Inverse relationship between peripheral insulin removal and action: studies with metformin

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Zhang, Z., and J. Radziuk. Inverse relationship between peripheral insulin removal and action: studies with metformin. Am J Physiol Endocrinol Metab 281: E1240–E1248, 2001.—The interaction of insulin with metformin on muscle glucose metabolism was examined in the perfused rat hindquarter. Glucose, lactate, and insulin were measured at the inflow and outflow from the hindquarter, which was perfused with human erythrocytes suspended in a Kreb’s-Ringer albumin buffer for 120 min. Perfusions were performed with no additions (I) and with insulin infusions targeted to concentrations of 175 (II) and 350 pmol/l (III) as well as infusions targeted to levels of 0 (IV), 70 (V), and 175 pmol/l (VI) but in the presence of metformin (90 μg/ml). In the presence of metformin, identical infusion rates of insulin yielded higher insulin concentrations, namely 283 ± 19 vs. 202 ± 31 pmol/l for VI and II, respectively (P < 0.05). Glucose uptake (GU) increased correspondingly to 79.8 ± 0.8 in VI from 60.8 ± 2.1 for IV and 50.1 ± 1.3 for II and 46.1 ± 2.7 mg/120 min for I (P < 0.05). This enhanced GU was matched by increasing insulin levels using only a higher rate of its infusion (III): GU of 70.2 ± 2.4 mg/120 min with insulin of 334 ± 26 pmol/l (P > 0.05). The simple concurrent presence of metformin and insulin [matching insulin concentrations in II rather than infusion rates (IV)] demonstrated no additional effect on GU above that of metformin. The synergistic effects of metformin and insulin could thus be explained by a metformin-mediated decrease in the extraction of insulin by the hindquarter (4.8 ± 0.4% vs. 8.6 ± 0.9%, P < 0.05). This increases interstitial insulin (and, in a closed system, perfusate insulin), which acts on cell surface receptors to increase glucose uptake. The results demonstrate that the extracellular insulin concentration, rather than insulin internalization and degradation, is the primary determinant of insulin action on GU in muscle and that changes in tissue insulin extraction may alter local concentrations and, therefore, systemic insulin sensitivity. This provides both a physiological mechanism and a possible therapeutic target for improving insulin sensitivity.

Insulin kinetics; biguanides; glucose metabolism

Insulin action is initiated by insulin-receptor binding at the plasma membrane of cells (9). This triggers a cascade of events that determine the various subsequent actions of insulin. It has been hypothesized that the endocytosis of the insulin-receptor complex is an integral part of this sequence and that it is internalized insulin (the endosomal insulin-receptor complex) that signals the actions of insulin (e.g., Ref. 26). Simultaneously, in this sequence, the degradation of the intracellular insulin allows for an efficient termination mechanism for insulin action (22, 40). Other work, however, indicates that it is the binding event that alone is responsible for at least some of the actions of insulin, including specifically that of glucose uptake via the translocation of glucose transporters (20). The signal generated by a specific insulin molecule could thus be terminated either by degradation or by dissociation of the molecule from the receptor and its reentry into the circulation. Under these circumstances, the concentration of insulin locally, within the interstitial space, and its equilibration with its specific binding sites (the receptors) would determine the strength of the insulin signal for these processes. Other processes, usually characterized by a longer response time (e.g., protein metabolism and mitogenesis) could be regulated by endosomal insulin (14, 25, 33). Endocytosis, particularly in the muscle, would also lead to local insulin removal, a not insignificant contributor to systemic insulin clearance. In theory, therefore, target tissue removal of insulin could contribute to a dual regulation of local insulin concentrations and therefore signal strength: local removal balanced by the supply of insulin from the circulation. Because insulin is removed as the insulin-receptor complex, its removal could also contribute to the determination of receptor number on the cell surface, adding yet another degree of control at this level (e.g., Ref. 29).

