IN VIVO KINETICS OF 123 IODINE-LABELLED INSULIN IN SKELETAL MUSCLE OF PATIENTS WITH TYPE 2 DIABETES. EFFECT OF METFORMIN

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SUMMARY - Background: The capillary filtration of albumin (CFA) is often increased in diabetic patients. However, the transcapillary transfer of insulin is considered to be a key stage in insulin action. The aim was to study the in vivo kinetics of 123 I-labelled human insulin in the skeletal muscle of type 2 diabetic patients with an increase in CFA and to evaluate the effect of metformin, using a noninvasive method.

Methods: Ten type 2 diabetic patients and 6 healthy control subjects were investigated. After an i.v injection of 123 I-labelled insulin, venous samples were drawn during 75 minutes and radioactivity was counted externally by a gammacamera on the contralateral forearm before, during and after venous compression. The changes in the serum percentage of bound and free 123I were followed during the entire test and the forearm iodine bound to insulin was estimated by multiplying the forearm counted radioactivity by the serum percentage of bound iodine at the same time.

Results: In the diabetic patients the maximal increase in the forearm iodine bound to insulin during venous compression was lower (p = 0.06), and 10 minutes after removal of venous compression the forearm retention of labelled insulin was significantly lower (p < 0.0005) than in controls. After one month of metformin treatment, retention of labelled insulin significantly increased (p < 0.001) but was still significantly lower than in the controls (p < 0.001).

Conclusion: The in vivo kinetics of 123 I-labelled insulin procedure allows the study of skeletal muscle metabolism provided venous compression is exerted. In type 2 diabetic patients a reduction of insulin transfer from capillary to tissue despite an increase in CFA, and a reduction of the time spent by insulin in the tissues contribute to insulin resistance. The latter disorder may be improved by metformin.

Key-words: type 2 diabetes, capillary filtration, insulin resistance, labelled insulin, metformin.

RÉSUMÉ - Cinétique in vivo de l’insuline marquée à l’iode 123 dans le muscle squelettique de patients diabétiques de type 2. Effets de la metformine.

Contexte : La filtration capillaire d’albumine (FCA) est souvent augmentée chez les patients diabétiques. Cependant, le transfert transcapillaire de l’insuline est considéré comme une étape clé pour l’action de l’hormone.

Méthodes : Dix diabétiques de type 2 et six sujets normaux (témoins) ont été explorés. Après injection intra-veineuse d’insuline marquée à l’iode 123, des échantillons veineux ont été prélevés pendant 75 min et la radioactivité a été comptée sur l’avant-bras opponé, avant, pendant et après compression veineuse.

Résultats : Chez les patients diabétiques, sur l’avant-bras, l’augmentation maximale de l’iode lié à l’insuline pendant la compression veineuse était réduite (p = 0,06), et 10 min après levée de la compression veineuse, la rétention d’insuline marquée était significativement plus faible (p < 0,0005) que chez les témoins. Après un mois de traitement par metformine, la rétention d’insuline marquée était significativement augmentée (p < 0,001) mais toujours significativement plus basse que chez les témoins (p < 0,001).


Mots-clés : diabète de type 2, filtration capillaire, insulinorésistance, insuline marquée, metformine.
Insulin action in vivo proceeds step by step, e.g. insulin transport across capillaries, receptor binding and phosphorylation, translocation of glucose transporters to the membrane and intra-cellular metabolic events. There is evidence supporting the concept that the movement of insulin into the interstitium, which precedes the binding of insulin to its receptor, is an important rate-determining stage in insulin action. Indeed a continuous capillary endothelium is a significant barrier to free diffusion from the blood into tissues such as skeletal muscle, adipose tissue and skin [1]. The glycocalyx, the basement membrane and the interstitium beyond the capillary contribute to reducing diffusion [2, 3] and thus gradients of insulin concentration from capillary to tissue are unavoidable when plasma insulin concentrations change. Rasio et al. have shown that there is a delay between changes in plasma versus lymph insulin concentrations during an intravenous glucose tolerance test [4-6]. The movement of insulin across the capillary endothelial cell in vitro is a rapid, receptor-mediated and transendocytotic process [7-9], and this transport is saturable [10].

