Improvement of Insulin Sensitivity by Chelation of Intracellular Ca^{2+} in High-Fat–Fed Rats

Y.J. Jang, H.J. Ryu, Y.O. Choi, C. Kim, C.H. Leem, and C.S. Park

It has been postulated that sustained high levels of intracellular calcium concentration ($[Ca^{2+}]_i$) in the insulin target cells may cause insulin resistance. We evaluated this hypothesis by examining the effect of an intracellular Ca^{2+} chelator, 5,5′-dimethyl derivative of bis (*o*-aminophenoxy) ethane-N,N,N′,N′ tetraacetic acetoxymethyl ester (dimethyl-BAPTA/AM), on insulin resistance. Insulin resistance was induced in rats by feeding a high-fat diet for 3 to 4 weeks. The whole body insulin sensitivity was determined by the steady state glucose infusion rate (GIR) under euglycemic hyperinsulinemic (6 mU · kg^{-1} · min^{-1}) clamps. Compared with control rats, the high-fat diet (HFD) fed rats showed significantly lower GIR (12.2 ± 0.7 *v* 20.2 ± 0.9 mg · kg^{-1} · min^{-1}; $P < .01$). In the HFD rats, an intravenous injection of dimethyl-BAPTA/AM (6 mg/kg) 90 minutes before the clamps significantly increased GIR to 16.3 ± 0.9 mg · kg^{-1} · min^{-1} ($P < .02$), reversing insulin resistance by about 50%; but this intervention had no effect in the controls. This increase in GIR by dimethyl-BAPTA/AM was observed without an increase in femoral artery blood flow, indicating that the chelator increased GIR directly through improving cellular responsiveness to insulin. The stimulatory effect of insulin on 2-deoxy glucose (2-DG) uptake by the isolated epididymal adipocytes was reduced by 35% in the HFD rats compared with the control rats ($P < .01$). Pretreatment of the HFD rats with dimethyl-BAPTA/AM restored 2-DG uptake to the level in the control rats. The direct measurement of $[Ca^{2+}]_i$ using fura-2/AM in isolated adipocytes showed that basal $[Ca^{2+}]_i$ was significantly higher in the HFD rats than in the control rats (145 ± 11 *v* 112 ± 9 nmol/L; $P < .05$). An injection of dimethyl-BAPTA/AM in the HFD rats lowered $[Ca^{2+}]_i$ to 127 ± 11 nmol/L, which did not differ from the level in the control rats ($P > .2$). The present study clearly demonstrates that an injection of intracellular Ca^{2+} chelator in the HFD rats reverses insulin resistance, as well as normalizes elevated $[Ca^{2+}]_i$ in the insulin target cells. The results strongly support that sustained high levels of $[Ca^{2+}]_i$ in the insulin target cells may play an important role in insulin resistance, at least in the HFD rats.

INSULIN RESISTANCE IS of major pathogenic importance in several common human disorders, including type 2 diabetes, obesity, hypertension, and atherosclerotic cardiovascular disease.[1] However, its underlying mechanism is not fully understood. One of the potential factors that may account for or contribute to insulin resistance is a sustained high level of intracellular calcium concentration ($[Ca^{2+}]_i$) in the insulin target cells.[2-5]

Draznin et al[6] demonstrated an optimal range of $[Ca^{2+}]_i$ for the stimulatory effect of insulin on glucose transport in isolated rat adipocytes, with the levels beyond this range causing marked decrease in insulin sensitivity. Similarly, elevations of $[Ca^{2+}]_i$ induced by incubation of adipocytes with depolarizing concentrations of K^+ or parathyroid hormone resulted in diminished cellular responsiveness to insulin.[7] High levels of the steady state $[Ca^{2+}]_i$ have been observed in adipocytes of patients with obesity,[8] type 2 diabetes,[9] skeletal muscles of agouti rats,[10] and hepatocytes of diabetic rats.[11] Consistently, an inverse correlation between the whole body insulin sensitivity and the $[Ca^{2+}]_i$ of peripheral blood cells has been observed in human studies.[12,13]

The 5,5′-dimethyl derivative of bis (*o*-aminophenoxy) ethane-N,N,N′,N′ tetraacetic acetoxymethyl ester (dimethyl-BAPTA/AM), an effective intracellular Ca^{2+} chelator,[14] rapidly enters cells where it is hydrolyzed to yield an impermeable tetracarboxylate form of dimethyl-BAPTA, which binds to intracellular Ca^{2+} with a high affinity (k_d = 40 nmol/L).[14] Thus, dimethyl-BAPTA/AM can function as an intracellular Ca^{2+} buffer and stabilize $[Ca^{2+}]_i$ at a physiologic or subphysiologic level.

The purpose of this study was to examine if a sustained elevation of $[Ca^{2+}]_i$ in the insulin target cells contributes to insulin resistance of the high-fat diet (HFD) fed rats. To assess this possible mechanism, we examined the effect of dimethyl-BAPTA/AM on insulin resistance and $[Ca^{2+}]_i$ in the HFD rats.

