon, Wilts, U.K.) were inserted into a right antecubital vein for insulin and glucose infusion, and retrogradely into a vein draining the left hand, which was kept in a box heated to 60–70°C to provide samples of arterialized blood [9]. The sampling cannula was kept patent by flushing with saline (1 50 mmol/l NaCl).

After cannulation, the subjects rested for at least 30 min. Four baseline samples were taken at 5 min intervals before starting a primed infusion of insulin (35 m-units/l) over the last hour of the infusion. Plasma was separated within 15 min and was stored at $-20^\circ$C. The arterialized venous plasma glucose concentration was measured every 5 min on a Beckman Model 2 Glucose Analyser and was clamped at the fasting level as described previously [10, 11]. Plasma insulin concentration was measured by a double-antibody r.i.a. [12]. Plasma P$_i$ concentration was measured by a u.v. phosphomolybdate method on a Technicon RA1000 analyser.

$^{31}$P n.m.r.

A Bruker Biospec spectrometer (Bruker Spectrospin Ltd, Coventry, U.K.) interfaced with a 1.9 T, 30 cm bore superconducting magnet (Oxford Magnet Technology, Oxford, U.K.) was used as previously described [13]. The subject lay supine with the right arm abducted into the bore of the magnet and with a 25 mm double-turn surface coil placed over flexor digitorum superficialis of the right arm. Spectra were collected using a 20 µs pulse length (90° pulse length at coil centre, 16 µs) and a 2 s interpulse delay. The collection time for each spectrum was 128 s immediately before and during insulin and glucose infusion, giving a time resolution for changes in metabolite ratios and pH$_i$ of 2.1 min. Spectra collected before the glucose–insulin infusion was begun served as baseline values for each individual study. In one subject, spectra were collected for 70 min. In three subjects the arm was released from the magnet 40–50 min after the beginning of the infusion and was replaced after about 100 min (no exercise was permitted).

Matched control studies were carried out on the same four subjects without infusion and without the use of the thermostatted box (in two of the subjects, control studies performed using the box made no difference to the results, so only the paired results from all four controls, i.e., without the box, are presented). Spectra were collected from the right arm for at least 50 min. Investigations on any individual were performed 1–2 weeks apart.

Metabolite ratios were calculated from the uncorrected triangulated peak areas of phosphocreatine (PCr), the $\beta$-phosphate of ATP (\$P_{\beta}$-ATP), P, and phosphomonoesters (PME). The absolute concentration of P, was calculated from the P$_i$/ATP ratio, and the pH$_i$ was determined from the chemical shift difference at the P$_i$ and PCr peaks as previously described [13]. The free ADP concentration was calculated from the creatine kinase equilibrium expression (also as in [13]):

$$\text{PCr} + \text{ADP} + H^+ \leftrightarrow \text{ATP} + \text{creatinine}$$  \hspace{1cm} (1)

**Statistical analysis**

All data are expressed as means ± SEM. Slopes were calculated by linear regression over 50 min, because after this time data collection was discontinuous in some subjects. The significance of differences was assessed by using Student’s t-test.

**RESULTS**

Values of plasma insulin and glucose concentrations were almost identical to those obtained previously by using the same technique [11]. Plasma insulin concentration was 5.6 ± 0.9 m-units/l before the start of the infusion and increased 10-fold, reaching a mean value of 54 ± 5 m-units/l over the last hour of the infusion. Plasma glucose concentration was 5.4 ± 0.2 mmol/l at the start of the infusion and 5.5 ± 0.1 mmol/l over the last hour of the infusion. The glucose infusion rate was 35 ± 5 g/h over the last hour, and a total of 55 ± 10 g of glucose was infused overall.