Evidence has been obtained in dogs [11] and also in humans [12] that insulin penetration into tissues from the blood was rate-limiting for insulin action. In these studies lymph insulin concentrations were found to be far lower than arterial insulin concentrations and the strongest correlation with glucose uptake during the euglycemic hyperinsulinenic clamp was found with lymph insulin and not with arterial insulin [11, 12]. The lymphatic insulin concentration seems to determine in vivo insulin action. Measurements in the interstitial fluid by the microdialysis technique have shown that subcutaneous interstitial insulin is about 50% of the plasma concentration [13]. These findings reveal that the transcapillary transport of insulin may constitute a possible factor in the pathogenesis of glucose intolerance, diabetes and other diseases with insulin resistance.

$^{125}$I-labelled insulin has been used several times in rats and humans to study whole-body kinetics, the greatest advantage being its short half-life time and the possibilities of direct imaging of insulin binding to the target-organ site [14-17]. However, to our knowledge, this tracer has never been used to study the in vivo muscle metabolism of insulin.

Capillary permeability to small molecules and to albumin has been shown to be increased in diabetes in several studies [18, 19]. We have previously described an isotopic test using technetium-labelled albumin and a procedure derived from the Landis method [20, 21]. These investigations have shown that in more than half of the diabetic patients the capillary filtration of albumin was increased and the lymphatic uptake of interstitial albumin was reduced [22]. Both of these disorders contribute to interstitial retention of albumin. In these patients, interstitial retention of insulin is likewise expected to be increased. If such is not the case, a reduction in transcapillary transfer or an exaggerated lymphatic uptake of insulin might be suspected. Therefore an in vivo method investigating these phenomena and testing drug effects in type 2 diabetic patients would be useful. The present work aimed to study the in vivo kinetics of $^{125}$I-labelled human insulin in skeletal muscle of type 2 diabetic patients with an increased capillary filtration of albumin and in control subjects, and to carry out a pilot open trial evaluating the effect of metformin, an agent widely known to improve insulin sensitivity and insulin binding [23-25].

**Subjects and Methods**

**Subjects**

Six healthy control subjects, four men and two women, aged 28 to 58 years, with normal body weight (mean BMI = $20.9 \pm 3.0$ kg/m$^2$), were volunteers participating in the study.

Ten type 2 diabetic patients, six men and four women, aged 37 to 62 years were included. Mean duration of diabetes was 7.7 years (range 1-22). Mean body mass index was 26.9 kg/m$^2$ (range: 24.7-28.7). Hypoglycemic oral treatment was withdrawn at least one month before inclusion and none of these patients was taking any other drug. None of them had a disease which could induce abnormal capillary permeability, such as hypertension, nephrotic syndrome, cyclic oedema, thyroid failure, heart or liver disease [19]. They were all moderately hyperglycemic at the time of the investigation (fasting blood glucose = 5.2-9.4 mmol/l) and under a controlled diet. An isotopic test was performed in these patients as previously described [20-22] and showed an increase in albumin retention (14.1% ± 2.8 SD) as compared with 0.4% ± 0.2 in control subjects, which suggests a marked increase in capillary filtration of albumin.

**Methods**

**Isotopic test**

**Labelling of insulin**

Labelling of insulin was carried out as previously described [14, 26]. Human regular lyophilised insulin was a gift of Organon Laboratories (Gilly, France). Insulin was monooiodinated in the tyrosin A14 position with $^{125}$I. The iodination was started by adding lactoperoxidase. $^{125}$I-labelled insulin was purified by chromatography. The final solution was then sterileized by ultrafiltration through a 0.22 µm millipore filter and was made ready for injection by diluting with normal saline (total volume of the injectant = 4 to 5 ml).
Preliminary studies to assess the *in vivo* biological potency of $^{123}$I-labelled insulin were carried out using a blood glucose depression test in normal rats [26]. The labelling efficiency rate was evaluated by a second chromatography: $73.6\% \pm 11.0$ (SD), and it was stable for 24 hours.

