Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 59 (2010) 1784 – 1793
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Enhanced long-chain fatty acid uptake contributes to overaccumulation of triglyceride in hyperinsulinemic insulin-resistant 3T3-L1 adipocytes

Ying-Hsiu Lai[a,b,1], Yueh Chien[a,b,1], Ching Fai Kwok[c,d], Low-Tone Ho[a,b,c,d,*]

[a]*Institute of Physiology, National Yang-Ming University, Taipei 11221, Taiwan*
[b]*Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei 11217, Taiwan*
[c]*Faculty of Medicine, School of Medicine, National Yang-Ming University, Taipei 11221, Taiwan*
[d]*Division of Endocrinology and Metabolism, Department of Medicine, Taipei Veterans General Hospital, Taipei 11217, Taiwan*

Received 24 September 2009; accepted 11 May 2010

Abstract

The precise pathogenesis of obesity remains controversial. In obesity, diminished adipose glucose utilization suggests that some other substrates may be responsible for the adipose triglyceride (TG) overaccumulation. Here we attempted to evaluate if long-chain fatty acid (LCFA) flux was modulated by a physiologically relevant condition of hyperinsulinemia in 3T3-L1 adipocytes and if the altered LCFA influx might eventually contribute to the TG overaccumulation in obesity. The effects of prolonged insulin exposure to adipocytes on basal, insulin-stimulated LCFA uptake as well as intracellular LCFA metabolism were measured. Prolonged insulin exposure was found to induce insulin resistance (IR) yet enhance basal and insulin-stimulated LCFA uptake in normoglycemic condition, and the addition of high glucose exacerbated these abnormalities of both glucose and LCFA influx. Along with the enhanced LCFA uptake was an increase in the rates of intracellular LCFA deposition and incorporation into TG; but a decrease was found in basal and insulin-suppressive LCFA oxidation, as well as in isoproterenol-induced fatty acid efflux. Inhibition of either phosphatidylinositol 3-kinase or mitogen-activated protein kinase (MAPK) pathway did not prevent the induction of IR, whereas the enhanced basal and insulin-stimulated LCFA uptake was abrogated by inhibition of MAPK pathway. In hyperinsulinemic insulin-resistant 3T3-L1 adipocytes, basal and insulin-stimulated LCFA uptake tends to increase via a MAPK-dependent mechanism. The increment of LCFA influx predominantly accounts for TG overaccumulation, but not for mitochondrial oxidation, and is prone to retain within adipocytes. These findings may interpret the plausible mechanism of pathogenesis for obesity in hyperinsulinemia-associated IR.

1. Introduction

Obesity increases the risks of type 2 diabetes mellitus, hypertension, and cardiovascular diseases and significantly reduces the average life expectancy [1]. Serving as an anabolic hormone, insulin has multiple effects such as promoting glucose uptake, lipogenesis, and antilipolysis. Many studies have reported that the sensitivity of glucose uptake to insulin is diminished, whereas that of antilipolysis to insulin is normal [2-4]. These alterations in insulin actions have also been reported to be associated with the development of obesity [5]. Nevertheless, the pathophysiology of obesity remains uncertain.

The plasma levels of both glucose and nonesterified fatty acids tend to elevate during the postprandial state [6]. In adipocytes, the stimulatory effect of insulin on glucose uptake has been well studied; and more than 90% of the long-chain fatty acid (LCFA) uptake is via a transporter-mediated process [7]. After entering the adipocytes, most of the glucose and LCFAs are incorporated into triglyceride (TG). A recent study further demonstrated the stimulatory effect of insulin on LCFA uptake and the concomitant translocation of fatty acid transport proteins to plasma membranes in adipocytes [8]. Accordingly, there is little doubt that insulin serves a prominent role in the regulation of glucose and LCFA uptake in adipocytes.

Obesity is generally considered to induce insulin resistance (IR) and hyperinsulinemia by secreting excess

* Corresponding author. Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan. Tel.: +886 2 2875 7393; fax: +886 2 2872 4982.
E-mail address: ltho@vghtpe.gov.tw (L.-T. Ho).
[1] Y-H Lai and Y Chien contributed equally to this work.

0026-0495/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2010.05.007

free fatty acids and cytokines that impair insulin actions [5], but some investigations have questioned if IR and hyperinsulinemia per se also contribute to the development of obesity [5]. For example, these 2 factors are the root causes of IR syndrome that usually includes obesity [9]; and their existence has already been observed during weight gain in subjects who are not obese [10]. Nevertheless, it appears that adipose IR is not the primary cause of obesity because adipose-specific glucose transporter (GLUT) 4 abrogation induces IR without developing obesity [11]. Considering the lipogenic effect of insulin, hyperinsulinemia seems to serve as an inducer of obesity or an important factor for deteriorating this pathologic state.

The observation that adipose TG stores keep increasing despite the decreased influx of glucose raises the possibility that there might be some other substrates responsible for the overproduction of adipose TG in obesity, and LCFA can be the candidate substrate. Interestingly, elevated cardiac LCFA uptake and TG storage are observed in genetic [12] or diet-induced [13] models of IR. Therefore, we hypothesized that, in the IR state, hyperinsulinemia can stimulate the increase of adipose LCFA uptake to render overproduction of TG and eventually the development of obesity.

Prolonged exposure to insulin impairs the insulin-stimulated glucose uptake in rats [14] and in 3T3-L1 adipocytes [15]. Numerous studies have further reported that 3T3-L1 adipocytes develop IR in response to physiologically relevant concentrations of insulin [16-18]. In the present study, we attempted to assess whether continual exposure to physiologic doses of insulin could enhance LCFA uptake in 3T3-L1 adipocytes and, if so, whether this enhancement could account for the overproduction of TG, but not for further oxidation of LCFA. Meanwhile, we also evaluated the mechanism for altered LCFA uptake and the mechanism underlying the selectivity of substrate utilization.

2. Research design and methods

2.1. Materials

Dulbecco modified Eagle medium (DMEM) and penicillin-streptomycin-glutamine solution were purchased from Gibco BRL (Gaithersburg , MD). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). [9, 10(n)-^{3}H]-palmitate, [1-^{14}C]-palmitate, and 2-deoxy-D-[1-^{3}H]-glucose were purchased from Amersham Biosciences (Aylesbury, United Kingdom). Fatty acid–free bovine serum albumin (BSA), isobutylmethylxanthine (IBMX), dexamethasone, insulin, 2-deoxyglucose, palmitic acid, and other lipid standards were obtained from Sigma (St Louis, MO). LY294002 and U0126 were obtained from Biomol International (Plymouth Meeting, PA).

2.2. Cell culture

3T3-L1 fibroblasts (American Type Culture Collection, Rockville, MD) were maintained in growth medium containing DMEM, with low glucose (5.5 mmol/L) supplemented with 10% FBS and 1% penicillin-streptomycin-glutamine solution in incubators equilibrated with 10% CO_2, as described by Ho et al [19] with modifications. Two days after confluence, differentiation was induced by IBMX (0.5 mmol/L), dexamethasone (0.25 μmol/L), and insulin (1 μg/mL) in growth medium. Another 2 days later, the medium was replaced by growth medium containing insulin (1 μg/mL) for another 2 days. Thereafter, cells were fed with growth medium every 2 days. Eight to 12 days after the initiation of differentiation, cells exhibiting 90% to 95% adipocyte phenotype, as evaluated by light microscopy, were used in all experiments.

