

## Possible role of cytosolic free calcium concentrations in mediating insulin resistance of obesity and hyperinsulinemia.

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### Research Article

Insulin- and glyburide-stimulated changes in cytosolic free calcium concentrations ( $[Ca^{2+}]_i$ ) were studied in gluteal adipocytes obtained from six obese women (139  $\pm$  3% ideal body wt) and six healthy, normal weight age- and sex-matched controls. Biopsies were performed after an overnight fast and twice (at 3 and 6 h) during an insulin infusion (40 mU/m<sup>2</sup> per min) (euglycemic clamp). In adipocytes obtained from normal subjects before insulin infusion, insulin (10 ng/ml) increased  $[Ca^{2+}]_i$  from 146  $\pm$  26 nM to 391  $\pm$  66 nM. Similar increases were evoked by 2  $\mu$ M glyburide (329  $\pm$  41 nM). After 3 h of insulin infusion, basal  $[Ca^{2+}]_i$  rose to 234  $\pm$  21 nM, but the responses to insulin and glyburide were completely abolished. In vitro insulin-stimulated 2-deoxyglucose uptake was reduced by insulin and glucose infusion (25% stimulation before infusion, 5.4% at 3 h, and 0.85% at 6 h of infusion). In obese patients, basal adipocyte  $[Ca^{2+}]_i$  was increased (203  $\pm$  14 nM, *P* less than 0.05 vs. normals). The  $[Ca^{2+}]_i$  response demonstrated resistance to insulin (230  $\pm$  23 nM) and glyburide (249  $\pm$  19 nM) stimulation. Continuous insulin infusion increased basal  $[Ca^{2+}]_i$  (244  $\pm$  24 nM) and there was no response to either insulin or glyburide at 3 and 6 h of study. Rat adipocytes were preincubated with 1-10 mM glucose and 10 [...]

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# Possible Role of Cytosolic Free Calcium Concentrations in Mediating Insulin Resistance of Obesity and Hyperinsulinemia

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## Abstract

Insulin- and glyburide-stimulated changes in cytosolic free calcium concentrations ( $[Ca^{2+}]_i$ ) were studied in gluteal adipocytes obtained from six obese women ( $139 \pm 3\%$  ideal body wt) and six healthy, normal weight age- and sex-matched controls. Biopsies were performed after an overnight fast and twice (at 3 and 6 h) during an insulin infusion ( $40 \text{ mU/m}^2$  per min) (euglycemic clamp). In adipocytes obtained from normal subjects before insulin infusion, insulin ( $10 \text{ ng/ml}$ ) increased  $[Ca^{2+}]_i$  from  $146 \pm 26 \text{ nM}$  to  $391 \pm 66 \text{ nM}$ . Similar increases were evoked by  $2 \mu\text{M}$  glyburide ( $329 \pm 41 \text{ nM}$ ). After 3 h of insulin infusion, basal  $[Ca^{2+}]_i$  rose to  $234 \pm 21 \text{ nM}$ , but the responses to insulin and glyburide were completely abolished. In vitro insulin-stimulated 2-deoxyglucose uptake was reduced by insulin and glucose infusion (25% stimulation before infusion, 5.4% at 3 h, and 0.85% at 6 h of infusion).

In obese patients, basal adipocyte  $[Ca^{2+}]_i$  was increased ( $203 \pm 14 \text{ nM}$ ,  $P < 0.05$  vs. normals). The  $[Ca^{2+}]_i$  response demonstrated resistance to insulin ( $230 \pm 23 \text{ nM}$ ) and glyburide ( $249 \pm 19 \text{ nM}$ ) stimulation. Continuous insulin infusion increased basal  $[Ca^{2+}]_i$  ( $244 \pm 24 \text{ nM}$ ) and there was no response to either insulin or glyburide at 3 and 6 h of study.

Rat adipocytes were preincubated with 1–10 mM glucose and 10 ng/ml insulin for 24 h. Measurements of 2-deoxyglucose uptake demonstrated insulin resistance in these cells. Under these experimental conditions, increased levels of  $[Ca^{2+}]_i$  that were no longer responsive to insulin were demonstrated. Verapamil in the preincubation medium prevented the development of insulin resistance.