MATERIALS AND METHODS

Materials

Recombinant human insulin (Humulin, regular) was obtained from Daewoong Lilly, Korea. Dimethyl-BAPTA/AM was purchased from TEFLABS (Austin, TX), fura-2 and its acetoxymethyl ester (fura-2/AM) from Molecular Probes, (Eugene, OR), collagenase from Worthington Biochemical (Freehold, NJ), vitamin mixture and mineral mixture from ICN (Costa Mesa, CA), 2-deoxy-D-[1-^{3}H]glucose ([^{3}H]2-DG) from Amersham (Arlington Heights, IL), safflower seed oil, 2-DG, cytochalasin B, and phloretin from Sigma (St Louis, MO).

Animals and Diets

Male Sprague-Dawley rats, 6 weeks of age, were used for all studies. The rats were housed 4 per cage in a room with a 12/12-hour light/dark cycle and an ambient temperature of 22°C to 25°C. Animals were fed on either a standard rat chow consisting (as a percentage of total kcal) of 24% fat, 52% carbohydrate, and 24% protein, (Sam Yang, Wonju,

From the Department of Physiology and the Institute for Calcium Research, University of Ulsan College of Medicine, Seoul, Korea.

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Address reprint requests to Y.J. Jang, MD, PhD, Department of Physiology, University of Ulsan College of Medicine, 388-1, Poongnapdong, Songpagu, Seoul 138-736, Korea.

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Table 1. Composition of the HFD

Ingredients	g/kg of Diet
Casein	254
Safflower seed oil	339
Sucrose	85
Corn starch	169
Cellulose	51
Methionine	3
Gelatin	19
Vitamin mixture*	13
Mineral mixture†	67

*Per kilogram of vitamin mixture: 1.8 g vitamin A acetate, 0.125 g vitamin D_2, 22 g DL-α-tocopherol acetate, 45 g ascorbic acid, 5 g inositol, 75 g choline chloride, 2.25 g menadione, 5 g-p-aminobenzoic acid, 4.25 g niacin, 1 g riboflavin, 1 g pyridoxine hydrochloride, 1 g thiamine hydrochloride, 3 g calcium pantothenate, 0.02 g biotin, 0.09 g folic acid, and 0.00135 g vitamin B_1.

†Per kilogram of mineral mixture: 500 g $CaHPO_4$, 74 g NaCl, 220 g $KC_6H_7O_7 \cdot H_2O$, 52 g K_2SO_4, 24 g MgO, 3.5 g $MnCO_3$, 6 g $FeC_6H_5O_7$, 1.6 g $ZnCO_3$, 0.3 g $CuCO_3$, 0.01 g KIO_3, 0.01 g Na_2SeO_3, 0.55 g $CrK(SO_4)_2$, and 118 g sucrose.

Korea) or a HFD consisting of 59% fat, 20% carbohydrate, and 21% protein. The HFD, formulated as described by Storlien et al[15] (Table 1), was freshly made every 3 to 4 days and stored at 4°C. Rats were fed ad libitum on an assigned diet for 3 to 4 weeks before the experiments.

Euglycemic Hyperinsulinemic Glucose Clamps

The whole body insulin sensitivity was measured by euglycemic hyperinsulinemic clamps. To investigate the effect of intracellular Ca^{2+} chelator on insulin sensitivity, the HFD rats (n = 25) and the control rats (n = 24) were divided into 3 groups (Table 2). The first group was injected with dimethyl-BAPTA/AM (6 mg/kg in 250 μL of dimethyl sulfoxide [DMSO] 1:3 diluted with distilled water) and the second group with the same volume of vehicle 90 minutes prior to the initiation of insulin infusion. The third group was injected intraperitoneally (IP) with dimethyl-BAPTA/AM at 6:00 PM on the preceding day of glucose clamps (20 hours before the experiment). The dose of dimethyl-BAPTA/AM was chosen based on the results from several preliminary experiments.

The glucose clamp experiments were performed on the conscious rats as described by Buchanan et al.[16] After an overnight fast, at 9:00 AM, each animal underwent with brief restraint the placement of a tail artery catheter for blood sampling and 2 tail vein catheters for infusion. The animals were then returned to their cages and were free to move about during the experiments. Patency of the arterial catheter was maintained by a slow infusion (0.03 mL/min) of heparinized saline (50 U/mL).

Following at least 2 hours of stabilization, blood samples (0.6 mL) were taken to measure the basal plasma glucose and insulin concentrations. After basal sampling, dimethyl-BAPTA/AM or vehicle was injected through a tail vein catheter to the assigned rats 90 minutes prior to the start of insulin infusion (-90 minutes). In some of the rats injected with dimethyl-BAPTA/AM, a blood sample was taken again at time -1 minute to check if the chelator changes basal plasma glucose and/or insulin concentrations. Human insulin was infused at 6 mU $\cdot$ $kg^{-1} \cdot min^{-1}$ from time 0 to 120 minutes. Plasma glucose concentration was measured every 5 minutes for the first 30 minutes and then every 10 minutes for the rest of insulin infusion period. Plasma glucose level was clamped at 100 mg/dL by a variable glucose infusion according to the PACBERG program.[17] The average glucose infusion rate (GIR) during the final 30 minutes of the glucose clamp (steady state) was used as an estimate of the whole body insulin sensitivity. Blood samples for the measurement of the steady state plasma insulin concentration were taken at time 90, 100, 110, and 120 minutes.