**Protocol**

Each day during the study, just before the injection and in the evening following it, each subject received 500 mg of potassium perchlorate orally to block thyroid uptake of iodine. The investigations started at 08.00 hr on a subject fasting for 12 hours. The experimental procedure was based on the protocol developed by Sodoyez et al. with specific modifications for the study of the muscle kinetics of insulin [14, 15].

Following the injection of 37 MBq (1mCi) $^{123}$I-labelled insulin (3 to 4 U) in an antecubital vein underneath a gammacamera, dynamic gammacamera images were done over the upper abdominal area for 40 minutes. Then areas of interest were defined over the heart, liver, spleen and thyroid gland. Two ml venous samples taken through an intravenous cannula placed in the homolateral hand were drawn at 1, 3, 5, 10, 15, 17.5, 20 minutes and every five minutes thereafter, until 45 minutes after the injection, since it has been shown that at 45 minutes half the plasma radioactivity is bound to insulin and that this proportion of iodinated insulin remains stable for the next 30 minutes [16, 17]. The 1 min value of serum radioactivity was taken in each case as the maximum reference value for the injected dose.

Forty-five minutes after the injection, the protocol was similar to the isotopic test for capillary filtration of albumin, derived from the Landis method we have been using for many years [20, 21]. Briefly, venous compression (80 mmHg) was exerted for 12 minutes on the arm contralateral to the injection and venous sampling. Radioactivity was counted externally by a gammacamera on the forearm homolateral to venous sampling. Radioactivity was counted on the forearm was $\frac{\text{activity in counts per second}}{\text{activity}}$ and in the subcutaneous tissue of the forearm was $\frac{\text{activity in counts per second}}{\text{activity}}$. The changes in the percentages of bound and free $^{123}$I were thus followed during the entire isotopic test. In order to determine the forearm iodine bound to insulin (FIBI), radioactivity counted on the forearm was first corrected by physical decay percentage of $^{123}$I (with half-life = 13 hours and energy X-rays = 159 keV) and then multiplied by the percentage of bound iodine measured in the serum at the same time. The maximal increase of FIBI during venous compression was expressed as a percentage of the basal level before compression. Forearm retention of insulin was evaluated 10 minutes after the removal of venous compression as previously reported for albumin retention [20-22] by calculating the percentage $[(\text{Residual FIBI at } 10\text{ min } - \text{Basal FIBI})/(\text{Maximal FIBI } - \text{Basal FIBI})] \times 100$.

**Metformin trial**

Eight of the 10 diabetic patients agreed to participate in an open trial with 1.700 mg metformin/day for one month and to have a second isotopic test 24 hours after the last tablet of metformin. During this month diet and body weight remained unchanged.

Before and at the end of the trial, fasting and post-prandial blood glucose, serum cholesterol and triglycerides, fructosamine, fasting and post-prandial plasma insulin were measured. Serum fructosamine was measured by a colorimetric method with nitrilotetrazolium blue (Roche Diagnostic System, Neuilly sur Seine, France) (normal $< 285 \mu\text{mol/l}$) and HbA1c was measured on microcolumn chromatography (normal $< 6\%$). Plasma insulin concentrations were determined by radio immunoassay (Behring Institute, Mahrburg, Germany) (Normal range at fasting: 20-130 pmol/l).

Approval to perform the study was given by the local Committee for the Protection of Persons in Research in Biological Trials of Aulnay sous Bois and each subject signed their written consent before being included in the study.

**Statistical analyses**

Results are given as mean ± sd or mean (range) values. Comparisons of quantitative parameters were performed by Wilkoxon tests for paired and unpaired series.

■ **RESULTS**

**Study on type 2 diabetic patients**

On the day of the isotopic test, glycemic control was good in most of the patients as supported by fasting blood glucose below 10 mmol/l and normal or nearly normal serum fructosamine levels and HbA1c either normal or slightly elevated. No adverse effect occurred during the isotopic test and blood glucose never decreased below 4 mmol/l.