## Introduction

The role of intracellular calcium as a mediator of insulin action was originally proposed by Clausen et al. in 1974 (1) and by Kissebah et al. in 1975 (2). Since then, considerable evidence favoring this hypothesis has been accumulated (3–5). Although some investigators failed to observe a relationship between calcium and insulin action (6, 7), diverse aspects of insulin action have been demonstrated to be dependent upon extracellular and cytoplasmic  $Ca^{2+}$  (8–14).

Using a new calcium indicator (fura-2), we recently demonstrated that insulin and glyburide are capable of increasing cytosolic free calcium concentrations ( $[Ca^{2+}]_i$ )<sup>1</sup> in isolated rat adipocytes, primarily by enhancing  $Ca^{2+}$  transport across plasma membranes (15).

In the present study we have attempted to answer three questions: (a) do insulin and glyburide increase  $[Ca^{2+}]_i$  in adipocytes obtained from normal subjects; (b) are similar effects of insulin and glyburide observed in adipocytes isolated from patients with moderate obesity; and (c) does in vivo hyperinsulinemia alter the cellular response to insulin and glyburide stimulation. In particular, we focused on the role of  $[Ca^{2+}]_i$  in modulating cellular sensitivity. The latter studies were performed during a 6-h insulin infusion (euglycemic clamp).

## Methods

**Materials.** Porcine insulin was a gift from Eli Lilly Co. (Indianapolis, IN) and glyburide was generously supplied by Upjohn Co. (Kalamazoo, MI). Fura-2 and fura-2AM were purchased from Behring Diagnostics (San Diego, CA) and collagenase was obtained from Worthington Biochemical Corp. (Freehold, NJ).

**Experimental design.** Gluteal adipose tissue was obtained by needle biopsy in six obese women ( $139 \pm 3\%$  ideal body wt) and six healthy, normal weight age- and sex-matched controls. The biopsies were performed after an overnight fast and twice (at 3 and 6 h) during insulin infusion ( $40 \text{ mU/m}^2$  per min) (euglycemic clamp) as previously described (16). Blood glucose levels in all patients and control subjects were maintained in the range of 85–95 mg/dl. Adipocytes were isolated by the method of Rodbell (17). Obese subjects were studied before and 3 mo after moderate weight loss (down to  $127 \pm 4\%$  ideal body wt). During these 3 mo they were maintained on an isocaloric weight maintenance diet. Because weight reduction did not alter basal or insulin- and glyburide-stimulated  $[Ca^{2+}]_i$ , the results were combined for the sake of clarity.

**Measurements of  $[Ca^{2+}]_i$ .** These measurements were performed as previously described (15, 18) using a spectrofluorometer (model 340; Turner Designs, Mountain View, CA). During fura-2 loading (45 min at  $37^\circ\text{C}$ ) and  $Ca^{2+}$  measurements the cells were incubated in 2.4 ml of Krebs-Hepes buffer containing 1 mM  $CaCl_2$ , 118.4 mM NaCl, 4.69 mM KCl, 1.2 mM  $MgCl_2$ , 1.18 mM  $KH_2PO_4$ , 1.25 mM  $NaHCO_3$ , 20 mM Hepes, 5 mg/ml BSA, and 30 mg/dl glucose at pH 7.4. The final cell concentration was  $\sim 2 \times 10^5$  cells/cuvette (or  $8 \times 10^4$  cells/ml) and the measurements were obtained before and 10 min after additions of either insulin (10 ng/ml) or glyburide (2  $\mu\text{M}$ ). The fluorescence of the extracellular fura-2 was estimated by adding  $MnCl_2$  (50  $\mu\text{M}$ ), which quenches extracellular fura-2.  $MnCl_2$  was then chelated by the addition of 100  $\mu\text{M}$  pentetic acid. The estimate of extracellular fura-2 was made before stimulation of the cells with insulin, glucose, or glyburide. The fluorescence of either the buffers used in these studies or of tissues (without fura-2) was 10–13% of that observed with the cells loaded

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1. Abbreviations used in this paper:  $[Ca^{2+}]_i$ , cytosolic free calcium concentrations.