Dose-dependent effects of the chelator on insulin resistance were evaluated with a decreased and increased dose of dimethyl-BAPTA/AM. Thus, in addition to 6 mg/kg of dimethyl-BAPTA/AM mentioned previously, 3 mg/kg (n = 7) or 9 mg/kg (n = 8) of the chelator was injected at time –90 minutes in the HFD rats.

Plasma glucose concentrations were measured by the glucose oxidase method (Beckman Glucose Analyzer II; Beckman Instruments, Fullerton, CA). Basal and steady state plasma insulin concentrations were measured with the radioimmunoassay kit using rat (Linco Research, St Charles, MO) and human insulin (Coat-A-Count Insulin; DPC, Los Angeles, CA) standard, respectively.

Femoral Artery Blood Flow

To examine if an injection of dimethyl-BAPTA/AM influences blood flow to the peripheral insulin target tissues, the effect of the chelator on femoral artery blood flow was assessed using VF-1 Pulsed Doppler Flow System (Crystal Biotech, Hopkinton, MA) in combination with the data acquisition software, Dataflow (Crystal Biotech, Hopkinton, MA). The rats were anesthetized with the constant infusion (0.08 mg/min) of pentobarbital sodium (Entobar; Hanlim Pharmaceutical, Seoul, Korea) through a catheter in the contralateral femoral vein. The high frequency (20 MHz) pulsed Doppler flow probe was positioned on the femoral artery. Body temperature was maintained at

Table 2. Effects of Dimethyl-BAPTA/AM on Insulin Sensitivity in HFD Rats and Control Rats

	No.	Fasting Glucose (mg/dL)	Fasting Insulin (μU/mL)	Steady-State Insulin (μU/mL)	Steady-State GIR (mg $\cdot$ $kg^{-1} \cdot min^{-1}$)
HFD rats					
Dimethyl-BAPTA/AM at −90 minutes	8	92 ± 2	25 ± 3	197 ± 16	16.3 ± 0.9*†
Vehicle at −90minutes	9	90 ± 2	19 ± 4	184 ± 13	12.2 ± 0.7*
Dimethyl-BAPTA/AM at −20 hours	8	92 ± 2	24 ± 5	165 ± 30	16.2 ± 0.5*†
Control rats					
Dimethyl-BAPTA/AM at −90 minutes	8	92 ± 2	17 ± 2	213 ± 23	20.7 ± 0.4
Vehicle at −90 minutes	7	94 ± 4	12 ± 3	170 ± 8	20.2 ± 0.9
Dimethyl-BAPTA/AM at −20 hours	9	88 ± 2	21 ± 5	153 ± 23	19.2 ± 0.7

NOTE. Human insulin was infused at 6 mU $\cdot$ $kg^{-1} \cdot min^{-1}$. Dimethyl-BAPTA/AM (6 mg/kg in 250 μL of DMSO 1:3 diluted with distilled water) or the same volume of the vehicle was injected through an intravenous catheter 90 minutes prior to the initiation of glucose clamp experiment. In some of the rats, dimethyl-BAPTA/AM was injected 20 hours before the glucose clamp.

*$P < .01$ *v* the vehicle-injected control rats.

†$P < .01$ *v* the vehicle-injected HFD rats.

37°C ± 0.2°C using a heating lamp throughout the anesthesia. The blood flow rate through the femoral artery was continuously recorded, and the mean flow rate was calculated every 30 seconds. After a 2-hour stabilization period, the basal flow rate was recorded for an hour from -150 to -90 minutes. At time -90 minutes, either dimethyl-BAPTA/AM (6 mg/kg) or vehicle was injected through a femoral vein catheter. Blood flow was recorded for 210 minutes thereafter to mimic the glucose clamp experiments used in the present study (90 minutes of equilibration period after the injection plus 120 minutes of clamp period).

2-DG Uptake in Adipocytes

The control rats (n = 9) and the HFD rats with (n = 11) or without (n = 11) an injection of dimethyl-BAPTA/AM (6 mg/kg, IP) on the preceding night (16 to 20 hours before the isolation of adipocytes) were used for the study. Epididymal adipocytes were isolated by the collagenase digestion method of Rodbell[18] as previously described.[19] Isolated cells were washed 3 times and resuspended at a dilution of 10^7 cells/mL in Krebs-Ringer/HEPES medium containing 130 mmol/L NaCl, 5 mmol/L KCl, 1.3 mmol/L $MgSO_4$, 1.3 mmol/L $CaCl_2$, 2.5 mmol/L NaH_2PO_4, 10 mmol/L HEPES, 0.5 mmol/L glucose, and 10 mg/mL bovine serum albumin, pH 7.4.