Metformin is one of the primary agents used in the treatment of type 2 diabetes (e.g., Ref. 3). It is a pleiotropic compound displaying a large number of actions resulting in the overall decrease of both fasting and postprandial glucose (3, 43). Most studies demonstrate a decrease in hepatic glucose output (2, 12, 24, 34, 36, 42, 47), specifically gluconeogenesis (2, 30, 34, 36, 42, 47). In addition, peripheral effects on both fat and muscle metabolism have been shown (17, 23, 28, 38). It has been suggested in some studies that the primary action is peripheral (1, 21). In particular, in vitro studies indicate both independent actions of metformin (2, 36) and the enhancement of insulin action...
(47). It has also been suggested that metformin may alter insulin clearance, thus contributing to the regulation of its systemic concentrations (5, 41). To date, such actions have been demonstrated only in the liver.

In light of the foregoing, the present studies were performed in the perfused rat hindquarter to 1) ascertain whether metformin has any effects on muscle insulin clearance and 2) determine any potential relationship between muscle insulin clearance and its action on glucose uptake.

If insulin clearance and action were positively related, this would support the concept that it is indeed endosomal insulin that exerts metabolic actions. If the relationship were inverse, a dissociation of insulin binding and its associated signaling in glucose metabolism and its (irreversible) removal would be indicated. The latter scenario would also be supportive of the possibility of improving insulin action by decreasing its peripheral removal.

**MATERIALS AND METHODS**

**Animals**

Male Sprague-Dawley rats weighing 252 ± 11 g were fed on standard lab chow and fasted for 24 h before surgery. Protocols were approved by the Ethics Committee of the Ottawa Civic Hospital and the *Principles of Laboratory Animal Care* (National Institutes of Health Publication No. 85–23, revised 1985) were followed.

**Materials**

Outdated human blood was obtained from Transfusion Medicine, Ottawa Civic Hospital, regular human insulin from Eli Lilly (Indianapolis, IN), and bovine serum albumin from Sigma.

**Hindlimb Perfusion Technique**

The apparatus used for perfusion of the rat hindlimb was similar to that previously used for liver perfusion in our laboratory (48). Surgical preparation of the rat hindlimb was modified from the procedure of Mondon et al. (31). The rats were anesthetized by intraperitoneal injection of pentobarbital sodium (65 mg/kg). The abdominal skin, which was incised at midline, was carefully removed over the lower part of the animal, and the tail was excised. The superficial epigastric vessels were then ligated, and the abdomen was incised from the pubic symphysis to the xiphoid process. The genitalia were removed. Three milliliters of heparin (1,000 units) were injected into the inferior vena cava before cannulation. The abdominal aorta was cannulated using an 18-gauge intravenous catheter (Johnson & Johnson), which was immediately attached to PE160 tubing (Becton-Dickinson) and connected to the perfusion apparatus. The perfusion medium was pumped through the tissue immediately after cannulation. The initial 120 ml of perfusate passing through the tissue was pumped through the tissue immediately after cannulation-connected to the perfusion apparatus. The perfusion medium, preperfused for 40 min at 37°C with a medium consisting of four parts of human erythrocytes washed three times with saline before the study and six parts Krebs-Ringer bicarbonate buffer with 3% BSA oxygenated with 95% O₂–5% CO₂ (1 l/min). The medium also contained 5.6 mmol/l glucose and 3.3 mmol/l lactate, as well as 90 µg/ml metformin when this was to be present during the experiment.

The recirculating perfusion procedure was initiated by exchange with the same medium and the additions indicated in Table 1 for the six different groups used. To compensate for some of the clearance of insulin and to achieve target levels more quickly, in the groups where insulin was present [II (I2), III (I3), V (MI1), VI (MI2)], the rate of insulin infusion was varied in a preset fashion. The average rates of insulin infusion are also indicated in Table 1. It should be noted that identical rates of insulin infusion were used for each insulin dose regardless of the presence of metformin. To further compensate for the glucose uptake by the muscle, variable amounts of glucose were infused into the perfusion medium to maintain the glucose concentration at ~5.6 mmol/l. The perfusion was continued for 2 h, and the flow rate was 11.5 ml/min. Perfusate samples of 0.8 ml were withdrawn at both the inflow to and outflow from the system at 0, 5, 10, 20, 30, 45, 60, 75, 90, 105, and 120 min from the start of the perfusion. Sample volume was not replaced.