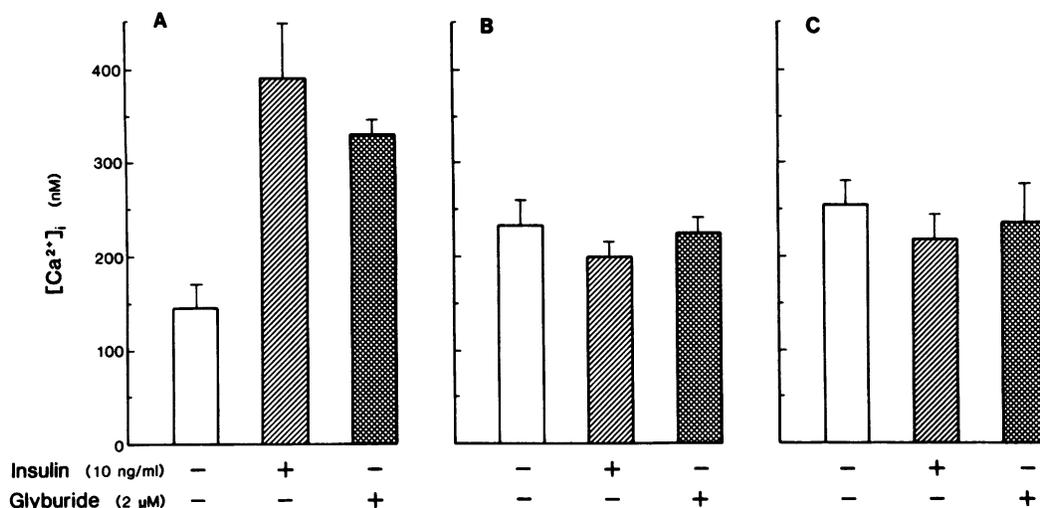


Figure 1. Effect of insulin and glyburide on  $[Ca^{2+}]_i$  in adipocytes obtained from six normal women after overnight fast (A), at the 3rd (B) and 6th (C) h of euglycemic clamp. Results represent mean  $\pm$  SEM. Control values for  $[Ca^{2+}]_i$  represent time controls; that is, the levels of  $[Ca^{2+}]_i$  at 0, 3, and 6 h of clamp but before in vitro additions of either insulin or glyburide.

with the probe. Cellular and buffer fluorescence did not change in response to either glucose, insulin, or glyburide. Results are presented as mean  $\pm$  SEM and compared using paired or unpaired *t* tests.

**2-Deoxyglucose uptake.** Adipocytes ( $2 \times 10^5$  cells) were incubated in the absence and in the presence of insulin (25 ng/ml) for 30 min at 37°C. Glucose uptake was initiated by the addition of  $[^3H]$ 2-deoxyglucose (0.2  $\mu$ Ci). After 3 min of incubation the reaction was terminated by transferring 200- $\mu$ l aliquots of incubation mixture to the microfuge tubes containing 100  $\mu$ l silicone oil and centrifuging the tubes in a microfuge (Beckman Instruments, Inc., Palo Alto, CA). The cell pellets were counted for radioactivity present in a liquid scintillation counter (Beckman Instruments, Inc.).

**In vitro studies with rat adipocytes.** Adipocytes isolated by the method of Rodbell (17) were preincubated for 24 h as previously described by Marshall et al. (19) and Garvey et al. (20). The incubation media contained either 1 or 10 mM glucose with or without 10 ng/ml insulin. After preincubation, the cells were washed three times and incubated for 40 min in glucose- and insulin-free medium to eliminate any possible influence of high glucose or insulin present during the 24-h preincubation. The cells were resuspended in KRB and divided into two groups for 2-deoxyglucose uptake and  $[Ca^{2+}]_i$  determinations as described above.

## Results

The basal, nonstimulated level of  $[Ca^{2+}]_i$  in adipocytes obtained from the control subjects before insulin infusion was  $146 \pm 26$  nM. Incubation of these adipocytes with insulin (10 ng/ml) for 10 min at 37°C resulted in an increase in  $[Ca^{2+}]_i$  to  $391 \pm 66$  nM (Fig. 1 A). A similar increase was evoked by 2  $\mu$ M glyburide ( $329 \pm 41$  nM). In adipocytes obtained after 3 h of insulin infusion (40 mU/m<sup>2</sup> per min), basal  $[Ca^{2+}]_i$  rose to  $234 \pm 21$  nM (Fig. 1 B), but responses of these adipocytes to both insulin and glyburide were completely abolished ( $197 \pm 13$  nM and  $224 \pm 5$  nM, respectively).  $[Ca^{2+}]_i$  continued to increase during insulin infusion, reaching  $255 \pm 24$  nM at the 6th h of the infusion (Fig. 1 C). There was no further stimulation by either insulin or glyburide ( $214 \pm 22$  nM and  $235 \pm 40$  nM, respectively).