Aliquots (100 μL) of the cell suspension were pipetted into polystyrene 75 × 12 mm tubes and incubated without shaking at 37°C for an hour. Insulin (100 nmol/L) was added for 30 minutes prior to the measurement of 2-DG uptake. Uptake measurements were initiated by the addition of [^{3}H]2-DG to a final concentration of 0.1 mmol/L (100 μCi/mmol) and terminated after 3 minutes by the addition of 400 μL of ice cold medium containing 0.2 mmol/L phloretin. Aliquots (200 μL) of the cell suspension were pipetted into 400-μL microcentrifuge tubes containing 500 μL of silicone oil and centrifuged for 30 seconds at 15,000 × *g*. The tubes were cut through the oil layer, and the radioactivity associated with the cells was measured by scintillation counting. Noncarrier-mediated transport was also assessed in parallel incubations containing 20 mmol/L cytochalasin B, and it was subtracted from each determination. Each measurement was made in quadruplicates.

Measurement of $[Ca^{2+}]_i$

Basal $[Ca^{2+}]_i$ was measured in the isolated single adipocytes using digital fluorescent imaging microscopy system (Merlin System; Life Sciences Resources, Cambridge, UK). Epididymal adipocytes were isolated from the control rats (n =7) and the HFD rats with (n = 6) or without (n = 7) dimethyl-BAPTA/AM injection (6 mg/kg, IP) on the preceding night. The cells attached to the perfusion chamber were loaded with fura-2/AM (5 μmol/L) for 15 minutes and subsequently washed by perfusing the chamber for 10 minutes with Krebs-Ringer/HEPES buffer at 37°C. A total of 4 or 5 cells from each animal was randomly selected for the measurement. The adipocytes loaded with fura-2 were alternatively illuminated at 340 and 380 nm. The ratio image (340/380) of fura-2 fluorescence emitted at 510 nm was sampled every 1 second. The ratio was calibrated with calcium standard (Molecular Probes), and $[Ca^{2+}]_i$ was calculated using the equation from Grynkiewicz et al.[20]

Statistical Analysis

Data are presented as mean ± SE. To test for differences between the cases and controls, we used the Student's paired or unpaired *t* test. Multiple comparisons were made by analysis of variance (ANOVA), followed by the Bonferroni test for individual differences. *P* values less than .05 were considered statistically different.

RESULTS

Effects of Dimethyl-BAPTA/AM on the Whole-Body Insulin Sensitivity

The whole body insulin sensitivity was assessed in the HFD rats (n = 25) and the control rats (n = 24) by euglycemic hyperinsulinemic (6 mU · kg^{-1} · min^{-1}) clamps with and without pretreatment of dimethyl-BAPTA/AM (6 mg/kg) (Table 2). Body weights of the HFD rats and the control rats were not significantly different (384 ± 12 *v* 356 ± 12 g; $P > .09$). The basal plasma concentrations of glucose ($P > .5$) and insulin ($P > .4$) were comparable in all 6 groups: they were not significantly influenced either by the HFD for 3 to 4 weeks or by the injection of dimethyl-BAPTA/AM at –20 hours (Table 2). In addition, the basal plasma glucose (92 ± 2 *v* 92 ± 2 mg/dL; n = 12; $P > .6$) and insulin concentrations (17 ± 3 *v* 18 ± 4 μU/mL; n = 9; $P > .8$) measured before and 90 minutes after the injection of dimethyl-BAPTA/AM were almost identical.

Infusion of insulin at 6 mU · kg^{-1} · min^{-1} increased plasma insulin levels to 184 ± 13 μU/mL and 170 ± 8 μU/mL at the steady state in the HFD rats and the control rats, respectively ($P > .9$; Table 2). An injection of dimethyl-BAPTA/AM either at –90 minutes or at –20 hours did not significantly affect the steady state insulin concentrations in either diet group ($P > .9$). During insulin infusion, plasma glucose was successfully clamped at 100 mg/dL with variable rates of glucose infusion: coefficients of variation of glucose measurements during the clamps were less than 5% in all the experiments.

Despite similar plasma insulin concentrations at the steady state, GIR to maintain euglycemia was markedly reduced in the HFD rats compared with the control rats (12.2 ± 0.7 *v* 20.2 ± 0.9 mg · kg^{-1} · min^{-1}; $P < .01$; Table 2), demonstrating 40% reduction of the whole body insulin sensitivity. In the HFD rats, an intravenous injection of dimethyl-BAPTA/AM at –90 minutes, significantly increased GIR to 16.3 ± 0.9 mg · kg^{-1} · min^{-1} ($P < .01$). The chelator injected at –20 hours also increased GIR in a similar magnitude. Thus, approximately 50% of reduction in insulin-mediated glucose uptake induced by high-fat feeding was restored by a single injection of the intracellular Ca^{2+} chelator, and this effect lasted at least for about 20 hours. In contrast, dimethyl-BAPTA/AM did not affect GIR in the control rats ($P > .9$).