The time-activity changes were obtained from the defined areas of interest (heart, liver and spleen), as shown for a control subject on Fig. 1. As the heart activity in counts per second here represents the total activity injected, the thyroid activity was less than one
percent of the heart activity, and in the liver, maximum activity (Amax) occurred 5 to 27 minutes (Tmax) after the injection. There was a trend (NS) to a higher mean value of the liver Amax/heart Amax ratio in the diabetic patients (17.9 ± 7.0%) than in the control subjects (8.5 ± 2.9%).

In the serum samples, the percentage of bound 123I decreased rapidly during the first 15 minutes after the injection of labelled insulin and then more slowly, and finally remained approximately constant between 45 and 75 minutes after injection (Fig. 2). In controls it decreased by 35% on average during the first 15 minutes, by 7% more between 15 and 45 min, and thereafter it was remarkably constant until 75 minutes. The same kinetic pattern was found in diabetic patients (Table I).

In each subject FIBI increased by the first 5 minutes following the exertion of venous compression and after 7 to 10 minutes it reached a plateau lasting 2 minutes at least. After removal of venous compression, FIBI decreased and remained higher than the basal radioactivity levels in each control subject whereas it decreased below the basal level in most of the diabetic patients (Fig. 3). The maximal FIBI increase during venous compression was found to be lower, although not statistically significant, in the diabetic patients than in the control subjects: 112 ± 15% vs 135 ± 25% (p = 0.06). Ten minutes after removal of venous compression the forearm retention of labelled insulin was significantly lower in the diabetic patients than in the controls: −20 ± 11% vs +21 ± 10%, p < 0.0005 (Fig. 4).

**Metformin trial on the type 2 diabetic patients**

Biological parameters remained unchanged after one month of metformin treatment (Table II). The ratio liver Amax/heart Amax decreased, although non significantly, to 11.9 ± 5.0% (p = 0.10 vs before treatment). In the serum samples, the kinetic decrease of serum insulin-bound 123I percentage during the isotopic test was very similar before and after metformin treatment (Table I). The maximal increase of FIBI during venous compression was unchanged: 112 ± 15%. The forearm retention of labelled insulin (+ 3 ± 4%) was significantly increased (p < 0.001 vs before metformin treatment), but remained significantly lower than in the controls (p < 0.001) (Fig. 4).

**DISCUSSION**

The transfer of insulin from the blood into tissues could be an important rate-limiting stage in insulin action. Endothelial cells can internalize and release insulin rapidly with minimal degradation [8]. Less than 10% insulin was thus found in the degradation compartment constituted by the lysosomes and at least
80% of the insulin was recovered intact [27]. Hence a hypothesis has been raised that this endothelium-stored insulin represents the first hormone pool available upon sudden increases in plasma glucose levels. The transport mechanisms in the endothelial cells could thus operate by modulating insulin delivery and action at target tissue sites [27-29]. However it has been shown in obese men that insulin penetration into tissues appears to be as rate-limiting as in the lean subjects [12]. Nevertheless insulin transfer across capillaries might substantially modify insulin sensitivity in diabetic patients. Defects in insulin receptor struc-
ture and function have been found in capillary endothelial cells from diabetic rats [30].

The kinetics of $^{123}$I-labelled insulin has been proposed to study in vivo insulin metabolism [14-17].

