Cellular ionic effects of insulin in normal human erythrocytes: a nuclear magnetic resonance study

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Summary. Elevated erythrocyte cytosolic free calcium, and suppressed free magnesium and pH values are associated with the hyperinsulinaemia and insulin resistance of hypertension, obesity, and Type 2 (non-insulin-dependent) diabetes mellitus. To determine the role of insulin in this process, we utilized ¹⁹F- and ³¹P-nuclear magnetic resonance spectroscopy to study the cellular ionic effects of insulin in vitro on normal human erythrocytes. Insulin elevated cytosolic free calcium levels in a dose- and time-dependent manner. The effect began at 10 μ U/ml, peaked at 200 μ U/ml, and continued at both the 500 μ U/ml and 1000 μ U/ml doses. At 200 μ U/ml, free calcium levels rose from 24.6 ± 2.5 nmol/l to a peak value at 120 min of 66.4 ± 11 nmol/l ($p < 0.05$ vs basal), levels remaining elevated throughout the incubation (45.7 ± 5.6 nmol/l at 60 min, and 47.9 ± 9.1 nmol/l at 180 min, $p < 0.05$ vs

basal, respectively). Similarly, insulin also increased intracellular free magnesium at all time points (basal: 177 ± 11 μ mol/l; 60 min: 209 ± 19 μ mol/l; 120 min: 206 ± 22 μ mol/l; and 180 min: 202 ± 12 μ mol/l; $p < 0.05$ vs basal at all times). No insulin-induced changes in pH were observed. We conclude (i) that insulin in physiological concentrations may participate in regulating divalent cations in the mature human erythrocyte, (ii) that insulin per se cannot account for the previously described cellular ionic lesions of hypertension and diabetes, and (iii) that future clinical studies of cell ion metabolism should be conducted in the fasting state, be controlled for ambient circulating insulin levels, or both.

Key words: Insulin, calcium, magnesium, pH, nuclear magnetic resonance spectroscopy, erythrocytes.

Hyperinsulinaemia and peripheral insulin resistance are associated with both essential hypertension and coronary heart disease [1–3], and numerous observations support the suggestion that insulin may directly contribute to the pathophysiology of these syndromes [4–8].

We have studied the relationship between insulin and hypertensive cardiovascular disease at the cellular ionic level, and have found intracellular ionic abnormalities in erythrocytes of subjects with hypertension, obesity, and Type 2 (non-insulin-dependent) diabetes mellitus, including higher cytosolic free calcium and reciprocally lower free magnesium levels [9]. Furthermore, these abnormalities were closely related to the increase in the blood pressure [10], to the degree of cardiac hypertrophy [11], and to the hyperinsulinaemia and insulin resistance of hypertension [12].

Thus, to determine the extent to which insulin itself might be responsible for these cellular ionic abnormalities, we utilized ³¹P- and ¹⁹F-nuclear magnetic resonance (NMR) spectroscopy to measure the effects of graded doses of insulin in vitro on normal erythrocytes. Our results document direct effects of insulin on ion levels in human

erythrocytes, resulting in the cellular accumulation of both free calcium and free magnesium. We suggest that these ionic effects, if also reflective of insulin action elsewhere, may underlie at least some of the tissue actions attributable to insulin [13].

Subjects and methods

After an overnight fast 50 ml of heparinized blood was drawn between 09.00–12.00 hours from non-diabetic normotensive otherwise healthy volunteers who were not taking any medication at the Cardiovascular Center of the New York Hospital-Cornell Medical Center. Blood for each sample ($n = 10$ for each study) was processed using multiple washings in isotonic Hanks' buffered salt solution (HBSS), pH 7.4, and subsequently utilizing ³¹P- and ¹⁹F-NMR techniques as described below, for analysis of intracellular cations Ca_i, Mg_i, and pH_i levels before (basal = 0 min) and 60, 120, 180 min after the addition of regular human insulin to isolated cells (200 μ U/ml). Incubation occurred at 37°C in the continuous presence of insulin.