The effect of 4 different doses (0, 3, 6, 9 mg/kg) of dimethyl-BAPTA/AM injected at -90 minutes on the whole body insulin sensitivity is shown in Fig 1. The steady state GIR was not affected by dimethyl-BAPTA/AM at a dose of 3 mg/kg (12.1 ± 0.6 *v* 12.2 ± 0.7 mg · kg^{-1} · min^{-1}), but was significantly increased at a dose of 6 mg/kg (16.3 ± 0.9 mg · kg^{-1} · min^{-1}; $P < .01$). Increasing the dose to 9 mg/kg did not raise GIR any further (16.2 ± 0.4 mg · kg^{-1} · min^{-1}).

Effects of Dimethyl-BAPTA/AM on Femoral Arterial Blood Flow

To check if dimethyl-BAPTA/AM increases blood flow rate to peripheral insulin-target tissues, we measured blood flow rates of femoral artery before and after the injection of the chelator in the anesthetized rats. As shown in Fig 2, the blood flow rate at each time point is expressed as the ratio to the mean

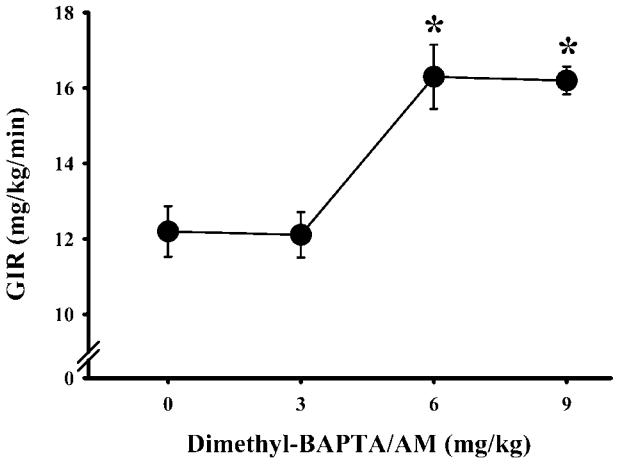

Fig 1. Dose-response curve of dimethyl-BAPTA/AM on the whole body insulin sensitivity in the HFD rats. The whole body insulin sensitivity is represented by the steady-state GIR during euglycemic hyperinsulinemic (6 mU · kg^{-1} · min^{-1}) clamps. Dimethyl-BAPTA/AM (3, 6, or 9 mg/kg in 250 μL DMSO 1:3 diluted with distilled water) or the same volume of the vehicle was injected 90 minutes prior to the initiation of insulin infusion. *$P < .01$ *v* the vehicle-injected rats.

flow rate during the basal period prior to the injection. Injection of dimethyl-BAPTA/AM (6 mg/kg) through a catheter in the contralateral femoral vein resulted in an abrupt and transient peak in the femoral arterial blood flow. After the peak, blood flow was returned to a level close to the basal rate and remained relatively constant during the whole observation period (Fig 2). The response of blood flow to vehicle was indistinguishable from that of the chelator. No difference between the control rats and the HFD rats were observed either.

Because insulin infusion was initiated 90 minutes after the injection of dimethyl-BAPTA/AM or vehicle, and lasted for 120 minutes thereafter, the blood flow data was analyzed according to these 2 periods (Table 3). Both dimethyl-BAPTA/AM and vehicle increased femoral arterial blood flow by 10% to 20% during the first 90 minutes of equilibration period. However, during the clamp period, the blood flow returned to the basal level: the mean values of the ratio of blood flow to the basal rate were 0.87 ± 0.06 and 0.97 ± 0.08 in dimethyl-BAPTA/AM- or vehicle-injected control rats, and 0.97 ± 0.11, and 0.91 ± 0.07 in the chelator- or vehicle-injected HFD rats, respectively. Thus, there was no significant statistical difference in blood flow response either between the chelator- and vehicle-injected rats or between the HFD rats and control rats ($P > .9$).

Table 3. Effects of Dimethyl-BAPTA/AM on the Femoral Arterial Blood Flow

	Equilibration Period (−90 to 0 min)	Clamp Period (0 to 120 min)
HFD rats		
Dimethyl-BAPTA/AM	1.16 ± 0.08	0.97 ± 0.11
Vehicle	1.11 ± 0.07	0.91 ± 0.07
Control rats		
Dimethyl-BAPTA/AM	1.22 ± 0.10	0.87 ± 0.06
Vehicle	1.17 ± 0.06	0.97 ± 0.08

NOTE. Either dimethyl-BAPTA/AM (6 mg/kg in 250 μL of DMSO 1:3 diluted with distilled water) or the same volume of the vehicle was injected intravenously at −90 minutes in the anesthetized rats. The blood flow rate is expressed as the ratio to the average flow rate during the basal period (−150 to −90 minutes) in the experiments described in Fig 2. Values represent the mean ± SE of 5 experiments.