Analogous perfusions (*n* = 3) were also performed without the hindquarter to estimate glucose utilization by the red cells between the two sampling sites.

**Chemical Analyses**

Inflow and outflow samples from the perfusate were analyzed for glucose and lactate with the use of a glucose-lactate analyzer (Yellow Springs Instrument, Yellow Springs, OH). Partial pressure and hemoglobin saturation of oxygen were measured using a blood gas analyzer and hemoximeter, respectively (Radiometer), and radioimmunoassay of insulin concentrations was as previously described (31).

**Calculations**

Net uptake of glucose, lactate, and insulin. Because glucose and insulin levels in these studies are nearly constant or change slowly, their uptake was calculated as the difference between their inflow and outflow concentrations multiplied by the perfusion rate. Fractional extraction corresponds to tissue uptake divided by the inflow rate. Statistical analysis. Curves of insulin and lactate concentration as well as glucose uptake vs. time were compared by using one-way ANOVA, treating time as a repeated measure. Derived values were compared among the different groups using one-way ANOVA. Within the analysis of variance, orthogonal contrasts were used to determine whether specific linear combinations of means were significantly different.

**Table 1. Additions to perfusion medium in the 6 experimental groups used**

<table>
<thead>
<tr>
<th>Group</th>
<th>Abbreviation</th>
<th>n</th>
<th>Metformin Concentration, µg/ml</th>
<th>Target Insulin Concentration, pmol/l</th>
<th>Insulin Infusion Rate, pmol/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>I0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>I2</td>
<td>9</td>
<td>0</td>
<td>175</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>III</td>
<td>I3</td>
<td>6</td>
<td>0</td>
<td>350</td>
<td>0.44 ± 0.03</td>
</tr>
<tr>
<td>IV</td>
<td>M10</td>
<td>6</td>
<td>90</td>
<td>0</td>
<td>0.125 ± 0.005</td>
</tr>
<tr>
<td>V</td>
<td>M11</td>
<td>5</td>
<td>90</td>
<td>70</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>VI</td>
<td>M12</td>
<td>7</td>
<td>90</td>
<td>175</td>
<td>0.29 ± 0.02</td>
</tr>
</tbody>
</table>

1. perfusions targeted to the insulin infusion rates indicated; MI, perfusions targeted to the insulin infusion rates indicated plus metformin. *Target insulin concentration is what would be achieved by the infusion rate indicated without the presence of metformin; e.g., target insulin concentration for an infusion rate of 0.28 pmol/min (designated I2) is 175 pmol/l.
from zero (39). The significance level was set at $P = 0.05$. Data are reported as means ± SE.

RESULTS

Biochemical and Physiological Profile of Hindquarter Donors for Muscle Perfusion Studies

The mean body weight of the donor rats in the six groups studied was near 250 g (Table 2). Plasma glucose levels were near 5 mmol/l and lactate near 2.5 mmol/l when measured in the anesthetized animals and in all six groups.