The dose of 200 μ U/ml was chosen after performing a dose-response curve experiment which showed that at this concentration the maximum cellular effect is reached. For this experiment increasing doses of insulin were added to separate tubes ($n = 6$ for each con-

Fig. 1. ^{19}F -nuclear magnetic resonance spectrum of QUIN-MF (right, second peak) and Ca-Quin-MF (left, first peak) in intact erythrocytes. Integration of the areas under the respective resonance peaks allows for a calculation of intracellular free calcium (see Subjects and Methods)

Fig. 2. Time course of the effect of insulin (200 $\mu\text{U}/\text{ml}$) on erythrocyte intracellular free calcium, Ca_i , and free magnesium, Mg_i , levels

centration), to achieve a final concentration range of 0 (basal, no insulin added), 10, 50, 100, 200, 500 and 1000 $\mu\text{U}/\text{ml}$. After 60 min of incubation at each concentration, ^{19}F -NMR analyses of cytosolic free calcium were repeated, and compared to the unsupplemented basal values. Our previous experience with the NMR measurements and those reported here demonstrated the stability of the basal values for the entire time course of the experiments.

^{31}P -NMR analysis of Mg_i and pH_i

The methods for ^{31}P -NMR analysis of Mg_i and pH_i have been described in detail elsewhere [10, 14]. The intracellular magnesium concentration was determined according to the equation [10]:

$$\text{Mg}_i = K_d(\text{MgATP}) \times (\Phi^{-1} - 1)$$

where $K_d(\text{MgATP})$ is the apparent dissociation constant for the reaction $\text{MgATP} = \text{Mg}^{2+} + \text{ATP} = 0.4 \times 10^{-5} \text{ mol/l}$ under physiological condi-

tions at 37°C and at $\text{pH } 7.2$; and $\Phi = (\text{ATP})_{\text{free}}/(\text{ATP})_{\text{total}}$ is determined from the chemical shift difference of the α - and β -phosphoryl group resonances of ATP in the ^{31}P -NMR spectrum.

The pH was determined by measuring the chemical shift difference of the 3 and 2 phosphoryl resonances of the 2,3 diphosphoglycerate (2,3 DPG) on the ^{31}P spectra [15]. ^{31}P -NMR and the chemical shift difference of the 3- and 2-phosphoryl resonances of 2,3-diphosphoglycerate were plotted against the pH value at which the spectrum was obtained. A titration curve, prepared by analysing spectra obtained at various known pH values, was linear within the pH range tested and was used to determine the pH of unknowns [14].

^{19}F -NMR analysis of Ca_i

Ca_i was determined in erythrocytes by a modification of the method of Levy et al. [16], using the fluorinated, membrane-permeant calcium chelator Quin-MF-AM. Blood (10 ml) was centrifuged at 2000 rev/min for 10 min to separate plasma from the packed cells. The packed erythrocytes were suspended in 100 ml of HBSS with 0.35 g NaHCO_3 at a pH of 7.4. Cells were loaded in a shaking water bath with 20 $\mu\text{mol/l}$ Quin-MF-AM, a membrane permeant Ca^{2+} -chelator, for 25 min at 37°C .

Loaded cells were subsequently centrifuged at 2000 rev/min for 10 min, the medium was aspirated, and the cells resuspended in 50 ml of fresh HBSS together with the subject's plasma (approximately 5 ml) which was then incubated at 37°C for 90 min. Cells were centrifuged, washed again with fresh HBSS together with another aliquot of 5 ml of plasma, and then recentrifuged and decanted into 10 mm NMR tubes for analysis. ^{19}F -NMR were run on Varian VXR-500 NMR spectrometer operating at 470 MHz and 37°C . Free cytosolic calcium was calculated according to the formula:

$$\text{Ca}_i = K_{d,\text{app}} \times (\text{Ca-Quin-MF}/(\text{Quin-MF})) [9],$$

where $K_{d,\text{app}}$ of Ca-Quin-MF was calculated as 139 nmol/l in erythrocyte haemolysates at 37°C , and where $\text{Ca-Quin-MF}/(\text{Quin-MF})$ was calculated from the ratio of areas of the Quin-MF vs Ca-Quin-MF resonances peaks on the ^{19}F -NMR spectrum (Fig. 1).