Insulin-Stimulated 2-DG Uptake by Adipocytes

Stimulatory effects of insulin on glucose transport were determined in the adipocytes isolated from the control rats (n = 8) and the HFD rats with (n = 11) or without (n = 11) an injection of dimethyl-BAPTA/AM (Fig 3). Insulin (100

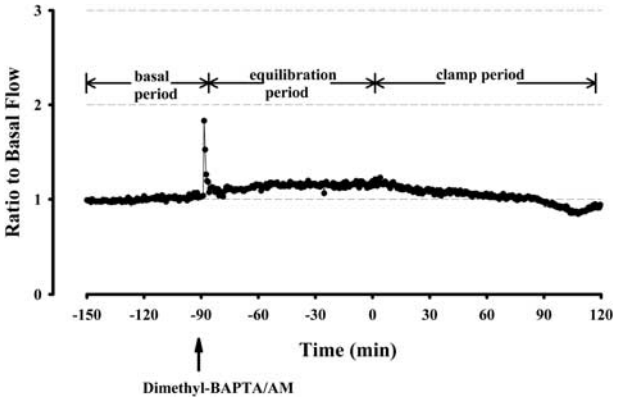

Fig 2. A typical pattern of changes in the femoral artery blood flow rate in response to dimethyl-BAPTA/AM. The blood flow rate through a femoral artery was measured using VF-1 Pulsed Doppler Flow System in the anesthetized rats. The blood flow rate is expressed as the ratio to the average flow rate during the basal period (-150 to -90 minutes). Dimethyl-BAPTA/AM (6 mg/kg) was injected at –90 minutes through a catheter in the contralateral femoral vein.

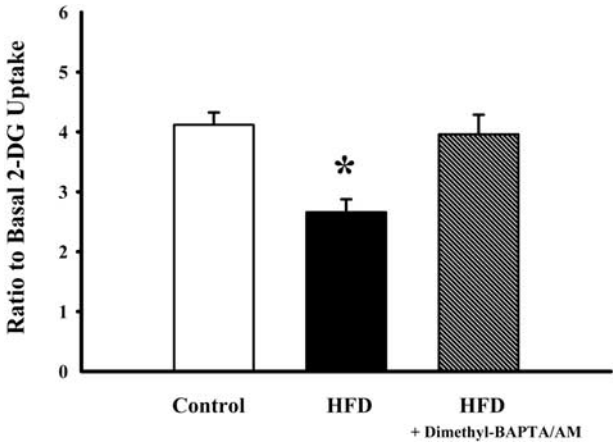

Fig 3. Effect of dimethyl-BAPTA/AM on insulin-stimulated 2-DG uptake in the isolated epididymal adipocytes. The control rats (n = 8), the HFD rats (n = 11), and the HFD rats injected with dimethyl-BAPTA/AM (6 mg/kg) at 6:00 PM on the preceding day of cell isolation were used. Effect of insulin (100 nmol/L) is expressed as the ratio of insulin-stimulated 2-DG uptake rate to a basal value. *$P < .05$ *v* the control rats.

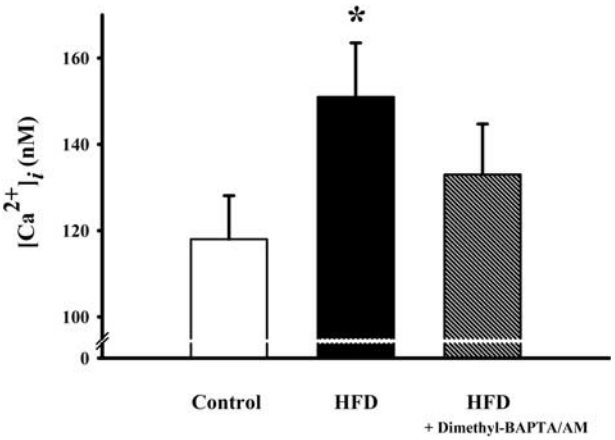

Fig 4. Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in isolated adipocytes. $[Ca^{2+}]_i$ in single adipocytes was measured using a digital fluorescence microscope. Epididymal adipocytes were isolated from the control rats (n = 7), the HFD rats (n = 7), and the HFD rats injected with dimethyl-BAPTA/AM (6 mg/kg) the night before the experiments (n = 6). A total of 4 or 5 cells from each animal was randomly selected for the measurement. *$P < .05$ *v* the control rats.

nmol/L) increased 2-DG uptake by 4.12 ± 0.21-fold of the basal level in the control rats, but only by 2.66 ± 0.22-fold in the HFD rats ($P < .01$). Pretreatment of the HFD rats with dimethyl-BAPTA/AM increased insulin-stimulated 2-DG uptake to 3.96 ± 0.33-fold of the basal level, which was almost identical to that in the control rats ($P > .9$).

$[Ca^{2+}]_i$ in Adipocytes

Basal $[Ca^{2+}]_i$ of epididymal adipocytes was 112 ± 9 nmol/L (n = 29) in the control rats, and it was significantly elevated in the HFD rats to 145 ± 11 nmol/L (n = 33; $P < 0.05$) (Fig 4). An injection of dimethyl-BAPTA/AM to the HFD rats the night before the cell isolation, reduced $[Ca^{2+}]_i$ to 127 ± 11 nmol/L (n = 23), which was not significantly different from the value in the control rats (112 ± 9 nmol/L; $P > .2$).