Metabolite Concentrations During Perfusion

Perfusate glucose concentrations remained nearly constant during the perfusions: I, 5.4 ± 0.2; II, 5.5 ± 0.1; III, 5.6 ± 0.2; IV, 5.6 ± 0.2; V, 5.9 ± 0.3; and VI, 5.8 ± 0.1 mmol/l (not significant). Lactate concentrations, on the other hand, increased to some extent during the perfusions, as shown in Table 4. Final ($t = 120$ min) lactate concentrations were 137 to 202% of initial levels. Although there were few differences in a pairwise comparison, contrast analysis indicated that lactate accumulated to a somewhat greater extent in the groups where metformin was present in perfusion medium (IV-VI) than in those where it was not ($P = 0.015$). Oxygen saturation was measured at both the influx into and the efflux from the perfusion system. The hemoglobin was 100% saturated at the influx. In those where metformin was present in perfusion medium, saturation at the end of the study was $84 \pm 6$% (MI2), indicating no differences in oxygenation between those with and without metformin (175 pmol/l). When metformin was present in the system, the equivalent saturations were 84 ± 3% (MI0) and 84 ± 3% (MI2), indicating no differences in oxygen uptake by the hindquarter whether metformin was present or not. Effluent PO$_2$ was also not different: 83 ± 6 mmHg for I0 and I2 and 70 ± 3 and 75 ± 6 mmHg for MI0 and MI2, respectively ($P > 0.05$), with identical PO$_2$ at the inflow to the liver. These data therefore indicate that oxygen utilization was the same under all these experimental circumstances.

Insulin Concentrations and Glucose Uptake in the Perfused Hindquarter

Figure 1A shows the time courses of insulin concentration during infusion at the high and intermediate rates. Figure 1B shows the concentrations when insulin is infused at the low and intermediate rates. The latter infusions, however, take place in the presence of metformin. The lower infusion rates of insulin in the presence of metformin yield concentrations of the hormone similar to those at higher rates of infusion but in the absence of metformin. Thus infusions at rates of 0.125 pmol/min (Table 1) in the presence of metformin yield a concentration curve that is the same as that during infusion at 0.28 pmol/min without metformin ($P < 0.05$); an infusion rate of 0.29 pmol/min with metformin is equivalent to 0.44 pmol/min ($P < 0.05$) without it. Similar concentrations with lower infusion rates imply lower clearance rates of insulin by the perfused hindquarter. This finding is reflected in the directly calculated rates of insulin extraction by the hindquarter (Table 3). Insulin extraction decreased from 6.1 ± 0.2% (V0) to 0.05. This decreased extraction rate was equivalent to that seen at higher insulin concentrations ($P > 0.05$). Interestingly, with the low rate of insulin administration to the perfusion system, the insulin extraction fell to 1.2% in the presence of metformin. Because insulin concentrations vary, insulin uptake became nearly the same ($P > 0.05$) for the three groups with higher insulin infusions but remained very low for the low insulin group (V). As seen in Fig. 1, when insulin was not added to the system, concentrations remained low (<30 pmol/l) with or without metformin, indicating that washout of insulin from the tissues remained minimal during the perfusion. There was an increase in basal glucose uptake in the presence of metformin (Fig. 2). When insulin was infused, the uptake of glucose paralleled the concentrations of insulin, not its infusion rates. Only the highest infusion rate [I3 (III)] and, in the presence of metformin, the intermediate infusion rate [MI2 (VI)] induced an uptake of glucose that rose >50% above the respective baselines. Both of these groups demonstrated analogous insulin concentrations.

The question was then asked: is this effect a direct effect of metformin on glucose uptake that simply requires the presence of insulin, or is this effect of metformin mediated primarily by its effect in reducing insulin clearance and, therefore, increasing insulin levels? These questions are addressed below by use of the summary of the results provided in Tables 4 and 5 and are graphically summarized in Fig. 3.

Table 2. Biochemical and physiological profiles of hindquarter donors

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Plasma Glucose, mmol/l</th>
<th>Plasma Lactate, mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>251 ± 4</td>
<td>4.9 ± 0.4</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>II</td>
<td>249 ± 3</td>
<td>5.5 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>III</td>
<td>261 ± 4</td>
<td>5.6 ± 0.5</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>IV</td>
<td>257 ± 7</td>
<td>5.4 ± 0.4</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>V</td>
<td>265 ± 8</td>
<td>5.6 ± 0.2</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>VI</td>
<td>249 ± 2</td>
<td>5.3 ± 0.2</td>
<td>2.3 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Values determined in the anesthetized animal.