Statistical analysis

Data for the responses of each cellular ion species to insulin were analysed for statistical significance using one-way analysis of variance and subsequent post-hoc *t*-test (Bonferroni Dunn) (SuperAnova, Abacus Concepts Inc., Berkeley Calif., USA) for the effects of insulin over time at 0, 60, 120, and 180 min, and over the range of concentrations tested at 10, 50, 100, 200, 500, 1000 $\mu\text{U}/\text{l}$. All values are reported as the means $\pm$ SEM.

Results

Compared to basal, unstimulated levels of intracellular free calcium (Ca_i , $24.6 \pm 2.5 \text{ nmol/l}$), intracellular free magnesium (Mg_i , $177 \pm 11 \mu\text{mol/l}$), and intracellular pH (pH_i , 7.26 ± 0.02), insulin (200 $\mu\text{U}/\text{ml}$) elevated Ca_i at 60 min ($45.7 \pm 5.6 \text{ nmol/l}$, $p < 0.05$ vs basal), 120 min ($66.4 \pm 11.0 \text{ nmol/l}$, $p < 0.05$ vs basal), and 180 min ($47.9 \pm 9.1 \text{ nmol/l}$, $p < 0.05$ vs basal) (Fig. 2). Similarly, Mg_i rose in response to insulin in all subjects. This effect peaked at 60 min of incubation ($209 \pm 19 \mu\text{mol/l}$, $p < 0.05$ vs basal), but persisted at 120 ($206 \pm 22 \mu\text{mol/l}$, $p < 0.05$ vs basal) and 180 min ($202 \pm 12 \mu\text{mol/l}$, $p < 0.05$ vs basal) (Fig. 2). No significant effect of insulin on pH_i levels was detected in this *in vitro* erythrocyte system. After 60, 120, and 180 min of incubation, pH_i values were, respectively, 7.24 ± 0.02 , 7.25 ± 0.02 , and 7.24 ± 0.02 ($p = \text{NS}$ vs basal at all time points).

Fig. 3. Dose response curve of the effect of insulin on erythrocyte intracellular free calcium, Ca_i

Insulin stimulated Ca_i levels in all subjects, an effect which was dose dependent. When increasing doses of insulin were added to different aliquots of blood from the same subject, Ca_i rose in a hyperbolic fashion, asymptotically approaching peak values at 200 $\mu\text{U/ml}$, and plateauing thereafter, identical results being obtained at 500 and 1000 $\mu\text{U/ml}$ concentrations. Conversely, even the lowest concentration of insulin tested, 10 $\mu\text{U/ml}$, significantly ($p < 0.05$) elevated Ca_i levels. At each dose, the Ca_i responses were: basal, 22.9 ± 2.1 nmol/l; 10 $\mu\text{U/ml}$, 37.1 ± 4.3 nmol/l; 50 $\mu\text{U/ml}$, 39.8 ± 7.1 nmol/l; 100 $\mu\text{U/ml}$, 46.3 ± 4.5 nmol/l; and 200 $\mu\text{U/ml}$, 64.8 ± 5.8 nmol/l. Further increases in insulin (500 and 1000 $\mu\text{U/ml}$) did not cause any further increase in Ca_i (Fig. 3).

Discussion

The clinical association of hypertension with obesity, insulin resistance, hyperinsulinaemia, with or without overt Type 2 diabetes, has long been appreciated, and a role for insulin itself in the hypertensive process has been postulated [1–8]. We have focussed on a role for altered cellular ion metabolism in mediating this association, and observed that the afore mentioned disorders in whole or in part, are characterized by increased cytosolic free calcium, and suppressed free magnesium and pH_i values [9, 17].