**Table I.** Changes in serum bound $^{123}$I fraction (%), 15, 45 and 75 min after injection of $^{123}$I labelled insulin and at the radioactivity forearm peak level. Venous compression was exerted from the 45th to the 59th minute.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>15 min</th>
<th>45 min</th>
<th>Radioactivity peak</th>
<th>75 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (6)</td>
<td>65 ± 15</td>
<td>58 ± 15</td>
<td>58 ± 15</td>
<td>58 ± 15</td>
</tr>
<tr>
<td>Type 2 diabetic patients (10)</td>
<td>62 ± 14</td>
<td>55 ± 15</td>
<td>54 ± 15</td>
<td>53 ± 15</td>
</tr>
<tr>
<td>Type 2 diabetic patients after metformin (8)</td>
<td>62 ± 8</td>
<td>56 ± 6</td>
<td>56 ± 6</td>
<td>56 ± 16</td>
</tr>
</tbody>
</table>

**Fig 3.** Forearm external counting of radioactivity (ips: impulses per second) by a gamma-camera in a normal subject (A) and a type 2 diabetic patient (B) during (45 to 57 minutes) and after (until 75 minutes) venous compression. Forearm external radioactivity was corrected by physical $^{123}$I decay and the percentage of bound $^{123}$I measured in the simultaneous serum samples. The radioactivity curve of the diabetic patient is shown before (●) and after (■) metformin treatment. The dotted lines correspond to the basal radioactivity level measured at 45 minutes. This level differed between both investigations, mainly due to the labelling efficiency rate. Tissue retention of labelled insulin was 34% in the control subject, ~40% and 4.5% in the diabetic patient before and after metformin treatment respectively.
trend towards a value of the liver Amax/heart Amax ratio found here to be higher in the diabetic patients than the controls has not been reported by other authors [16]. The increase in liver uptake of radiolabelled insulin is shown to be consistent with a lower uptake of insulin by skeletal muscles. This isotopic method has been used here for the first time in the study of the skeletal muscle metabolism of human insulin. Serum disappearance and the kinetics of tissue uptake of labelled insulin could be useful tools for the in vivo investigation of insulin resistance. We focused here on the insulin metabolism in the skeletal muscles since they represent the main site of insulin resistance in diabetes.

The isotopic test we describe in the present paper was derived from the technetium-labelled-albumin capillary filtration test [31]. This method was designed to investigate capillary permeability and uses venous

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**Table II.** Changes in biological parameters during the metformin trial in 8 type 2 diabetic patients. Day 0: at baseline. Day 30: after 30 days of metformin treatment. Data are given as mean (range) values.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 0</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glycemia (mmol/l)</td>
<td>6.68 (5.2; 9.4)</td>
<td>6.08 (4.9; 7.4)</td>
</tr>
<tr>
<td>Post-prandial glycemia (mmol/l)</td>
<td>8.76 (4.7; 13.4)</td>
<td>8.05 (4.3; 12.0)</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.31 (0.65; 2.10)</td>
<td>1.13 (0.58; 1.64)</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.54 (2.97; 8.13)</td>
<td>4.86 (2.91; 7.01)</td>
</tr>
<tr>
<td>Fructosamine (µmol/l)</td>
<td>265 (206; 297)</td>
<td>255 (230; 296)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.53 (4.2; 8.1)</td>
<td></td>
</tr>
<tr>
<td>Fasting plasma insulin (pmol/l)</td>
<td>69 (46; 93)</td>
<td>73 (50; 92)</td>
</tr>
<tr>
<td>Post-prandial plasma insulin (pmol/l)</td>
<td>300 (168; 459)</td>
<td>292 (142; 498)</td>
</tr>
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</table>
compression, which induces blood stasis and transcapillary escape of fluids and small and large molecules. As a result lymph flow usually increases but cannot overcome the important leakage of different micro and macromolecules in the interstitial space. We have shown with this method that during venous compression, the forearm radioactivity curve with labelled albumin increases sharply. Similarly, we have studied the radioactivity curve after $^{125}$I-labelled insulin iv injection, during and after venous compression. The percentage of serum insulin-bound $^{125}$I was quite stable during this period both in controls (at 58%) and diabetic patients (around 55%) (Table I). This result is very close to those previously shown by other investigators who found that 45 minutes after the injection half of the serum radioactivity was bound to insulin and this proportion remained stable during the next 30 minutes [16, 17]. This finding strongly suggests that venous compression does not influence the decrease in insulin-bound $^{125}$I. In order to determine the forearm radioactivity bound to insulin, we took the physical decay of $^{125}$I into account and we multiplied this corrected value by the percentage of $^{125}$I-bound measured in the serum at the same time. During venous compression, FIBI increases. This increase is too great to result only from blood stasis and very likely reflects the leakage of insulin. In the diabetic patients the maximal FIBI increase appeared to be reduced as compared to controls. Since the maximal increase was usually reached after 7 to 10 minutes, it is unlikely to depend on blood flow, all the more so since forearm blood flow is unchanged in diabetic patients [32] and during the test there was no hypoglycemic episode which might have induced changes in blood flow in diabetic patients and controls. The lower increase in FIBI more likely results from a reduction in the endothelial pool of labelled insulin and/or a decrease in the hormone diffusion process. The first mechanism may be due to abnormal insulin receptors in the capillary endothelial cells as shown in diabetic rats [30] or to a reduction in the capillary density in muscle fibers as shown in diabetes and hypertension [33, 34] or to the occupation of endothelial receptors by cold endogenous insulin. The second mechanism is linked to a reduction in insulin diffusion, possibly through a thickening of the capillary basement membrane [35]. On the other hand, the transcapillary escape of albumin which is often increased in diabetic patients [20, 36] was indeed increased in the patients presently investigated. This discrepancy is probably based on the transfer of albumin through different pores, and supports the view that the transfer of insulin may be selectively reduced even in patients with an increased capillary transfer of other proteins.