Table 3. Parameters of insulin metabolism by perfused hindquarter

<table>
<thead>
<tr>
<th>Group</th>
<th>Abbreviation</th>
<th>II</th>
<th>VI</th>
<th>III</th>
<th>IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average insulin extraction, %</td>
<td>8.6 ± 0.9*</td>
<td>4.8 ± 0.4†</td>
<td>5.7 ± 1.0 †</td>
<td>1.2 ± 0.8‡</td>
<td></td>
</tr>
<tr>
<td>Average insulin uptake, pmol/min</td>
<td>0.15 ± 0.02*</td>
<td>0.11 ± 0.01*</td>
<td>0.15 ± 0.01*</td>
<td>0.01 ± 0.01†</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. Values with different symbols are significantly different ($P < 0.05$).
It can be seen from Table 4 that the insulin concentrations attained during its infusion are the same when lower rates are used in the presence of metformin (M1 and M12 vs. I2 and I3, respectively). The corresponding glucose uptake or glucose infusion over the 120-min perfusion period indicates that only for the highest insulin concentration [reached during the highest insulin infusion rate without metformin (I3) and during the intermediate infusion rate with metformin (M12)] is there an increase in glucose uptake that can be attributed to insulin. Moreover, this change is dependent on the insulin concentration, not the infusion rate. This can be seen from the contrast analysis of Table 5.

The glucose uptake (infusion) assignable to the effect of metformin on insulin infused at the intermediate rate, I2, can be designated as M12-I2. The effect of metformin alone corresponds to M0-I0. The glucose uptake due to the interaction of insulin and metformin over and above the effect of either alone is, therefore, (M12-I2)-(M0-I0), which quantitatively is \( \sim 15 \text{ mg/120 min} \) \( (P = 0.0002) \). Moreover, the glucose uptake due strictly to the insulin infused at the rate I2 in the presence of metformin, M12-MI0, is not different \( (P = \) \( \) \( \))
The major observations made in these studies include the following. 1) A given dose of insulin is more effective in stimulating glucose uptake by the perfused hindquarter in the presence of metformin, implying a synergy in the combined action of insulin and metformin on glucose metabolism. 2) The synergy is highly consistent with a metformin-induced inhibition of insulin extraction from the interstitial space by muscle. This can be inferred from the fact that higher perfusate insulin concentrations ensue with a given insulin administration protocol when metformin is present. 3) The increase in glucose uptake at a given insulin dose in the presence of metformin is comparable to that observed when insulin is given alone at a higher dose that yields the same perfuse insulin concentration. The metformin-induced increase in insulin concentrations is, therefore, sufficient to explain the increase in glucose uptake. 4) Glucose uptake depends on the perfusate insulin concentration and not on the presence of metformin at a particular insulin concentration. This strongly suggests that the inhibition of insulin removal and the resulting increase in insulin concentrations comprise a necessary contribution to the stimulation of insulin-dependent glucose uptake by metformin. 5) Finally, these data provide confirmation that the given dose of metformin alone can stimulate glucose uptake by muscle. This stimulation is independent of the synergistic interaction of metformin with insulin.

The principal conclusion is the indication of a novel potential mechanism by which insulin action may be increased both physiologically and therapeutically. In vivo, however, increases in circulating insulin concentrations are not generally seen when metformin is administered acutely (e.g., Ref. 34). In the closed (recirculating) perfusion system used here, however, identical protocols for insulin infusion led to a doubling of insulin concentrations in the presence of metformin. Overall, less insulin was taken up by the muscle, and this was mediated by an extraction fraction that was reduced by at least one-half with metformin. Clearly, some part of this reduction could be caused by saturation phenomena as insulin concentration increases. Decreases in extraction, however, must initiate this increase, since insulin inputs were identical. Importantly, the phenomenon observed here is described in a closed system, where muscle is the major sink for insulin. There is little uptake of insulin by other tis-