2.3. Induction of IR and treatment

Insulin resistance in 3T3-L1 adipocytes was induced following the procedure described by Thomson et al [16] with modifications. Briefly, 3T3-L1 adipocytes were incubated in DMEM containing 1 nmol/L insulin, 5 or 25 mmol/L glucose, as well as 1% FBS for 16 hours. Krebs-Ringer HEPES buffer (KRH buffer; 128 mmol/L NaCl, 4.7 mmol/L KCl, 1.25 mmol/L $CaC1_2$, 1.25 mmol/L $MgSO_4$, 6.24 mmol/L Na_2HPO_4, 3.76 mmol/L Na_2HPO_4, and 20 mmol HEPES, pH 7.4) was used during the following washing procedures and metabolic assays. To remove insulin before the determination of glucose transport activity or the conduction of other assays, cells were extensively washed by warm KRH buffer containing 5 mmol/L glucose and 0.1% bovine serum albumin at 20-minute intervals over 2 hours.

Next, adipocytes were acutely stimulated with insulin for 30 minutes and assigned to glucose uptake, LCFA uptake, or other LCFA metabolic assays, in which 1 μmol/L and 100 nmol/L insulin was used, respectively. The Δ insulin-stimulated deoxyglucose or LCFA uptake was calculated as the absolute difference between insulin-stimulated state and basal state [18]; the area under the curve (AUC) was calculated by the trapezoid rule. Fifty micromoles per liter palmitate bound to BSA resulting in a palmitate-to-BSA ratio of 2 was used in all LCFA metabolic studies.

2.4. Glucose uptake

2-Deoxyglucose uptake was measured as previously described [20,21] with some modifications. Adipocytes were washed 3 times with 2 mL of warm KRH buffer containing 0.1% BSA. It was initiated by an addition of 0.2 mmol/L 2-deoxy-D-[1-^{3}H]-glucose (0.5 μCi/mL) at 37°C for 10 minutes. The reaction was terminated by buffer aspiration and 3 times of washing with 2 mL ice-cold KRH buffer/0.1% BSA, and then dissolved in 0.1% sodium dodecyl sulfate for scintillation counting.

2.5. LCFA uptake

Long-chain fatty acid uptake was measured as previously described [20] with some modifications. Adipocytes were washed 3 times with 2 mL of warm KRH buffer containing 5 mmol/L glucose and 0.1% BSA. Long-chain fatty acid uptake was initiated by adding transport buffer containing 50 μmol/L [^{3}H]-palmitate bound to BSA (0.5 μCi/mL) at 37°C for 5 minutes. The reaction was also terminated by buffer aspiration and 3 times of washing with 2 mL ice-cold KRH buffer/0.1% BSA, and then dissolved in 0.1% sodium dodecyl sulfate for scintillation counting.

2.6. Pretreatment of pharmacologic inhibitors

To assess the involvement of phosphatidylinositol 3-kinase (PI3K) pathway and mitogen-activated protein kinase (MAPK) pathway in the accompanied responses after the induction of IR, cells were first preincubated in 5 mmol/L glucose serum-starved DMEM containing LY294002 (10 μmol/L) or U0126 (25 μmol/L) for 1 hour before the IR induction. Afterward, the cells were refed with 25 mmol/L glucose serum-starved DMEM containing 1 nmol/L insulin in the continual presence of inhibitors for 16 hours, followed by 2-hour extensive washing.

2.7. Determination of LCFA incorporation into lipid

Long-chain fatty acid incorporation into lipid was determined as previously described [20]. Adipocytes were washed 3 times with 2 mL of warm KRH buffer containing 5 mmol/L glucose and 0.1% BSA. After pretreatment of hormones for indicated times, adipocytes were incubated in the same buffer containing 50 μmol/L [^{3}H]-palmitate bound to BSA (0.5 μCi/mL) for 2 hours. The reaction was terminated by buffer aspiration and 3 times of washing with 2 mL ice-cold KRH buffer/0.1% BSA. The cells were then scraped into 300 μL KRH buffer and taken for Folch extraction. Folch extracts were analyzed by thin-layer chromatography on Silica Gel 60A plates (Merck, Darmstadt, Germany) using a 2-solvent system [22]. The first solvent (diethyl ether:benzene:ethanol:acetic acid, 40:50:2:0.2) was run up to three fourths of the plate. Plates were air dried, heated briefly to remove traces of acetic acid, and run in the second solvent (diethyl ether:hexane, 6:94) to 1 cm from the top. The thin-layer chromatography plates were again dried for 30 minutes. The spots of each lipid class, visualized by spraying the plates with 0.0001% rhodamine 6G, were scraped and counted.

2.8. LCFA oxidation

The rate of fatty acid oxidation was estimated by measuring CO_2 production as previously described [23,24] with some modifications. Briefly, the monolayer cells were trypsinized, washed twice with warm KRH buffer containing 0.1% BSA and 5 mmol/L glucose, and suspended in the same buffer. Afterward, the cell suspensions were transferred into plastic vials. After pretreatment of hormones for indicated times, transport buffer containing 50 μmol/L [^{14}C]-palmitate bound to BSA (0.5 μCi/mL) was added; and the vial was immediately sealed with a rubber stopper that was attached to a central well (Kontes, Vineland, NJ; respectively, no. 882310-0000 and no. 882320-0000). Cells were incubated at 37°C during the experiment with constant shaking (60 strokes per minute). At the end of a 2-hour incubation, 0.5 mol/L perchloric acid was injected into the incubation vials through the rubber stopper to destroy the cells and terminate the metabolic reaction. To trap $^{14}CO_2$ produced during the incubation period, 0.3 mL of 2 N NaOH was injected into the suspended central well. Trapping of $^{14}CO_2$ was completed overnight with gentle shaking at 37°C. Eventually, NaOH containing the trapped $^{14}CO_2$ was collected and counted for radioactivity.

2.9. Fatty acid efflux

During the course of IR induction, 50 μmol/L ^{14}C-palmitate bound to BSA (0.5 μCi/mL) was concomitantly added into DMEM of both control and chronically insulin-treated cells. After a 16-hour incubation, unincorporated fatty acids were removed by extensive washing as described in IR induction. Cells were then incubated in KRH buffer containing 5 mmol/L glucose and 0.1% BSA at 37°C. After pretreatment of hormones for indicated times, supernatant and monolayers were collected and counted for radioactivity. No significant difference was found in the incorporated ^{14}C-radioactivity between control and insulin-resistant cells (283969 ± 5125 vs 282082 ± 5685 cpm per well, P = .809; results are the means ± SE from 4 independent experiments). Fatty acid efflux was expressed as the ratios of radioactivity in the supernatant to the total radioactivity found in the supernatant and the cells, as described previously [8].