To examine how this acquired inability of insulin to increase  $[Ca^{2+}]_i$  related to insulin action on glucose uptake, we studied basal and insulin-stimulated 2-deoxyglucose uptake in the fat cells obtained from three normal individuals at 0, 3, and 6 h of euglycemic clamp. The gluteal biopsies were obtained in the manner identical to those used to study  $[Ca^{2+}]_i$ .

Before insulin and glucose infusion, insulin increased in vitro 2-deoxyglucose uptake by 24% (Fig. 2). At the 3rd and 6th h of euglycemic glucose clamp, the unstimulated (basal) levels of 2-deoxyglucose uptake were unchanged, but insulin-stimulated values were significantly reduced when compared with preclamp studies (Fig. 2).

In obese patients before insulin infusion, basal adipocyte  $[Ca^{2+}]_i$  increased compared with normal subjects ( $203 \pm 14$  nM,  $P < 0.05$ ). The  $[Ca^{2+}]_i$  response of these adipocytes demonstrated resistance to both insulin ( $230 \pm 23$  nM, 12% increase) and glyburide ( $249 \pm 19$  nM, 16% increase) (Fig. 3). The comparison of these results with those obtained in normal individuals is shown in Fig. 4. Continuous infusion of insulin in obese patients further increased basal  $[Ca^{2+}]_i$  ( $244 \pm 24$  nM at 3 h and  $252 \pm 32$  nM at 6 h) and, similar to the controls, there was no response of the isolated adipocytes to either insulin or glyburide at 3 and 6 h of study.

If glucose and/or insulin infusion induce insulin resistance in adipocytes within 3 h, this phenomenon should be reproducible in vitro. To examine this possibility, we incubated freshly isolated rat adipocytes for 24 h in the presence of either 1 or 10 mM glucose with or without 10 ng/ml insulin. A similar experimental approach was previously used by Garvey et al. (20), who studied rat adipocytes, and by Sinha et al. (21), who studied human adipocytes. After a 24-h preincubation period, the adipocytes were washed with insulin- and glucose-free medium to deactivate the glucose transport system. These cells were then used to determine 2-deoxyglucose uptake and

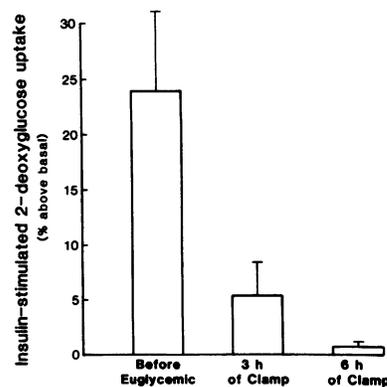


Figure 2. Insulin-stimulated 2-deoxyglucose uptake in adipocytes obtained by gluteal biopsy from three normal individuals before and during euglycemic clamp. Results represent mean  $\pm$  SEM of three determinations.

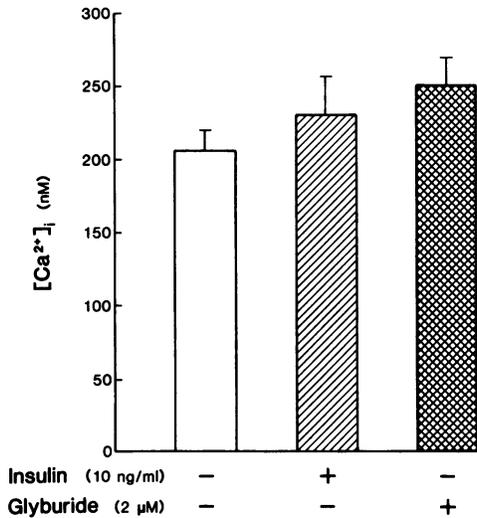


Figure 3. Effect of insulin and glyburide on  $[Ca^{2+}]_i$  in adipocytes obtained from six obese women after overnight fast. Results represent mean $\pm$ SEM.

$[Ca^{2+}]_i$ . In adipocytes preincubated with 1 mM glucose, both in the presence and in the absence of insulin, subsequent addition of insulin stimulated 2-deoxyglucose uptake by ~100–115% (Fig. 5). The presence of insulin (10 ng/ml) in the 24-h preincubation media did not alter either basal or insulin-stimulated 2-deoxyglucose uptake.