### Table 4. Metabolic parameters during insulin and metformin perfusions of rat hindquarter

<table>
<thead>
<tr>
<th>Group Abbreviation</th>
<th>I0</th>
<th>I2</th>
<th>I3</th>
<th>M10</th>
<th>M11</th>
<th>M12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final insulin levels, pmol/l</td>
<td>25.7 ± 4.9§</td>
<td>202 ± 31†</td>
<td>334 ± 26¶</td>
<td>15.3 ± 4.2§</td>
<td>190 ± 13†</td>
<td>283 ± 19§</td>
</tr>
<tr>
<td>Glucose uptake, mg/120 min</td>
<td>46.1 ± 2.7§</td>
<td>50.1 ± 1.3§</td>
<td>70.2 ± 2.4¶</td>
<td>60.8 ± 2.1¶</td>
<td>63.1 ± 1.2‡</td>
<td>79.8 ± 0.8§</td>
</tr>
<tr>
<td>Glucose infusion, mg/120 min</td>
<td>45.4 ± 2.3§</td>
<td>50.4 ± 1.2§</td>
<td>69.0 ± 2.8¶</td>
<td>58.5 ± 1.4‡</td>
<td>63.3 ± 1.3¶</td>
<td>75.6 ± 1.1*</td>
</tr>
<tr>
<td>Initial lactate concn, mmol/l</td>
<td>3.3 ± 0.3</td>
<td>4.0 ± 0.2</td>
<td>4.2 ± 0.5</td>
<td>3.6 ± 0.2</td>
<td>3.8 ± 0.2</td>
<td>4.1 ± 0.2</td>
</tr>
<tr>
<td>Final lactate concn, %initial</td>
<td>158 ± 17</td>
<td>134 ± 7</td>
<td>149 ± 21</td>
<td>175 ± 16</td>
<td>202 ± 17</td>
<td>164 ± 11</td>
</tr>
</tbody>
</table>

Values are means ± SE. Concentration. In each row, quantities with the same symbol are not significantly different (P > 0.05).

### Table 5. Contrast analysis of glucose uptake, infusion, and glycogen: glucose parameters

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Estimate for Glucose Uptake, mg/120 min</th>
<th>P (estimate &gt;0)</th>
<th>Estimate for Glucose Infusion, mg/120 min</th>
<th>P (estimate &gt;0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[M12-I2]–[M10-I0]</td>
<td>15.0 ± 3.5</td>
<td>0.0002</td>
<td>12.1 ± 3.3</td>
<td>0.0011</td>
</tr>
<tr>
<td>[M12-M10]–[I3-I0]</td>
<td>5.1 ± 3.7</td>
<td>0.18</td>
<td>6.5 ± 3.5</td>
<td>0.08</td>
</tr>
<tr>
<td>M11-M10</td>
<td>2.3 ± 2.8</td>
<td>0.42</td>
<td>4.8 ± 2.7</td>
<td>0.08</td>
</tr>
</tbody>
</table>

M12-I2, effect of metformin on insulin infused at the intermediate rate I2; M10-I0, effect of metformin alone; I3-I0, effect of insulin infused at the higher rate; see RESULTS for details.
sues, and any lymphatic return is also to the perfusion medium. Accumulation of insulin will therefore take place in the perfusate. Such a system will, in the perfusate, also reflect the events that occur at the cell surface or interstitial level. In vivo, on the other hand, interstitial increases in insulin concentrations, such as are inferred from these data, would be dissipated from a systemic perspective. For example, insulin would be transferred via the lymph back to the circulation, where it would be avidly cleared by the liver, kidney, and other organs (15). It would be anticipated that few of the local, interstitial changes would be reflected systemically, because in vivo, clearance in muscle is a relatively minor component of overall insulin removal.