2.10. Statistical analysis

Data were expressed as means ± SE. Differences between control and IR cells were determined with an unpaired Student 2-tailed test. Differences in the results between basal and insulin-stimulated state, as well as between control and IR cells, were determined by 1-way analysis of variance, followed by Tukey post hoc analysis. A value of $P < .05$ was considered statistically significant.

3. Results

3.1. Insulin-stimulated deoxyglucose and LCFA uptake in differentiated 3T3-L1 adipocytes cultured in growth media at physiologically relevant glucose levels

3T3-L1 adipocyte as a prominent tool is widely used for investigation of adipocyte metabolism. To reduce the possibility that high glucose or insulin concentrations potentially modify the adipocyte metabolism, we used a

standard IBMX, dexamethasone, and insulin differentiation protocol, which contained 1 μg/mL insulin in the differentiation cocktail and 5.5 mmol/L glucose, as described by Ho et al [19]. Hence, a normoglycemic environment that is physiologically relevant could be established during the whole culture period. Using such a protocol, stable differentiation of adipocytes was reproducibly achieved, characterized by neutral fat accumulation and the morphologic changes into adipocyte phenotype (Fig. 1A). In serum-starved adipocytes, acute insulin stimulation induced a 4.7-fold ($P < .001$) increase in deoxyglucose uptake and a 1.5-fold ($P < .05$) increase in LCFA uptake (left and right side of Fig. 1B, respectively), assuring us of a regular ability for substrate uptake in response to acute insulin treatment.

3.2. Diminishment of insulin-stimulated glucose uptake and enhancement of insulin-stimulated LCFA uptake in 3T3-L1 adipocytes through chronic exposure to insulin and glucose

To evaluate the consequence of chronic exposure to physiologically relevant levels of insulin in our experiments, adipocytes were preincubated in serum-starved media containing 1 nmol/L insulin for 16 hours, in the presence of 5 or 25 mmol/L glucose (denoted as *HI/5mM Glc* or *HI/25mM Glc*, respectively). Subsequently, cells were extensively washed and assigned to deoxyglucose or LCFA uptake assays. Basal deoxyglucose uptake was not affected, whereas insulin-stimulated deoxyglucose uptake was significantly decreased by chronic insulin exposure in normoglycemic environment (Fig. 2A, $P < .05$ vs "acute insulin" in control) and was further exacerbated in the concomitant challenge of 25 mmol/L glucose (Fig. 2A, $P < .001$ vs "acute insulin" in control and HI/5mM Glc). The finding that glucose down-regulates deoxyglucose uptake was consistent with that of the previous reports [14,16]. Interestingly, the same chronic insulin treatment increased both basal and insulin-stimulated LCFA uptake in normoglycemic condition (Fig. 2B, $P < .001$ vs "basal" and $P < .001$ vs "acute insulin" in control, respectively); and the increments in basal and insulin-stimulated LCFA uptake were additionally augmented in the continual presence of 25 mmol/L glucose (Fig. 2B, $P < .001$ vs "basal" in control and HI/5mM Glc; $P < .001$ vs "acute insulin" in control and HI/5mM Glc). These results revealed that chronic treatment to physiologic concentration of insulin enhances basal and insulin-stimulated LCFA uptake, and glucose acts synergistically with the hyperinsulinemic environment by a pattern similar to that in IR induction [18].

3.3. Alterations in the dose-response curves for insulin-stimulated glucose uptake and LCFA uptake after chronic exposure to HI/25mM Glc

To precisely assess the influence of prolonged exposure of insulin and glucose on the acute insulin actions on glucose and LCFA uptake, we compared the alterations of dose-response curves for deoxyglucose uptake and LCFA uptake after the chronic treatment of HI/25mM Glc. As for deoxyglucose uptake, in addition to the decreased responsiveness to maximal insulin stimulation by preincubation with HI/25mM Glc, the dose-response curve was shifted to the right by an order of magnitude (Fig. 3A). These insulin-resistant adipocytes remained unresponsive to 10 nmol/L insulin acute challenge (Fig. 3A, C); and the AUC of Δ insulin-stimulated deoxyglucose uptake was reduced (Fig. 3C, 1.77 ± 0.06 vs 1.15 ± 0.07 arbitrary units, $P < .05$ vs control), indicating the development of IR.

In control adipocytes, insulin elicited LCFA uptake in a regular dose-related manner. A mild response was observed

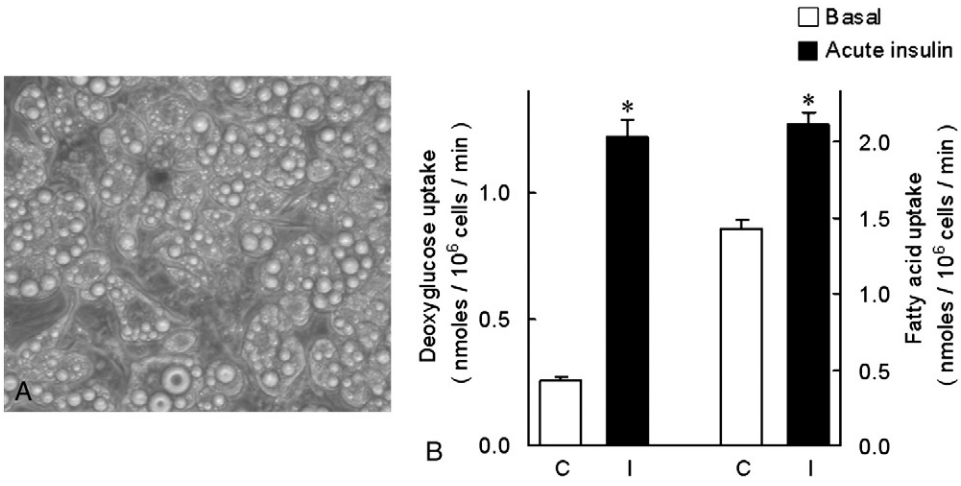

Fig. 1. Characterization of adipocyte differentiation and the stimulatory effect of insulin on deoxyglucose and LCFA uptake in 3T3-L1 adipocytes. A, Neutral fat accumulation and morphologic changes of differentiated 3T3-L1 adipocytes were examined by light microscopy (200×). B, Serum-starved cells were washed and treated with vehicle (white squares) or insulin (black squares) for 30 minutes, and then cellular uptake of 2-deoxyglucose and palmitate was measured. The results are mean ± SE from 3 independent experiments. *Significantly different from basal (no insulin additions, $P < .05$).