In contrast to preincubations with 1 mM glucose, the presence of 10 mM glucose for 24 h significantly reduced insulin-stimulated 2-deoxyglucose uptake ( $P < 0.01$ ). Insulin in the preincubation media reduced both basal and insulin-stimulated glucose uptake (Fig. 5).

To examine whether this glucose- and insulin-induced insulin resistance was related to the levels of  $[Ca^{2+}]_i$ , we measured  $[Ca^{2+}]_i$  in adipocytes preincubated under the same conditions used in the experiments with glucose uptake described above (Fig. 5). Insulin increased  $[Ca^{2+}]_i$  in adipocytes preincubated with low glucose (1 mM) in the presence or in the absence of insulin. Basal  $[Ca^{2+}]_i$  was not increased by the 24-h preincubation with 10 mM glucose, but in these adipocytes acute addition of insulin failed to stimulate  $[Ca^{2+}]_i$ . Preincubation of adipocytes with both 10 mM glucose and 10 ng/ml

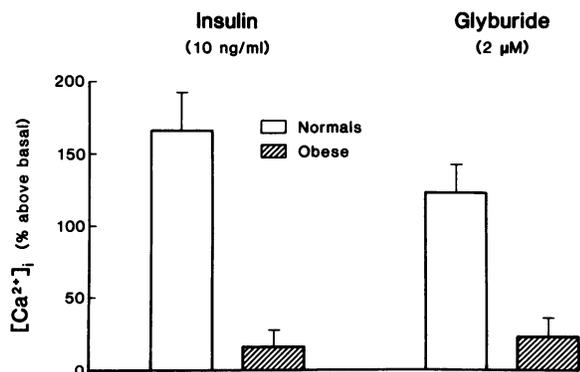


Figure 4. Effect of insulin and glyburide upon  $[Ca^{2+}]_i$  in adipocytes obtained from six normal weight controls and six obese women after overnight fast. Results represent mean $\pm$ SEM;  $P < 0.01$  (obese vs. normals).

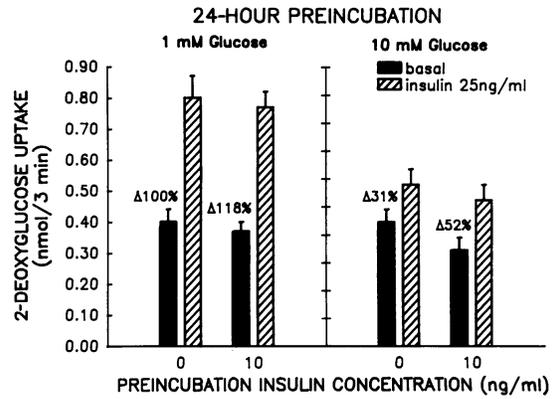


Figure 5. Effect of 24-h preincubation with 1 and 10 mM glucose (with or without 10 ng/ml insulin) on basal and insulin-stimulated 2-deoxyglucose uptake in rat adipocytes. Results represent the mean $\pm$ SEM of five to eight experiments.  $\Delta\%$ , % stimulation by insulin.

insulin raised basal  $[Ca^{2+}]_i$  and eliminated the effect of insulin in increasing  $[Ca^{2+}]_i$  (Fig. 6), inducing a picture similar to that observed in adipocytes obtained during euglycemic clamp.

If changes in  $[Ca^{2+}]_i$  are related to the glucose- and insulin-induced insulin resistance, then inhibiting an increase in  $[Ca^{2+}]_i$  may prevent insulin resistance. Since insulin increases  $[Ca^{2+}]_i$  by enhancing  $Ca^{2+}$  influx via voltage-dependent  $Ca^{2+}$  channels (15), we incubated adipocytes as described above in the presence of 30 μM verapamil. This  $Ca^{2+}$  channel blocker reduced the levels of  $[Ca^{2+}]_i$  from 162 $\pm$ 15 nM to 99 $\pm$ 13 nM with 10 mM glucose alone and from 239 $\pm$ 31 to 128 $\pm$ 14 nM with glucose and insulin ( $P < 0.05$ ), and restored cellular responsiveness to insulin (Fig. 7), suggesting that glucose- and insulin-induced  $Ca^{2+}$  influx is responsible for the development of insulin resistance. Similar restoration of adipocyte response to insulin was achieved in preliminary experiments with nifedipine (25 μM) or cobalt (0.5 mM) (not shown), suggesting that a blockage of  $Ca^{2+}$  influx may ameliorate glucose- and insulin-induced insulin resistance.