The mean muscle pH$_i$ immediately before the infusion was 7.07 ± 0.01 and was not significantly different in the control studies (7.05 ± 0.01) at the same time point during the experiment. The $^{31}$P n.m.r. data revealed no significant change in muscle pH$_i$ in any subject in the first 15 min after the infusion of insulin and glucose was begun. During this period the pH$_i$ was never more than 0.02 unit higher or 0.04 unit lower than the pre-infusion
value in any subject. The results show clearly, however, that over a longer period insulin did have an effect on pH_i. Fig. 1 shows all of the data points for all four subjects. In each of the four clamp studies the slope of pH_i versus time was significantly negative (P < 0.01). The mean slope for pH_i versus time was \(-0.027 \pm 0.007\) unit/h (P < 0.01) for the four subjects (individual slopes are shown in Fig. 2a). In contrast, in the absence of the insulin–glucose infusion the mean slope was positive at \(0.046 \pm 0.005\) unit/h (P < 0.001). Thus, over a period of 50 min, the infusion resulted in a mean muscle pH_i that was about 0.06 unit lower than that of control muscle.

There was no significant change with time in muscle PCr, \(\beta\)-ATP or PME signal intensities or their ratios in either control or hyperinsulinaemic studies. In the control experiments there was also no significant change in \(P_i\) (Fig. 2). However, during insulin infusion, muscle \(P_i\) increased significantly by 18%/h as shown by the increases in \(P_i/ATP\) and \(P_i/PCr\) (Fig. 2). The normal value of intracellular \(P_i\) concentration measured in this muscle by n.m.r. is 4 mmol/l [13], so the change that we found in the \(P_i\) signal represents a mean rate of increase in absolute muscle \(P_i\) concentration of 0.7 mmol h\(^{-1}\) l\(^{-1}\). Over the time of the infusion, plasma \(P_i\) concentration decreased (Fig. 3). The mean plasma \(P_i\) concentration before infusion was 1.16 ± 0.05 mmol/l, and this decreased by a mean rate over the first hour of 0.14 ± 0.02 mmol h\(^{-1}\) l\(^{-1}\) (P < 0.01).

The muscle PCr and ATP concentrations remained unaltered throughout the experiment, so the changes in pH_i during the clamp led to changes of <4 μmol/l from the pre-infusion ADP concentration of 13 ± 2 μmol/l. At 40–50 min of infusion it was 82 ± 4% of the pre-infusion value (P < 0.05), whereas in control experiments, the ADP

![Graph](image)

**Fig. 1.** Change in pH_i in forearm muscle during insulin–glucose infusion (●) and control experiments (○). Data points from all four subjects are shown. The slopes are given in Fig. 2a. The regression lines of the mean pH_i values for insulin–glucose infusion and control experiments have intercepts of 7.07 and 7.05, respectively, which are identical with the mean pH_i values at time 0.

![Graph](image)

**Fig. 2.** Individual rates of change in parameters measured in insulin–glucose infusion and control experiments. All rates were measured over the first 50 min. (a) Rates of change in pH_i. The slope of mean pH_i was \(-0.027 \pm 0.007\) unit/h (P < 0.01) during insulin–glucose infusion and \(0.046 \pm 0.005\) unit/h (P < 0.001) in control experiments. The mean paired difference in slope between insulin–glucose infusion and control experiments was \(-0.06 \pm 0.02\) (P < 0.05). (b) Rates of change of \(P_i/ATP\). The slope of mean \(P_i/ATP\) was \(14 \pm 4\%\) (P < 0.01) during insulin–glucose infusion, but was not significantly different from zero in control experiments. (c) Rates of change in relative \(P_i/PCr\). The slope of mean \(P_i/PCr\) was \(19 \pm 4\%\) (P < 0.01) during insulin–glucose infusion, but was not significantly different from zero in control experiments.
concentration rose by 29 ± 9% (P < 0.05). During insulin infusion, the intramuscular Pi concentration rose as the ADP concentration decreased, so the phosphorylation potential [(ATP)/(ADP-Pi)], a measure of the energy available to the cell from ATP hydrolysis, did not change significantly.