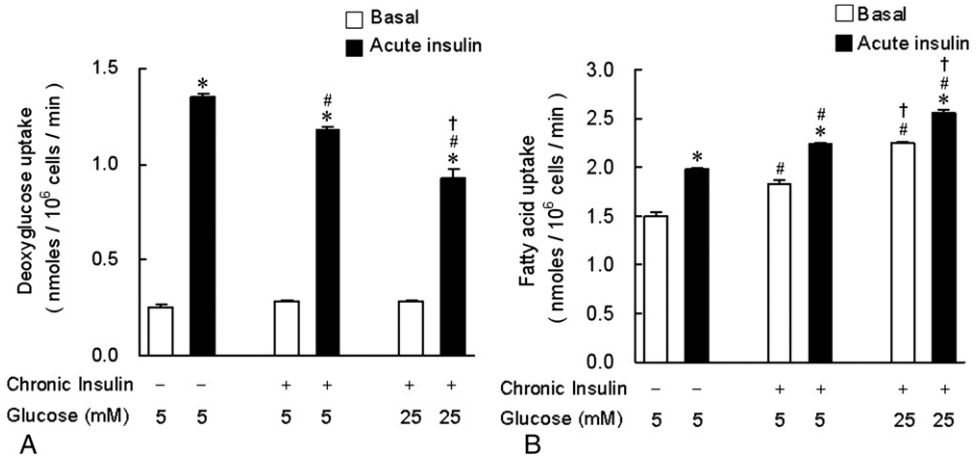

Fig. 2. Effect of chronic insulin exposure and glucose on insulin-stimulated deoxyglucose uptake and LCFA uptake in 3T3-L1 adipocytes. After incubation in serum-starved media containing HI/5mM Glc or HI/25mM Glc, adipocytes were extensively washed and stimulated with insulin for 30 minutes; and then cellular uptake of 2-deoxyglucose (A) or palmitate (B) was measured. Each panel represents the mean ± SE from 3 independent experiments. *Significantly different from "basal" (no insulin additions) in control ($P < .05$); #significantly different from the same state ("basal" or "acute insulin") in control ($P < .05$); †significantly different from the same state ("basal" or "acute insulin") in HI/5mM Glc ($P < .05$).

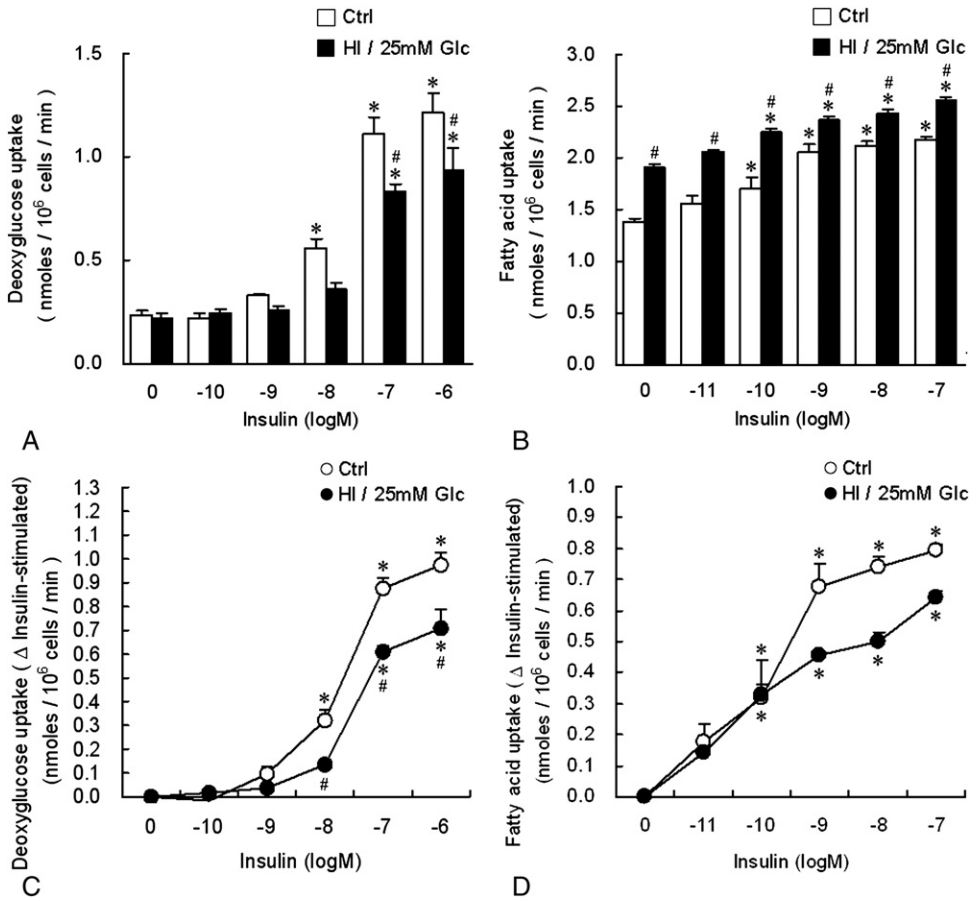

Fig. 3. Dose-response curves for insulin-stimulated deoxyglucose uptake and LCFA uptake after chronic exposure to insulin at high glucose concentrations. After incubation in serum-starved media containing HI/25mM Glc, adipocytes were extensively washed and assigned to deoxyglucose and LCFA uptake assays. Cells were then washed and stimulated with insulin for 30 minutes, and then cellular uptake of 2-deoxyglucose (A) and palmitate (B) was measured. The Δ insulin-stimulated deoxyglucose (C) and LCFA uptake (D) were calculated as the absolute difference between insulin-stimulated state and basal state. Each panel represents the mean ± SE from 4 independent experiments. *Significantly different from the vehicle alone in corresponding dose-response curve ($P < .05$); #significantly different from each corresponding dose of insulin stimulation in control ($P < .05$).

in LCFA uptake at 0.1 nmol/L, and maximal response was reached at 1 nmol/L (Fig. 3B, white squares). Interestingly, to elicit an observable or the maximal response, the doses of insulin were much lower in LCFA uptake than those in deoxyglucose uptake (Fig. 3, compare black squares in panels A and B). Sixteen-hour preincubation of HI/25mM Glc substantially elevated the basal uptake and insulin-stimulated LCFA uptake, at any given dose of acute insulin (Fig. 3B, black squares). Notably, there was also a decrease in the AUC of Δ insulin-stimulated LCFA uptake in HI/25mM Glc–pretreated adipocytes (Fig. 3D, 2.31 ± 0.21 vs 1.75 ± 0.03 arbitrary units, $P < .05$ vs control), suggesting that insulin-stimulated LCFA uptake was blunted by HI/25mM Glc. Taken together, chronic exposure to HI/25mM Glc led to a remarkable elevation of LCFA influx accompanied by a tendency of loss of the capacity for insulin-stimulated LCFA uptake, reflecting that the majority of LCFA uptake elevation virtually originated from up-regulation of basal LCFA uptake.