## Discussion

The present data directly demonstrate that both insulin and glyburide increase  $[Ca^{2+}]_i$  in human adipocytes. These find-

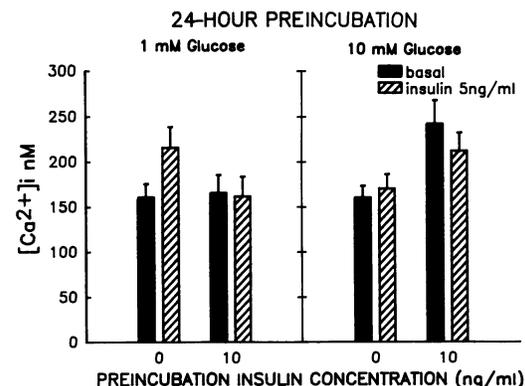


Figure 6. Effect of 24-h preincubation with 1 and 10 mM glucose (with or without 10 ng/ml insulin) on basal and insulin-stimulated  $[Ca^{2+}]_i$  in rat adipocytes. Results represent the mean $\pm$ SEM of five to eight experiments.

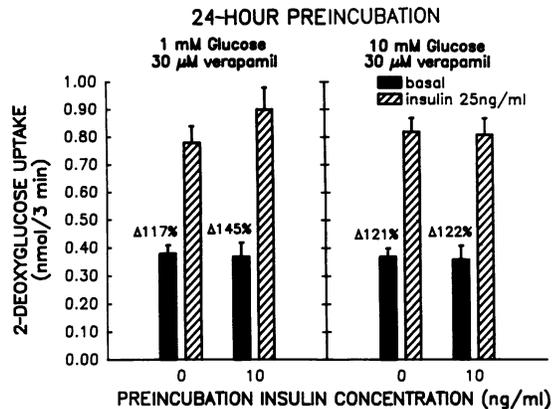


Figure 7. 2-Deoxyglucose uptake in adipocytes preincubated for 24 h with 1 and 10 mM glucose (with or without 10 ng/ml insulin) in the presence of  $\text{Ca}^{2+}$  channel blocker verapamil (30  $\mu\text{M}$ ). Results represent the mean  $\pm$  SEM of three to five experiments.  $\Delta\%$ , % stimulation.

ings support our previous observations using rat adipocytes (15), where both insulin and glyburide enhanced calcium influx via voltage-dependent calcium channels.

In this study, hyperinsulinemia induced by 3 and 6 h of insulin (and glucose) infusion not only increased  $[\text{Ca}^{2+}]_i$  in adipocytes obtained from normal volunteers, but made them unresponsive to either insulin or glyburide. This is not simply a desensitization of insulin action, because the effect of glyburide was also eliminated. The diminished responsiveness of adipocytes to insulin was also manifested by the reduced insulin-stimulated glucose uptake. Although the coexistence of these two phenomena does not prove causality, it is conceivable that impaired intracellular  $\text{Ca}^{2+}$  homeostasis contributes to the diminished cellular responsiveness to insulin.

It is interesting that in normal subjects hyperinsulinemia of relatively short duration induced the same degree of insulin and glyburide resistance as that seen in obese individuals. Indeed, obese patients demonstrated higher  $[\text{Ca}^{2+}]_i$  in the basal state and a lack of response to either insulin or glyburide in the adipocytes obtained even before the insulin infusion. During the course of the insulin infusion,  $[\text{Ca}^{2+}]_i$  remained elevated and unresponsive to the acute influence of either insulin or glyburide. These observations may indicate that obesity-associated high levels of intracellular free  $\text{Ca}^{2+}$  produce cellular resistance to insulin and glyburide. It is possible that these represent coexistent abnormalities that are not necessarily causally related.