DISCUSSION

It has been postulated that high $[Ca^{2+}]_i$ in the insulin target cells may diminish cellular responsiveness to insulin and thus contribute to the development of insulin resistance.[2,6,21] The present study with the use of dimethyl-BAPTA/AM to lower $[Ca^{2+}]_i$ provides direct evidence supporting this hypothesis. Basal levels of adipocyte $[Ca^{2+}]_i$ were significantly higher in the HFD rats than in the control rats (Fig 4). Pretreatment of the HFD rats with dimethyl-BAPTA/AM, an intracellular Ca^{2+} chelator, lowered $[Ca^{2+}]_i$ to the levels indistinguishable from those in the control rats (Fig 4). The same maneuver not only reversed impairments in insulin action stimulating glucose transport in isolated adipocytes (Fig 3), but also ameliorated whole body insulin resistance in the HFD rats (Table 2).

HFD for 3 to 4 weeks in rats induced profound whole body insulin resistance, which was manifested in the 40% reduction in the steady state GIR during euglycemic hyperinsulinemic (6 $mU \cdot kg^{-1} \cdot min^{-1}$) clamps (Table 2). A single injection of dimethyl-BAPTA/AM (6 mg/kg) in the HFD rats prior to clamps, restored the reduction in GIR by about 50% (Table 2). Although we did not measure glucose turnover rate during glucose clamps, the steady state GIR in this study estimated principally the rate of glucose uptake by peripheral tissues. This estimation is based on the observation by Fryer et al[22] that after an overnight fast in high-fat fed rats as well as control rats, hepatic glucose production is completely suppressed during euglycemic hyperinsulinemic clamps with an insulin infusion rate lower than that used in the present study. In addition, the preliminary studies in our laboratory showed that hepatic glucose output was totally suppressed during the clamps in the HFD rats and was not affected by the injection of dimethyl-BAPTA/AM (data not shown). Accordingly, our results of GIR measured under the present experimental conditions with an intracellular Ca^{2+} chelator in the HFD rats are likely to reflect an increased insulin-stimulated glucose uptake by the peripheral insulin target tissues.

An intracellular Ca^{2+} chelator can increase peripheral glucose uptake during hyperinsulinemic glucose clamps via (1) improving cellular responsiveness to insulin by lowering abnormally elevated $[Ca^{2+}]_i$ in the insulin target tissues and (2) enhancing blood flow to those tissues by dilating the peripheral blood vessels by lowering $[Ca^{2+}]_i$ in the vascular smooth muscles. In the HFD rats, dimethyl-BAPTA/AM at the dose of 6 mg/kg increased GIR significantly (Fig 1 and Table 2) without changes in femoral arterial blood flow (Fig 2 and Table 3). Even at a higher dose (9 mg/kg), GIR was not increased any further (Fig 1), and femoral arterial blood flow was increased only by 18% ± 3% (data not shown). Hence, there was a clear dissociation between the effects of the dimethyl-BAPTA/AM on GIR and peripheral blood flow. This dissociation, as well as the marked effect of the chelator on 2-DG uptake in isolated adipocytes (Fig 3), where the blood flow factor was excluded, support the possibility that the enhancing effect of the chelator on GIR in vivo is likely through its direct effect on the cellular response to insulin.

To prove that a sustained elevation of $[Ca^{2+}]_i$ contributes to the development of insulin resistance, it is desirable to test the causal relationship between basal $[Ca^{2+}]_i$ and insulin action in the main insulin target tissues, principally skeletal muscles.[23] It is, however, technically problematic to measure basal levels of cytosolic Ca^{2+} concentrations in the skeletal muscle cells isolated from adult rats due to the contractility and the size of cells. As an alternative, we utilized adipocytes, which is another insulin target cell that has traditionally been used for in vitro studies of insulin action.

The present study represents the first report demonstrating that a HFD increases basal levels of $[Ca^{2+}]_i$ in insulin target cells (Fig 4). $[Ca^{2+}]_i$, measured in the fura-2-loaded adipocytes with a digital fluorescence microscope, was about 30% higher in the HFD rats than in the control rats (145 ± 11 *v* 112 ± 9 nmol/L; $P < .05$; Fig 4). The mechanism by which a HFD causes an elevation of $[Ca^{2+}]_i$ is unknown. One potential underlying mechanism is that elevated free fatty acids (FFA) may increase $[Ca^{2+}]_i$. It has been demonstrated in several types of cells that FFA increases $[Ca^{2+}]_i$ by mobilizing Ca^{2+} from intracellular stores[24,25] or by stimulating Ca^{2+} influx to the cells.[26] FFA has also been reported to inhibit Ca^{2+} uptake and enhance Ca^{2+} efflux in sarcoplasmic-reticulum vesicles.[27] Al-

ternatively, it is possible that changes in membrane fatty acid composition, induced by a HFD,[28,29] may alter Ca^{2+} fluxes. It is well recognized that the fatty acid composition of membrane lipids can influence a range of cellular functions, including ion channel activities.[29-32]