3.4. Elevation of LCFA influx accounted for adipose TG overaccumulation but not for LCFA oxidation

The elevation of LCFA influx could be further partitioned into esterification and/or the mitochondrial machineries for LCFA oxidation. To clarify the metabolic fate of LCFA after the transmembrane uptake, adipocytes chronically exposed to HI/25mM Glc were pretreated with 100 nmol/L insulin for 30 minutes; and then LCFA oxidation and esterification were measured after a 2-hour incubation. In control adipocytes, insulin exhibited a remarkable suppressive effect on the rate of LCFA oxidation (Fig. 4A, $P < .001$ vs "basal" in control). In HI/25mM Glc–pretreated adipocytes, basal LCFA oxidation was substantially reduced ($P < .001$ vs "basal" in control); and insulin still suppressed the rate of LCFA oxidation (Fig. 4A, $P < .001$ vs "basal" in HI/25mM Glc). Notably, insulin suppressed LCFA oxidation by 47% in control adipocytes, whereas only a 24% reduction of LCFA oxidation by insulin was

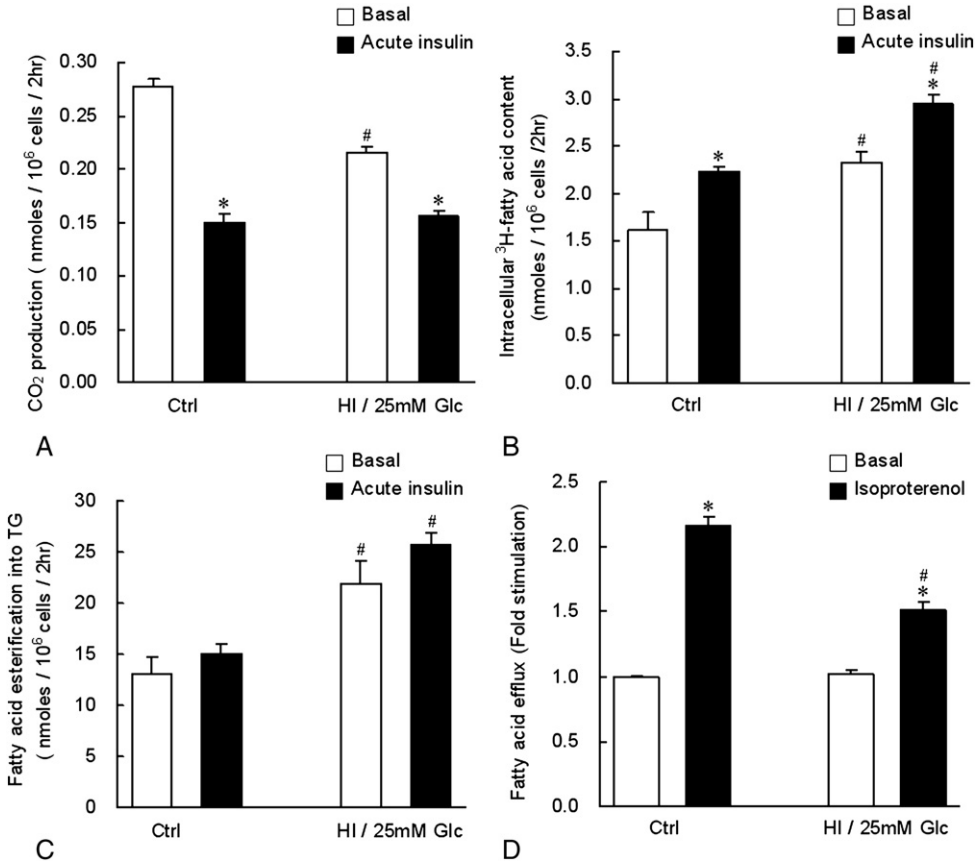

Fig. 4. Alterations in intracellular LCFA metabolism after chronic exposure to insulin at high glucose concentrations. A to C, After incubation in serum-starved media containing HI/25mM Glc, adipocytes were extensively washed and assigned to various assays. Cells were then washed and stimulated with insulin for 30 minutes; and then the rate of LCFA oxidation (A), intracellular ^{3}H-LCFA accumulation (B), and LCFA incorporation into TG (C) was measured. D, Fifty micromoles per liter ^{14}C-palmitate bound to BSA was concomitantly added into DMEM of both control and HI/25mM Glc–pretreated adipocytes during the course of IR induction. Unincorporated ^{14}C-palmitate was removed by extensive washing. Cells were then washed and stimulated with 1 μmol/L isoproterenol for 1 hour, and then fatty acid efflux was measured. In panels A, C and D, the results are mean ± SE from 4 independent experiments. In panel B, the results are mean ± SE from 3 independent experiments. *Significantly different from corresponding "basal" (no insulin or isoproterenol additions) ($P < .05$); #significantly different from the same state ("basal" or "acute insulin") in control ($P < .05$).

observed in HI/25mM Glc–pretreated adipocytes, indicating that the regular suppressive effect of insulin on LCFA oxidation was impaired by such a treatment. Acute insulin moderately increased the rate of ^{3}H-labeled palmitate deposition into the intracellular unesterified LCFA pool in both control and HI/25mM Glc–pretreated adipocytes (Fig. 4B, $P < .05$ vs "basal" in control and $P < .05$ vs "basal" in HI/25mM Glc, respectively). In these HI/25mM Glc–pretreated adipocytes, the magnitude of intracellular LCFA deposition was significantly increased by 1.45- and 1.32-fold in basal and insulin-stimulated states, as compared with that in control adipocytes (Fig. 4B, $P < .05$ vs each corresponding states in control). The rate of LCFA esterification into TG was unresponsive to acute insulin addition with or without any chronic treatment (Fig. 4C). Interestingly, consistent with the trend of LCFA deposition, the rate of esterification into TG was increased in all HI/25mM Glc–pretreated adipocytes (Fig. 4C, $P < .05$ vs each corresponding states in control). To exclude the possibility for discharge of the accumulated LCFA within the adipocytes, we compared the ability of fatty acid efflux between control and HI/25mM Glc–pretreated adipocytes. After chronic exposure to HI/25mM Glc, the maximal fatty acid efflux induced by 1 μmol/L isoproterenol was significantly impaired (Fig. 4D, $P < .001$ vs "isoproterenol" in control). Considering all of these findings, prolonged exposure to HI/25mM Glc resulted in several defects in mitochondrial LCFA oxidation and hormone-induced lipolysis; and the intracellular LCFA from enhanced influx tended to accumulate within the cells, which eventually contributed to overaccumulation of adipose TG.

3.5. Increment of LCFA influx in both basal and insulin-stimulated states was governed by the MAPK pathway of insulin signaling cascades

Phosphatidylinositol 3-kinase–dependent and MAPK-dependent pathways are 2 major branches of insulin