In obese patients, mild to moderate weight loss (8–12%) failed to reduce basal  $[\text{Ca}^{2+}]_i$  or restore adipocyte responsiveness to insulin and glyburide. The lack of improvement was not due to the levels of fasting insulinemia, since insulin levels in the obese patients were not different from controls ( $6.5 \pm 1 \mu\text{U/ml}$ ) either before ( $7.2 \pm 1 \mu\text{U/ml}$ ) or after ( $6.8 \pm 2 \mu\text{U/ml}$ ) weight reduction. However, plasma insulin levels in obese patients 2 h after ingestion of 75 g glucose were significantly higher than in controls ( $50 \pm 12 \mu\text{U/ml}$  before and  $39 \pm 12 \mu\text{U/ml}$  after weight loss vs.  $19 \pm 6 \mu\text{U/ml}$  in normals). It is rational to postulate that postprandial hyperinsulinemia remains a sufficient stimulus in maintaining high levels of intracellular  $\text{Ca}^{2+}$ .

The *in vitro* experiments with glucose- and insulin-induced insulin resistance reported in this communication are in

agreement with the *in vivo* data. In both cases, exposure of adipocytes to high glucose and insulin concentrations resulted in insulin resistance and increased levels of  $[\text{Ca}^{2+}]_i$ . It would appear, therefore, that if hyperglycemia and hyperinsulinemia induce insulin resistance, this insensitivity must develop in part as the consequence of direct action of glucose and insulin on peripheral tissues (in this case, adipocytes).

It has been previously shown, both *in vivo* (22) and *in vitro* (20, 21, 23), that exposure of adipocytes to high ambient insulin levels (particularly in the presence of higher levels of glucose) results in cellular insensitivity to subsequent insulin stimulation. The decrease in cellular sensitivity and responsiveness to insulin were attributed to insulin receptor and postreceptor defects, with the latter playing a predominant role (19–21, 23–26). Our data suggest that sustained high levels of intracellular  $\text{Ca}^{2+}$  may contribute to, if not initiate, the postreceptor defects. The fact that hyperinsulinemia and high levels of  $[\text{Ca}^{2+}]_i$  induced cellular resistance to glyburide suggests that  $[\text{Ca}^{2+}]_i$  may alter the cellular response to multiple agents. However, we did not study the gamut of agents stimulating glucose transport in adipocytes and therefore cannot draw any definitive conclusions.

Insulin has been shown to increase  $\text{Ca}^{2+}$  influx via voltage-dependent  $\text{Ca}^{2+}$  channels (15, 18), and its effect was potentiated by higher ambient glucose concentrations (15). Insulin can also affect cellular  $\text{Ca}^{2+}$  homeostasis by inhibiting  $\text{Na}^+ - \text{K}^+$  ATPase and  $\text{Ca}^{2+} - \text{Mg}^{2+}$  ATPase (for review, see references 4 and 11). The fact that verapamil restored the adipocytes' responsiveness to insulin does not necessarily imply that  $\text{Ca}^{2+}$  influx via voltage-dependent channels is the only mechanism whereby insulin and glucose increase  $[\text{Ca}^{2+}]_i$ . The loci of cellular insulin and glucose action in increasing and maintaining high levels of  $[\text{Ca}^{2+}]_i$  need to be further investigated.

The precise mechanism whereby higher  $[\text{Ca}^{2+}]_i$  induces insulin resistance is unknown. We observed that in normal rat adipocytes, insulin-stimulated transport of 2-deoxyglucose was inhibited at both low (with calcium channel blockers) and high (with ionophore) concentrations of intracellular  $\text{Ca}^{2+}$  (18). Similarly, Bonne et al. (27) and Taylor et al. (28) have previously shown that excessive concentrations of calcium (above 5 mM) inhibited the effect of insulin on glucose transport in isolated adipocytes. These observations are consistent with the possibility that persistently high  $[\text{Ca}^{2+}]_i$  may contribute to the overall reduction in cellular response. The influence of changes in  $[\text{Ca}^{2+}]_i$  on both receptor and postreceptor steps of insulin action has not been studied. Further investigations in this area may provide new insights into the pathogenesis of insulin resistance.

The present data demonstrate that insulin and glyburide are capable of increasing  $[\text{Ca}^{2+}]_i$  in isolated human adipocytes. In a relatively short interval of time, using an insulin infusion, it is possible to induce resistance to the action of insulin and glyburide in adipocytes of normal subjects. These findings also suggest that under certain circumstances (hyperinsulinemia and/or obesity) increased  $[\text{Ca}^{2+}]_i$  may be a factor in inducing insulin resistance.

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