The second obvious difference between diabetic patients and controls concerns the level of the forearm labelled insulin after removal of venous compression. In diabetic patients, FIBI decreased more quickly and reached levels below basal line, which suggests an accelerated disappearance of insulin whereas in control subjects radioactivity remained at levels higher than basal line until 20 minutes after removal of venous compression, which is consistent with tissue insulin stasis. Tissue stasis of labelled insulin is located either in the interstitial space, in the skeletal muscle cells or in adipose cells. In diabetic patients a reduction in both the interstitial stasis and cell uptake of insulin may be involved and this disorder may result from an accelerated lymphatic uptake of interstitial insulin. The acceleration of lymphatic transport of insulin may be due to the increase in lymph flow as previously shown in diabetes [37] or to an increased uptake of free insulin remaining in interstitial space.

In the present open design trial of metformin, the maximal increase of FIBI and therefore the transfer of insulin through capillaries was not modified by metformin, while “retention” of insulin after the removal of venous compression was significantly higher after one month of metformin treatment. This result is in variance with the improvement in the lymphatic uptake of interstitial albumin we previously found with metformin treatment [38, 39]. This discrepancy strongly suggests that after metformin muscle fiber uptake of insulin is enhanced. The trend to a decrease in the liver Amax/heart Amax ratio is consistent with this hypothesis. The increase in muscle uptake of insulin could be related to a metformin-induced increase in insulin receptors [23, 24]. Finally it is noticeable that the metformin effects were not associated with a significant change in glycemia and therefore appear to be independant of the changes in glycemic control.

In conclusion, the in vivo kinetics of $^{125}$I-labelled insulin clearly show abnormalities in the muscle metabolism of insulin in type 2 diabetic patients. The transfer of insulin through the capillary wall is reduced despite an increase in capillary filtration of albumin. The time insulin remains in the tissues is also reduced. These abnormalities might contribute to insulin resistance. However additional experiments are required to confirm this hypothesis. The time insulin remains in the tissues seems to be prolonged by metformin treatment and this very likely results from an increase in muscle fiber uptake of insulin. Finally the noninvasive scintigraphic procedure using $^{125}$I-labelled insulin with the venous compression which we have added to the procedure previously described by other authors might be one attractive way to investigate both peripheral insulin resistance in vivo and a drug effect on the capillary transfer and cell uptake of insulin. The effect of metformin on the capillary transfer of insulin to tissues needs to be confirmed in a placebo-controlled trial. Whether such an effect may play an important part in the long-term improvement of glycemic control remains to be determined.
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