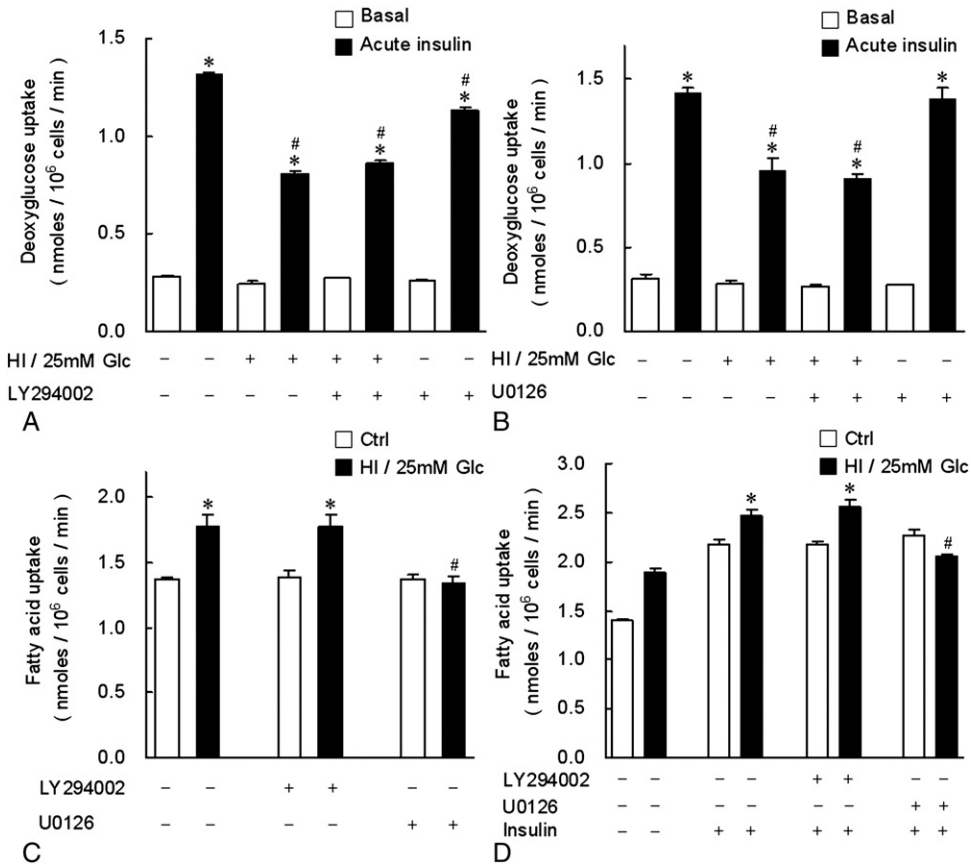

Fig. 5. Effect of pharmacologic inhibition of PI3K pathway and MAPK pathway on the HI/25mM Glc–induced abnormalities in glucose and LCFA influx. After incubation with 5 mmol/L glucose serum-starved DMEM containing PI3K inhibitor LY294002 (10 μmol/L) or MAPK kinase inhibitor U0126 (25 μmol/L) for 1 hour, 3T3-L1 adipocytes were refed with serum-starved DMEM containing HI/25mM Glc in the continual presence of inhibitors for 16 hours, followed by 2-hour extensive washing. Cells were then washed and stimulated with insulin for 30 minutes, and then cellular uptake of 2-deoxyglucose (A and B) and palmitate (C and D) was measured. In panels A and B, the results are mean ± SE from 8 independent experiments. *Significantly different from "basal" (no insulin additions) ($P < .05$); $^{\#}$significantly different from the "acute insulin" in control ($P < .05$). In panel C, the results are mean ± SE from 3 independent experiments. *Significantly different from vehicle only in control (no inhibitor additions) ($P < .05$); $^{\#}$significantly different from vehicle only in HI/25mM Glc (no inhibitor additions) ($P < .05$). In panel D, the results are mean ± SE from 8 independent experiments. *Significantly different from "insulin" in control (no inhibitor additions) ($P < .05$); $^{\#}$significantly different from "insulin" in HI/25mM Glc (no inhibitor additions) ($P < .05$).

signaling cascades after insulin binding to its receptor and the subsequent receptor autophosphorylation. Phosphatidylinositol 3-kinase is a key molecule mediating most of metabolic actions of insulin [25,26], and the MAPK pathway is primarily associated with the regulation of mitogenesis [27]. Although MAPK pathway is not responsible for the insulin-stimulated glucose uptake and glycogen synthesis [28], Stahl et al reported that both PI3K and MAPK mediate the insulin-stimulated LCFA uptake [8]. Insulin can rapidly activates both PI3K and MAPK pathways within few minutes [26] and has been reported to chronically induce the desensitization of insulin signaling at multiple levels [29]. Hypothetically, if IR and elevated LCFA influx, which was induced by HI/25mM Glc, originated from chronic stimulation (and/or desensitization) of PI3K and MAPK pathways, then the inhibition of these pathways would prevent the metabolic abnormalities they make. To test this hypothesis, we pretreated adipocytes with pharmacologic inhibitors against PI3K or MAPK activation for 1 hour and then exposed them chronically to HI/25mM Glc in the continual presence of inhibitors, followed by extensive washing and assays. The HI/25mM Glc–pretreated adipocytes consistently exhibited an impaired insulin-stimulated deoxyglucose uptake (Fig. 5A, $P < .001$; Fig. 5B $P < .001$ vs "acute insulin" in control). LY294002 is a potent PI3K inhibitor that can rapidly abolish insulin-stimulated glucose uptake. In the present study, continual exposure to LY294002 (10 μmol/L) alone did not affect basal deoxyglucose uptake but mildly suppressed insulin-stimulated deoxyglucose uptake (Fig. 5A), possibly because of the residual effect of LY294002 after extensive washing. The MAPK kinase inhibitor (U0126; 25 μmol/L) alone showed no effect on basal and insulin-stimulated deoxyglucose uptake (Fig. 5B). Neither LY294002 nor U0126 pretreatment abrogated the impaired insulin-stimulated deoxyglucose uptake by HI/25mM Glc (Fig. 5A, B); thus, the possibility of chronic activation of PI3K or MAPK pathway to mediate the development of HI/25mM Glc-induced IR can be excluded. On the other hand, chronic exposure to HI/25mM Glc elevated basal LCFA uptake as expected (Fig. 5C, $P < .05$ vs vehicle only in control); and this elevated LCFA uptake was not affected by LY294002, but was fully blocked by U0126 ($P < .05$ vs vehicle only in HI/25mM Glc). Acute insulin treatment consistently stimulated LCFA uptake in all control adipocytes (Fig. 5D, $P < .001$ vs vehicle only in control) and HI/25mM Glc–pretreated adipocytes ($P < .05$ vs vehicle only in HI/25mM Glc). In HI/25mM Glc–pretreated adipocytes, insulin-stimulated LCFA uptake was also elevated ($P < .05$ vs "insulin" in control); and the elevation in insulin-stimulated LCFA uptake was prevented by U0126 ($P < .05$ vs "insulin" in HI/25mM Glc), but not LY294002. Taken together, this indicated that chronic activation of MAPK pathway mediates the HI/25mM Glc–induced elevation of LCFA uptake in both basal and insulin-stimulated states.

4. Discussion

Obesity has been regarded as one of the components of IR syndrome [30], although the interrelationship between obesity and IR remains controversial. The predominant explanation was that obesity causes IR and compensatory hyperinsulinemia [5]. Nevertheless, IR and hyperinsulinemia, the root causes of IR syndrome, have also been reported to contribute to the development of obesity [5]. Our present study demonstrated that continual exposure to a physiologically relevant concentration of insulin did induce IR, but this exposure inversely increased basal and insulin-mediated LCFA influx and subsequently contributed to TG overaccumulation. In this experimental model of adipose IR, the selectivity of substrate utilization indicated that LCFA could be the major substrate responsible for overproduction of TG in obesity.