To investigate the extent to which the hyperinsulinaemia characterizing each of these clinical states could itself be responsible for these ionic alterations, we utilized ^{19}F - and ^{31}P -NMR spectroscopic techniques to study the direct in vitro effects of insulin on Ca_i , Mg_i , and pH_i levels in human erythrocytes. We observed that at physiological concentrations insulin concurrently elevated Ca_i and Mg_i levels, but did not result in a significant change in pH_i . These results not only confirm previous reports [18], but suggest that hyperinsulinaemia per se cannot account for either the Mg_i or pH_i disturbances which we previously reported in hypertension and diabetes. However, our results demonstrate for the first time a functional role of insulin in the mature human erythrocyte, and suggest that insulin may participate in the regulation of erythrocyte ion homeostasis. Future studies of ions in the erythrocyte will

therefore require a consideration of the insulinaemic status of the subject, and whether blood was obtained in the fasted or fed state.

Insulin directly elevated Ca_i in the erythrocyte at all doses tested, from 10 $\mu\text{U/ml}$ to 1000 $\mu\text{U/ml}$, confirming a report in isolated adipocytes, where Ca_i was measured using fluorescent probe techniques [19]. The mechanism by which insulin increases Ca_i is still unknown, although its action to inhibit CaATPase [20, 21], as well as to stimulate protein kinase C and diacylglycerol formation [22, 23] may be involved. The latter mechanism, dependent on Ca_i release from intracellular stores, cannot account for the results observed here in the erythrocyte.

The action of insulin to increase Mg_i also confirms data obtained previously using different techniques, in which total intracellular magnesium content increased in response to insulin applied to erythrocytes [18] and uterine smooth muscle cells [24]. Possible mechanisms include its action on an ATPase -dependent Mg pump [25], as well as via an Na -stimulated Mg transport system recently described in human erythrocytes [26]. In the present study we were not able to detect any direct insulin effect on pH_i in vitro, despite evidence that insulin stimulates Na^+/H^+ exchange in vitro [27], and that pH_i rises following glucose loading in vivo [28]. Indeed, a reduction [29], no change [30], or an increase [31] in pH_i , have been observed with insulin, which may depend on the presence of other concurrent factors [22].

What are the possible clinical implications of these data? First, despite epidemiologic studies linking hyperinsulinaemia to elevated blood pressure [1, 3], insulin itself is a vasodilator, and may even induce hypotension in the absence of an adequate, compensatory rise in catecholamine activity [32]. Secondly, and in a parallel manner, the result of insulin action to increase both Ca_i and Mg_i concentrations may also appear antagonistic, these ions affecting blood pressure differently; the increase in Ca_i increasing peripheral vasoconstriction and possibly elevating blood pressure, while an increase in Mg_i having the opposite effect [33]. Thus, magnesium antagonizes calcium entry into vascular smooth muscle [34], competes for calcium binding on calmodulin [35], and may induce vasodilatation [36]. Mg_i may also influence the intracellular content and disposition of calcium, decreased Mg_i levels being associated with increased levels of Ca_i in both essential hypertension and Type 2 diabetes [9]. If insulin itself contributes to the hypertensive process, then our data as well as those of others [37, 38] suggest the hypothesis that a dissociation of this normal, balanced cellular ionic action of insulin may occur, in which insulin-stimulated calcium accumulation predominates vis-à-vis its action to promote cellular magnesium uptake, the result being enhanced vasoconstriction, platelet aggregation, and other consequences of excess cellular calcium accumulation.

In summary, this study supports the hypothesis that insulin is an important modulator of intracellular divalent cation metabolism, independently of glucose transport. Further studies are needed to clarify whether altered cellular ionic responses to insulin are present in insulin-resistant states. Lastly, regardless of other implications, this study indicates that all future clinical studies of cellular ion

metabolism should be performed in the fasting state, take into account circulating insulin levels, or both.

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