Adipocytes isolated from the HFD rats exhibited a 35% reduction in insulin-stimulated 2-DG uptake ($P < .01$; Fig 3) with elevated $[Ca^{2+}]_i$ ($P < .05$; Fig 4) compared with those from the control rats. An injection of dimethyl-BAPTA/AM in the HFD rats completely recovered the insulin-stimulated 2-DG uptake rate to the level in the control rats and reduced the elevated $[Ca^{2+}]_i$ to the level indistinguishable from those in control rats (Figs 3 and 4). These results support the possibility that sustained elevations of $[Ca^{2+}]_i$ in the insulin target cells may be responsible, in part, for insulin resistance, and dimethyl-BAPTA/AM restore it by lowering elevated $[Ca^{2+}]_i$ in the insulin target cells.

The mechanism whereby the high levels of $[Ca^{2+}]_i$ interfere with insulin action on glucose metabolism remains largely unknown. Insulin-receptor binding is not affected, and receptor tyrosine kinase activity is only minimally affected.[33] Recruitment of glucose transporter, GLUT4, is neither influenced.[33] Begum et al[7] have demonstrated in isolated rat adipocytes that an elevation in $[Ca^{2+}]_i$ induces inhibition of phosphoserine phosphatase activity through an activation of inhibitor 1. Accordingly, sustained elevations in $[Ca^{2+}]_i$ may result in insulin resistance by preventing dephosphorylation of insulin-sensitive target protein(s) within the cell. Reduction in insulin-induced dephosphorylation of glycogen[7,34] and GLUT4[35] supports this possibility. Further studies are being investigated to identify the target proteins involved in the action of insulin, of which phosphorylation/dephosphorylation is regulated by $[Ca^{2+}]_i$.

In the HFD rats, pretreatment of dimethyl-BAPTA/AM restored impairments in insulin-mediated glucose uptake by only about 50% (Table 2), while it almost normalized insulin-stimulated glucose transport in adipocytes (Fig 3). At present, we do not have a clear explanation for this differential effect of dimethyl-BAPTA/AM on insulin sensitivity in vivo and in vitro. It can be speculated that some circulating factor(s) in HFD rats may impair trans-membrane transport process of glucose in vivo, and this effect is independent of the level of $[Ca^{2+}]_i$. It is also possible that the underlying mechanism of insulin resistance in skeletal muscle cells and adipocytes may be different: resistance in skeletal muscles is caused by both $[Ca^{2+}]_i$-dependent and -independent pathways, but that in adipocytes is entirely by $[Ca^{2+}]_i$-dependent pathways.

One can raise the possibility that dimethyl-BAPTA/AM given in vivo is hydrolyzed by serum esterases into dimethyl-BAPTA, formaldehyde, and acetic acid, and thus these latter 2 compounds, rather than dimethyl-BAPTA in the cell, cause an improvement in GIR. However, this is an unlikely explanation. We observed in spontaneously hypertensive rats[36] that an injection of 6 mg/kg dimethyl-BAPTA/AM increased GIR during hyperinsulinemic clamps, whereas the same dose of other acetoxymethyl derivatives did not affect the GIR. This observation indicates that the improvement of GIR in the present study (Table 2) is not caused by the products of serum esterases, but by a specific effect of dimethyl-BAPTA, which enters the cells.

Dimethyl-BAPTA/AM is readily diffused into cells and then hydrolyzed by esterase to yield dimethyl-BAPTA, which is expected to be trapped inside the cell due to its multiple negative charges.[14] Thus, the intracellular Ca^{2+} buffering effect of the chelator is possibly quite prolonged, as our data showed that the improving effect of dimethyl-BAPTA/AM on insulin sensitivity lasted at least 20 hours after a single injection of the drug (Table 2).

Considering that Ca^{2+} is one of the most important mediators in the cellular signal transduction,[37] there is a possibility that buffering $[Ca^{2+}]_i$ may disrupt normal responses of the cells to a variety of physiologic stimuli. However, it has been shown that another Ca^{2+} chelator BAPTA/AM effectively lowers sustained increase in $[Ca^{2+}]_i$ near to a physiologic level without affecting transient increase in $[Ca^{2+}]_i$ responding to physiologic levels of stimuli.[38] The observation that dimethyl-BAPTA/AM does not affect the GIR in the control rats (Table 2) also dictates that the chelator may not disturb the normal signal transduction, including that mediated by Ca^{2+}.

In conclusion, the current results clearly demonstrated that in the HFD rats, $[Ca^{2+}]_i$ in adipocytes were significantly elevated, and that pretreatment of the intracellular Ca^{2+} chelator dimethyl-BAPTA/AM improved insulin sensitivity, as well as reduced $[Ca^{2+}]_i$ to the level indistinguishable from that in the control rats. The results thus support that the sustained high levels of $[Ca^{2+}]_i$ may play an important role in the development of insulin resistance.

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