The mechanisms required for IR induction caused by physiologic concentrations of insulin have been widely explored in 3T3-L1 adipocytes [16-18,31], whereas several unsolved issues need to be further investigated. Thomson et al [16] and Chen et al [31] reported that chronic exposure to physiologic concentrations of insulin impaired GLUT4 translocation. However, studies by Ross et al [17] and Nelson et al [18] demonstrated that GLUT4 protein amount [17,18] and GLUT4 translocation [18] were not affected by such chronic insulin exposure. In agreement with our findings (Fig. 2A) in the present study, Thomson et al [16] reported that the presence of high glucose concentrations exacerbated the IR induced by insulin; but the effect of high glucose concentrations was not remarkable in the study by Chen et al [31]. The study by Nelson et al [18] further confirmed that high glucose flux was not involved in the development of IR through hexosamine pathway. Nevertheless, the precise change in glucose metabolism in this state was not fully elucidated. Because 2-deoxyglucose may differ kinetically from glucose, the correction factor lumped constant [32] must be used to derive rates of glucose utilization from rates of 2-deoxyglucose uptake and phosphorylation. Consequently, to assess the glucose metabolism or to delineate the impact of glucose metabolism-mediated effects, D-[U-^{14}C]-glucose appears to be more useful and worth to be used in further studies. Phosphatidylinositol 3-kinase–dependent and MAPK-dependent pathways are 2 major branches of insulin signaling cascades; our findings (Fig. 5A, B) excluded that chronic activation of PI3K or MAPK pathway is essential for this induction of IR. Notably, several evidences [17,18,31] demonstrated that proximal insulin signaling remained intact after long-term exposure to physiologic concentrations of insulin, suggesting that if a signaling defect exists, it likely lies more distal in the insulin signaling pathway. The small GTP-binding protein TC10 pathway functions in parallel with PI3K to stimulate GLUT4 translocation in response to acute insulin [33]. Interestingly, hyperinsulinemic state caused a marked loss of cortical F-actin [31]; and TC10 may control the

dynamics of actin dynamics [34]. Future studies are required to confirm these speculations of the TC10 abnormalities in IR induction.

Another major finding in this study was that chronic exposure to physiologic concentrations of insulin also enhances basal and insulin-stimulated LCFA uptake (Figs. 2B and 3B) and that MAPK pathway plays a mediating role in enhancing the LCFA uptake, at both basal and insulin-stimulated conditions (Fig. 5C, D). Coort et al [12] reported that LCFA uptake is enhanced in cardiomyocytes from obese Zucker rats through redistribution of FAT/CD36, and they speculated that this enhanced LCFA uptake in cardiomyocytes is due to the physiologically high plasma insulin levels. In addition, these insulin-resistant cardiomyocytes from obese Zucker rats are unresponsive to acute insulin stimulation to elicit regular LCFA uptake [12]. In accordance with the findings and interpretation by Coort et al, our present study provided direct evidence that LCFA uptake is enhanced in response to chronic exposure to physiologic concentrations of insulin. Besides, in our dose-response experiments (Fig. 3B, D), the decreased AUC of Δ insulin-stimulated LCFA uptake also shows the diminished responsiveness for insulin-stimulated LCFA uptake after chronic exposure to insulin. However, in adipocytes, the respective transporters responsible for basal and insulin-stimulated LCFA uptake were not fully understood [8,35]; and the insulin-stimulated LCFA uptake could be fatty acid specific [35]. Further studies will be required to clarify the components mediating the alterations of LCFA uptake after chronic insulin exposure. Similar to the results of IR induction (Fig. 2A), the role of glucose that potentiates the enhancement in LCFA uptake (Fig. 2B) also needs further elucidation.

The influence of prolonged insulin exposure on intracellular LCFA metabolism is general (Fig. 4A-D). The deposition of intracellular unesterified LCFA (Fig. 4B), which was parallel to the trend for enhanced LCFA influx, has been reported to strongly interfere with the physiology of membrane functions and eventually lead to membrane instability and disruption [36]. Hence, the general effect of prolonged insulin exposure on intracellular LCFA metabolism might be secondary to the rise in intracellular unesterified LCFA. The apparently impaired basal and insulin-suppressed LCFA oxidation (Fig. 4A) suggested that some defects occur in the machineries of mitochondrial LCFA oxidation. The TG pool of these treated adipocytes exhibited a reduced lipolytic rate in response to adrenergic stimulation (Fig. 4D), as previously described by Olansky and Pohl [37]. This impaired isoproterenol-induced lipolysis could be due to that hyperinsulinemic conditions disrupt β-adrenergic signaling to protein kinase A in the study by Zhang et al [38]. The robust increase in the rate of LCFA incorporation into TG (Fig. 4C) reflected that intracellular LCFA deposit is extensively partitioned into esterification and is prone to retain within adipocytes chronically exposed to insulin.

In conclusion, chronic exposure to physiologic concentrations of insulin, potentiated by adding high glucose, may induce IR and inversely enhance basal and insulin-stimulated LCFA uptake in 3T3-L1 adipocyte. Insulin resistance is not mediated by the chronic activation of PI3K or MAPK pathway. However, MAPK pathway plays a role in enhancing LCFA influx in basal and insulin-stimulated states. The enhanced LCFA influx further induces intracellular LCFA deposition and TG overaccumulation and, meanwhile, impairs the mitochondrial LCFA oxidation and adrenergic agent–induced lipolysis. The present study demonstrated that, in the presence of hyperinsulinemia, LCFA serves as the substrate for TG storage during the development of IR-associated obesity. Targeting the MAPK branch of adipose insulin signaling may be considered as a therapeutic approach against hyperinsulinemia-associated obesity.

Acknowledgment

We thank Ms Yung-Pei Hsu for her outstanding secretarial work, Ms Su-Jane Chen for her critical review of the manuscript, and Dr Shu-Chiung Chiang for the valuable assistance in statistical analyses. These studies were supported in part by serial research grants from the National Science Council of Taiwan (NSC 96-2314-B-075-003; NSC 97-2314-B-075-013-MY3).