**DISCUSSION**

$pH_i$

The hyperinsulinaemic clamp technique used in our experiments raises the insulin concentration acutely to a new plateau within the physiological range while maintaining euglycaemia. This allows the tissue response to insulin to be studied without the complications of a varying glucose or insulin concentration. Plasma insulin concentration and glucose consumption increase rapidly and there is an increase in glycolytic flux, yet we found no increase in muscle $pH_i$ during the experiment. In fact, muscle $pH_i$ declined significantly over the infusion period. Thus the action of insulin on glucose uptake or metabolism could not have been due to an increase in $pH_i$. Under certain conditions, insulin obviously can increase $pH_i$ and stimulate glycolysis concomitantly [3, 5], but, as discussed in the Introduction, these results may not be relevant to the muscle in vivo. Our results also show that the effects of insulin on glycolytic flux in muscle cannot be due to a decrease in the ATP/ADP ratio.

What are the possible reasons for the decrease in $pH_i$ in the infused muscle? (1) Insulin increases the resting membrane potential [14]. This change is in the right direction and of the right magnitude to produce an increase in $H^+$ concentration in the cell, but only if the final $pH$ remains above the set point of activation of the Na+/H+ antiport by $H^+$ (in the absence of $H^+$ extrusion, muscle $pH_i$ would be approximately 6.3). (2) The rate of glycolysis is increased. This would lead to more lactic acid production if the pyruvate produced were not oxidized mitochondrially. Previous results suggest that there is little or no net lactate production in forearm muscle during the clamp [15, 16], but little would be required for the small decrease in $pH_i$ observed in our experiments, provided the $H^+$ produced was not exported. Complete oxidation of glucose to CO$_2$ also has an acidifying effect on the myoplasm, and sufficient glucose would be oxidized under the conditions of our experiments [16, 17] to produce the observed changes in $pH_i$ given the known buffering capacity of skeletal muscle [18]. However, the $pH$ of resting muscle is very well regulated [19, 20], so the control of $H^+$ efflux would have to change if $H^+$ were to be retained in the cell (discussed below). (3) Temperature change in the muscle. The cytosolic $pH$ of vertebrate cells decreases as the temperature rises [18]. As measured by indirect calorimetry, increasing glucose metabolism has a thermogenic effect that may be insulin dependent [21], but the measured rise in core temperature and skin temperature from insulin stimulation of glucose metabolism is not significant [22]. Thus, the decrease in $pH_i$ that we find in the hyperinsulinaemic clamp studies is not likely to be due to an increase in muscle temperature. (4) A change in $pH_i$ regulation by the cell membrane. Results of experiments carried out in the past few years suggest that maintenance of $pH_i$ in mammalian cells is likely to be complex. Four systems involving Na$^+$, H$^+$, HCO$_3^-$ and Cl$^-$ have now been identified in mammalian cells [8]. At least two are present in skeletal muscle: the Na$^+/H^+$ antiporter and Na$^+$-independent Cl$^-$/HCO$_3^-$ exchange (see [23]). It has been shown that differential stimulation of the three acid–base transport systems in mesangial cells can cause $pH_i$ to rise or fall depending on the experimental conditions [8], and that the alkalizing effect of growth factors in fibroblasts is not found when HCO$_3^-$ is present in the medium [24]. Such differential effects would help to explain the contradictory findings on the effects of insulin on the $pH_i$ of different muscle preparations. However, the differences cannot be explained simply by the presence or absence of HCO$_3^-$, because insulin-induced rises in $pH_i$ have been found in the presence of HCO$_3^-$ [3] as well as in its absence [5]. Even if insulin had activated Na$^+/H^+$ exchange, our findings of intracellular acidification would suggest that if insulin increased Cl$^-$/HCO$_3^-$ exchange, the activation of this anion exchange would have to exceed that of the Na$^+/H^+$ antiport. There is, however, no evidence for stimulation of Cl$^-$/HCO$_3^-$ exchange by insulin.