The data therefore indicate that metformin induces a reduced net uptake of insulin by the myocytes, increasing its concentration at the cell surface, which in turn increases its action on glucose uptake. The physiological implication of this observation is that the extracellular insulin concentration, rather than its rate of internalization and clearance, is the primary effector of its action on glucose metabolism. That is, although both insulin clearance and the stimulation of glucose uptake are triggered by insulin binding, these two events are not necessarily linked. These data are consistent with previous observations that inhibitors of insulin degradation do not block acute effects of insulin such as those on glucose metabolism (19). More recently, it was demonstrated that overexpression of dynamin in 3T3-L1 adipocytes inhibited insulin internalization also without affecting the effects of insulin on glucose metabolism (8). The data are also compatible with the independent, and therefore additive, effects of metformin and insulin concentrations previously described (16, 38), because the enhancement of insulin action described here occurs indirectly by alterations specifically in the local insulin concentrations.

These observations are also reminiscent of those made previously with chloroquine, which is known to inhibit insulin degradation in muscle (32). It has been used to treat insulin resistance in a patient with accelerated insulin degradation (4). The effectiveness of the treatment suggests that by increasing interstitial insulin concentrations, insulin action can be increased. The data presented here indicate that metformin may have chloroquine-like effects. Interestingly, methylamine, which is used in the synthesis of metformin, has similar effects (33) as well as structural similarities to chloroquine. It has been further suggested that the inhibition of intracellular insulin degradation by chloroquine (11) or bacitracin (10) diverts the intact molecule to retroendocytotic pathways, thus inducing an “autocrine” action on the cellular receptors by such recycled insulin and leading to a decrease in its systemic clearance (35). Whether the effects of metformin are due to such an increase in retroendocytosis remains to be seen. An alternate mechanism could be based on interactions of metformin with membrane proteins (44), and insulin receptors in particular, thus directly inhibiting insulin uptake. Because it is the insulin-receptor complex that is internalized, such a
mechanism would also decrease the internalization of receptors, upregulating their number on the cell surface and potentially further improving sensitivity. This hypothesis is attractive because it could also provide a unified basis for the independent actions of metformin both on the insulin-receptor complex and on glucose transporters, which also have a finite residence time in the plasma membrane.

A number of studies (16, 17, 21, 23, 27, 28, 37, 38), both in vivo and in vitro, have demonstrated metformin action at peripheral sites, which occurred both independently of and in conjunction with insulin. The mechanisms that have been suggested for these interactions include an increase in the translocation to the plasma membrane of GLUT-1 and GLUT-4 (16, 28) in adipocytes, or GLUT-1 alone (23), when L6 myotubes were examined. An increase in the intrinsic activity of the membrane transporters has also been found (13) in Xenopus oocytes expressing GLUT-4, which would be particularly compatible with the hypothesized membrane effects of metformin (44). Ideally, therefore, membrane transporters would have been measured in the present studies. An increase in plasma membrane GLUT-4 when insulin and metformin were added would have further strengthened the argument that the effect of metformin is, to an important extent in these studies, mediated by the presence of additional undegraded insulin. This is, perhaps, particularly true because metformin and insulin displayed differential effects on GLUT-1 and GLUT-4, respectively (23), in L6 muscle cells, although this observation was not extended to cardiomyocytes (16) or adipocytes (28). By the same token, it is not likely that GLUT-1 translocation or, indeed, activity would have increased beyond that seen in the case of metformin addition alone, because in isolated cells the effect of metformin at higher concentrations was additive to, rather than synergistic with, insulin (16, 23). In addition, insulin and metformin could interact in other ways, such as by modifying the accumulation of glycogen (e.g., Ref. 37).

From the overall metabolic perspective, it is also interesting to note that, when metformin was present in the perfusate, there was a small increase in circulating lactate. This is likely because of an increased metabolism of glucose, whose uptake is increased by metformin also independently of insulin. An inhibition of lactate uptake by metformin, as previously postulated in the liver (36), could also contribute.