References

[1] Hainer V, Toplak H, Mitrakou A. Treatment modalities of obesity: what fits whom? Diabetes Care 2008;31:S269-77.
[2] Burns TW, Terry BE, Langley PE, Robison GA. In-vitro observations on isolated adipose tissue cells from hyperobese subjects. Diabetes 1977;26:657-62.
[3] Arner P, Bolinder J, Engfeldt P, Hellmer J, Ostman J. Influence of obesity on the antilipolytic effect of insulin in isolated human fat cells obtained before and after glucose ingestion. J Clin Invest 1984;73:673-80.
[4] Bolinder J, Lithell H, Skarfors E, Arner P. Effects of obesity, hyperinsulinemia, and glucose intolerance on insulin action in adipose tissue of sixty-year-old men. Diabetes 1986;35:282-90.
[5] Kahn BB, Flier JS. Obesity and insulin resistance. J Clin Invest 2000;106:473-81.
[6] Frayn KN. Non-esterified fatty acid metabolism and postprandial lipaemia. Atherosclerosis 1998;141:S41-6.
[7] Stump DD, Fan X, Berk PD. Oleic acid uptake and binding by rat adipocytes define dual pathways for cellular fatty acid uptake. J Lipid Res 2001;42:509-20.
[8] Stahl A, Evans JG, Pattel S, Hirsch D, Lodish HF. Insulin causes fatty acid transport protein translocation and enhanced fatty acid uptake in adipocytes. Dev Cell 2002;2:477-88.
[9] Kashyap SR, Defronzo RA. The insulin resistance syndrome: physiological considerations. Diab Vasc Dis Res 2007;4:13-9.
[10] Erdmann J, Kallabis B, Oppel U, Sypchenko O, Wagenpfeil S, Schusdziarra V. Development of hyperinsulinemia and insulin resistance during the early stage of weight gain. Am J Physiol Endocrinol Metab 2008;294:E568-E575.
[11] Abel ED, Peroni O, Kim JK, Kim YB, Boss O, Hadro E, et al. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. Nature 2001;409:729-33.

[12] Coort SL, Hasselbaink DM, Koonen DP, Willems J, Coumans WA, Chabowski A, et al. Enhanced sarcolemmal FAT/CD36 content and triacylglycerol storage in cardiac myocytes from obese Zucker rats. Diabetes 2004;53:1655-63.
[13] Ouwens DM, Diamant M, Fodor M, Habets DD, Pelsers MM, El Hasnaoui M, et al. Cardiac contractile dysfunction in insulin-resistant rats fed a high-fat diet is associated with elevated CD36-mediated fatty acid uptake and esterification. Diabetologia 2007;50:1938-48.
[14] Garvey WT, Olefsky JM, Matthaei S, Marshall S. Glucose and insulin co-regulate the glucose transport system in primary cultured adipocytes. A new mechanism of insulin resistance. J Biol Chem 1987;262:189-97.
[15] Flores-Riveros JR, McLenithan JC, Ezaki O, Lane MD. Insulin down-regulates expression of the insulin-responsive glucose transporter (GLUT4) gene: effects on transcription and mRNA turnover. Proc Natl Acad Sci U S A 1993;90:512-6.
[16] Thomson MJ, Williams MG, Frost SC. Development of insulin resistance in 3T3-L1 adipocytes. J Biol Chem 1997;272:7759-64.
[17] Ross SA, Chen X, Hope HR, Sun S, McMahon EG, Broschat K, et al. Development and comparison of two 3T3-L1 adipocyte models of insulin resistance: increased glucose flux vs glucosamine treatment. Biochem Biophys Res Commun 2000;273:1033-41.
[18] Nelson BA, Robinson KA, Buse MG. High glucose and glucosamine induce insulin resistance via different mechanisms in 3T3-L1 adipocytes. Diabetes 2000;49:981-91.
[19] Ho M, Foxall S, Higginbottom M, Donofrio DM, Liao J, Richardson PJ, et al. Leptin-mediated inhibition of the insulin-stimulated increase in fatty acid uptake in differentiated 3T3-L1 adipocytes. Metabolism 2006;55:8-12.
[20] Bastie CC, Nahle Z, McLoughlin T, Esser K, Zhang W, Unterman T, et al. FoxO1 stimulates fatty acid uptake and oxidation in muscle cells through CD36-dependent and -independent mechanisms. J Biol Chem 2005;280:14222-9.
[21] Frost SC, Lane MD. Evidence for the involvement of vicinal sulfhydryl groups in insulin-activated hexose transport by 3T3-L1 adipocytes. J Biol Chem 1985;260:2646-52.
[22] Freeman CP, West D. Complete separation of lipid classes on a single thin-layer plate. J Lipid Res 1966;7:324-7.
[23] Fong JC, Leu SJ, Chai SP. Differential inhibition of lipolysis by 2-bromopalmitic acid and 4-bromocrotonic acid in 3T3-L1 adipocytes. Biochim Biophys Acta 1997;1344:65-73.
[24] Rodbell M. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. J Biol Chem 1964;239:375-80.
[25] Shepherd PR, Withers DJ, Siddle K. Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. Biochem J 1998;333: 471-90.
[26] Sakaue H, Ogawa W, Takata M, Kuroda S, Kotani K, Matsumoto M, et al. Phosphoinositide 3-kinase is required for insulin-induced but not for growth hormone- or hyperosmolarity-induced glucose uptake in 3T3-L1 adipocytes. Mol Endocrinol 1997;11:1552-62.
[27] Denton RM, Tavare JM. Does mitogen-activated-protein kinase have a role in insulin action? The cases for and against. Eur J Biochem 1995;227:597-611.
[28] Kayali AG, Austin DA, Webster NJ. Stimulation of MAPK cascades by insulin and osmotic shock: lack of an involvement of p38 mitogen-activated protein kinase in glucose transport in 3T3-L1 adipocytes. Diabetes 2000;49:1783-93.
[29] Inoue G, Cheatham B, Kahn CR. Different pathways of postreceptor desensitization following chronic insulin treatment and in cells overexpressing constitutively active insulin receptors. J Biol Chem 1996;271:28206-11.
[30] Rao G. Insulin resistance syndrome. Am Fam Physician. 2001;63:1159-63, 65-6.
[31] Chen G, Raman P, Bhonagiri P, Strawbridge AB, Pattar GR, Elmendorf JS. Protective effect of phosphatidylinositol 4,5-bisphosphate against cortical filamentous actin loss and insulin resistance induced by sustained exposure of 3T3-L1 adipocytes to insulin. J Biol Chem 2004;279:39705-9.
[32] Russell III RR, Mrus JM, Mommessin JI, Taegtmeyer H. Compartmentation of hexokinase in rat heart. A critical factor for tracer kinetic analysis of myocardial glucose metabolism. J Clin Invest 1992;90: 1972-7.
[33] Chiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, et al. Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. Nature 2001;410: 944-8.
[34] Kanzaki M, Watson RT, Hou JC, Stamnes M, Saltiel AR, Pessin JE. Small GTP-binding protein TC10 differentially regulates two distinct populations of filamentous actin in 3T3L1 adipocytes. Mol Biol Cell 2002;13:2334-46.
[35] Lobo S, Wiczer BM, Smith AJ, Hall AM, Bernlohr DA. Fatty acid metabolism in adipocytes: functional analysis of fatty acid transport proteins 1 and 4. J Lipid Res 2007;48:609-20.
[36] van der Vusse GJ, Glatz JF, Stam HC, Reneman RS. Fatty acid homeostasis in the normoxic and ischemic heart. Physiol Rev 1992;72: 881-940.
[37] Olansky L, Pohl SL. beta-Adrenergic desensitization by chronic insulin exposure in 3T3-L1 cultured adipocytes. Metabolism 1984;33: 76-81.
[38] Zhang J, Hupfeld CJ, Taylor SS, Olefsky JM, Tsien RY. Insulin disrupts beta-adrenergic signalling to protein kinase A in adipocytes. Nature 2005;437:569-73.