The rise in the $pH_i$ of control muscle over the period of the experiment was unexpected. However, little is known about the handling of $H^+$ near the $pH_i$ of resting muscle because experiments investigating $pH_i$ regulation are generally performed by subjecting the tissue to an acid load. As with the hyperinsulinaemic clamp data, we can only speculate on the possible causes of the $pH_i$ change. Given the lack of muscle movement during the experiment, the superficial location of the muscle and the extended position of the arm, it is feasible that a temperature drop contributed to the increase in $pH_i$ by changing the $pK$ of intracellular buffers. For temperature to be entirely responsible, it would have had to decrease by approximately 3 K [18]. Other possible contributory factors are an increase in $H^+$ transport rate and/or a diminished production of metabolic acids.
Our results should be compared with those of Thomsen et al. [25], who observed no change in the \( p\text{H} \) of calf muscle after an oral glucose load. In those studies insulin levels must have increased but were not measured; in ours, glucose concentration was held constant. Thus, although the experimental conditions are not strictly comparable, both studies demonstrate an absence of the predicted rise in \( p\text{H} \).

**Intracellular \( P_i \)**

\( P_i \) uptake by skeletal muscle is thought to be important in insulin- and glucose-induced hypophosphataemia ([2, 26, 27] and reviewed in [1]), as well as in that associated with refeeding [28–30] and alkalosis [27]. Our results show clearly that the insulin-induced decrease in the plasma \( P_i \) concentration was accompanied by an increase in the \( P_i \) concentration in muscle [Figs. 2 and 3]. Such an increase has also been observed in human skeletal muscle after an oral glucose load [26]. What is the mechanism by which this occurs? It is widely believed that \( P_i \) shift hypophosphataemia of the kind we see in our experiments is secondary to increased incorporation of \( P_i \) into organic phosphate compounds [28, 30]. As has been discussed elsewhere [31], this requires a fall in the cytosolic \( P_i \) concentration, and the greater the permeability of the cell membrane to net \( P_i \) influx, the smaller this fall need be. While steady-state exchange fluxes are readily measured by isotopic techniques, this important net-flux permeability has rarely been studied. For human skeletal muscle the near-constancy of the sum of the concentration of \( P_i \) and P\text{Cr} during exercise [19], despite the many-fold rise in the cytosolic \( P_i \) concentration, suggests that the effective net flux permeability for \( P_i \) is low (less than 0.1 mmol h\(^{-1}\) kg\(^{-1}\)) as defined in [31]). If the observed fall in the plasma \( P_i \) concentration were due solely to muscle \( P_i \) uptake, it would mean that muscle uptake was approximately 0.1 mmol h\(^{-1}\) kg\(^{-1}\). This would require the cytosolic \( P_i \) concentration to fall by at least 25%, which would be detectable by n.m.r. spectroscopy. In contrast, the significant rise in the muscle \( P_i \) concentration observed here suggests a primary increase in \( P_i \) influx, perhaps due to an increase in Na\(^{+}\)-linked \( P_i \) uptake [32]. Also, no phosphate sink in the form of an n.m.r.-visible organic phosphate pool was seen. These results are therefore in accordance with earlier reports that insulin stimulates both \( P_i \) uptake in rat diaphragm in the absence of glucose and uptake of the non-metabolizable arsenate ion in rat soleus [reviewed in [1]].

In conclusion, we have found that, under euglycaemic conditions, insulin in physiological concentration does not increase the \( p\text{H} \) of normal human skeletal muscle *in vivo*, but in fact leads to a slow decrease in \( p\text{H} \). This finding refutes the suggestion that the action of insulin may be mediated by the effects of intracellular alkalization on cytoplasmic enzyme activity. We have also obtained evidence for a primary increase in net \( P_i \) flux across the plasma membrane of the muscle cell *in vivo*.

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