These studies were designed to obtain a first assessment of the interaction of insulin and glucose dynamics in the muscle with the use of metformin as a probe for this analysis. The dose of metformin (~0.5 mM) was therefore chosen to maximize metabolic effects based on published data, including other in vitro systems (skeletal muscle and cardiomyocytes; see, e.g., Refs. 16 and 23) but stopping short of alterations to oxidative phosphorylation/oxygen utilization, which might indicate incipient toxicity (2, 18, 23). In the liver, this has been indicated to occur at levels >1 mM (2, 16). Only at 5 mM was a small decrease in oxygen utilization seen in cardiomyocytes (16), and even then, no changes in the cardiomyocyte viability were seen. The dose chosen was also near that used to consistently elicit glucose transport effects in myocytes (16, 23).

Many in vitro effects of metformin, including those presented here, have been described at supratherapeutic doses and/or with extended incubation times with the drug in the case of cells. They therefore do not directly indicate mechanisms for a therapeutic effect of metformin, although they may suggest directions for future investigation. It is important to note, however, that the unchanged oxygen utilization by the muscle seen here speaks against a relative tissue hypoxia induced by metformin, which could in itself stimulate glucose transporter translocation, albeit from a distinct pool (7). In addition, the unchanged effect of given perfusate insulin concentrations on glucose uptake is also not consistent with an effect of metformin that could be characterized as toxic (18). One might add that, in the liver, metformin potentiates insulin action below 1 mM and that only when the concentration exceeds 1 mM are there suggestions that its effect may involve redox-state changes (47). The lack of toxicity generally would also be consistent with the very large (although transient) accumulations of metformin in vivo relative to circulating concentrations in the liver and particularly the intestine (45). In the presence of therapeutic circulating levels of metformin (~20–30 μM), the liver demonstrates concentrations of nearly 0.5 mM and the gut of 2–3 mM. In the skeletal muscle also, levels three- to sevenfold maximal circulating concentrations of metformin were found 2–4 h after its administration (45). These data therefore suggest that shorter exposure to higher concentrations may be an indicator, although not a demonstration, of potential mechanisms of the therapeutic action of metformin. All that being said, metformin remains a useful tool in dissecting out potential mechanisms for the modulation of insulin action.

Finally, whether any in vitro system, including the perfused hindquarter as used here, is representative of the in vivo situation can be questioned. Given the large number of systemic effects demonstrated by metformin, however, the isolation of a particular tissue, namely the muscle, does offer important advantages. Thus the accumulation of insulin seen here in the closed system amplifies the events occurring at the cell surface, allowing insight into these events (insulin removal) that might otherwise be difficult to detect. That the system is viable during the course of the study is attested to by consistent oxygen utilization and by glucose uptake, which is, moreover, responsive to physiological concentrations of insulin. It should be noted that the suitability of hindquarter/hindlimb perfusion systems for metabolic studies has been intensively investigated and found to yield results comparable to those in vivo when both could be measured (6, 46). To further strengthen the comparisons, all of the group data sets are compared simultaneously or as averages over the 120-min experimental period. Different extraction rates will lead to some differences in the interstitial gradients of insulin. Insulin action is neces-
MUSCLE INSULIN EXTRACTION AND SENSITIVITY

sarily delayed relative to its concentration if this is changing. Comparison of overall insulin kinetics and their biological effect on glucose uptake is therefore achieved by statistical comparisons of the entire curve or by averaging the data over the time course of studies performed under identical experimental conditions for the different protocols.

In summary, these data are consistent with the suggestion that extracellular insulin concentration may be a more important determinant of insulin action on glucose uptake than its internalization. This concentration can be altered at the target tissue level by modifying the extraction of insulin, suggesting a physiological mechanism for modulating the sensitivity to this hormone. This mechanism could, in turn, provide a target for the therapeutic modulation of insulin sensitivity and may potentially contribute to the action of metformin.

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REFERENCES


