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4-Bromocrotonic Acid Enhances Basal but Inhibits Insulin-Stimulated Glucose Transport in 3T3-L1 Adipocytes

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Inhibitors of fatty acid oxidation, 2-bromopalmitic acid (Br-C16) and 4-bromocrotonic acid (Br-C4) were examined for their effect on glucose transport in 3T3-L1 adipocytes. Whereas Br-C16 was without effect, Br-C4 augmented basal but inhibited insulin-stimulated 2-deoxyglucose uptake in a dose- and time-dependent manner. Immunoblot analysis indicated that following Br-C4 pretreatment, the content of GLUT1 in plasma membranes was increased whereas insulin-induced translocation of GLUT4 was greatly eliminated. The total cellular amount of GLUT1 or GLUT4, on the other hand, was not altered. Thus these results seem to suggest that Br-C4 has opposite effect on basal and insulin-stimulated glucose transport by a mechanism other than its inhibition of fatty acid oxidation. The translocation processes for both GLUT1 and GLUT4 transporters appears to be altered.

Glucose and fatty acids are two major substrates for energy production in animals. Coordination between their metabolism in providing energy is sophisticated and is regulated by many hormonal and metabolic factors. Disturbance of the energy homeostasis may cause serious clinical syndromes that are manifested by abnormal blood glucose or fatty acid levels. For instance, ingestion of hypoglycin, an inhibitor of acyl-CoA dehydrogenase and fatty acid oxidation, may cause hypoglycemia (1), whereas insufficient release of insulin from pancreatic β-cells as in diabetes mellitus may lead to hyperglycemia. Among the many hormonal factors involved in glucose homeostasis, insulin is considered the most important one for glucose disposal from the circulating blood. Glucose uptake by muscle and adipose tissues is greatly augmented when the serum insulin level is increased. Although adipose tissue accounts for only 5-20% of glucose disposal, much of the work on insulin-stimulated glucose transport has been performed in adipocytes, due to the fact that many mechanistic studies with regard to insulin's action have been easier to carry out in this tissue (2).

About thirty years ago, a glucose fatty-acid cycle model was proposed by Randle, et al.(3) to elucidate the competing nature of glucose and fatty acids for meeting the energy demand of the cells. Indeed, many cofactors such as CoASH and NAD^+ are shared by the oxidative process of glucose and fatty acid in mitochondria. Slowdown of either process may lead to accelerated process of the other one. Animal studies have shown that administration of nicotinic acid/phenylisopropyladenosine or etomoxir, inhibitors of lipolysis and fatty acid oxidation respectively, led to lowered blood glucose in diabetic rats(4,5). In vitro studies with isolated myocytes also indicated that increased rate of glucose oxidation and glucose uptake was induced by suppression of fatty acid oxidation (6,7). The underlying mechanism, however, remains unclear.

In the present study, we have attempted to further delineate the correlation between fatty acid oxidation and glucose uptake in differentiated 3T3-L1 adipocytes, a well established in vitro cell model for adipocytes (8,9). We treated cells with 2-bromopalmitic acid (Br-C16) and 4-bromocrotonic acid (Br-C4), specific inhibitors acting at carnitine palmitoyltransferase I (10) and thiolase (11), respectively, and compared their effects on fatty acid oxidation with those on glucose transport. Although both agents inhibited the rate of palmitate oxidation (12), only Br-C4 was found to augment basal while to inhibit insulin-stimulated 2-deoxyglucose uptake by the adipocytes. The mechanism of the action of Br-C4 on glucose uptake has been investigated.

MATERIALS AND METHODS

Materials. Dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), 2-deoxy-D-glucose (2-deoxyglucose), anisomycin, cycloheximide, pu-

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romycin, phenylmethylsulfonyl fluoride, cytochalasin B, bovine serum albumin (BSA, fatty acid-free) and insulin were obtained from Sigma Chemicals, St. Louis. 2-Bromopalmitic acid was purchased from Fluka, Switzerland. 4-Bromocrotonic acid was a generous gift from Dr. Horst Schulz, CUNY, NY.

Cell culture. 3T3-L1 cells, obtained from American Type Culture Collection, were grown in 6-well plates (Nunc) or 12-well plates (Costar) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO), glutamine, penicillin and streptomycin as described (13-15). The cultures were kept at 37°C in a humidified atmosphere of 10% CO_2, 90% air, and the medium was changed every 2 days. 3T3-L1 preadipocytes were differentiated to adipocytes according to the procedure as described by Carnicero (13) and Rubin, et al.(8) with modification. Briefly, 2 days after confluence (day 0), the medium was removed and fresh medium containing 0.5 mM IBMX and 0.25 mM dexamethasone was added. After another 3 days, the medium was replaced with fresh culture medium and the cultures were then maintained as described above.

Subcellular fractionation. Subcellular membranes were prepared as described by Simpson et al. (16) with modification (13). Briefly, after cells grown on 10-cm culture dishes were treated with various testing reagents, the cells were washed once with 10 ml of Buffer A (250 mM sucrose, 20 mM Hepes, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH7.4). Monolayers from two dishes were scraped into 24 ml of ice-cold Buffer A and were immediately homogenized with 15 strokes of a motor-driven Teflon pestle in a 40-ml homogenizing vessel. A 3-ml aliquot of the homogenate was subjected to centrifugation at 250,000 × g for 1.5 h to collect total cellular membranes which were resuspended to about 5 mg of protein/ml in buffer B (20 mM Hepes, 1 mM EDTA, pH7.4). The remainder of the homogenate was subjected to centrifugation at 16,000 × g for 20 min. The pellet was resuspended in 6 ml of Buffer B with a Dounce homogenizer, applied to a sucrose cusion (1.12 M sucrose in Buffer B), and subjected to centrifugation at 100,000 × g for 1h. Plasma membranes removed from the top of the cushion were resuspended in Buffer B, and centrifuged at 30,000 × g for 30 min. Plasma membranes collected from the pellet were resuspended in Buffer B to about 5 mg of protein/ml. The supernatant from the 16,000 × g centrifugation was subjected to centrifugation at 30,000 × g for 30 min. The resulting supernatant was then subjected to centrifugation at 250,000 × g for 1.5 h to collect the low density microsomes which were resuspended in Buffer B to about 5 mg of protein/ml. All final membrane suspensions include 1 mM phenylmethylsulfonyl fluoride.

Immunoblot analysis. For each experiment, membrane samples were subjected to SDS-polyacrylamide (10%) gel electrophoresis under reducing conditions as described by Laemmli (17), and transferred to a nitrocellulose membrane. The GLUT1 and GLUT4 transporter proteins were immunodetected by using polyclonal rabbit anti-GLUT1 (1:1000; East Acres Biologicals) and monoclonal mouse anti-GLUT4 (IF8, 1.3 μg/ml; Genzyme) antibodies respectively, and the blots were developed by the enhanced chemiluminescence method (ECL, Amersham) employing horseradish peroxidase-conjugated donkey anti-rabbit IgG (1:5000) for GLUT1 and sheep anti-mouse IgG (1:9000) for GLUT4, respectively. Quantitation of relative band intensity was performed by laser scanning densitometry.

Measurement of glucose transport activity. Glucose transport activity was analyzed by measuring uptake of 2-deoxyglucose into the cells as described previously (18,19). Briefly, after the cells were treated with the tested reagents for appropriate times, [^{3}H]2-deoxyglucose(final conc. 0.2 mM, 7.5 mCi/umol) was added and incubation was continued for another 5 min. The reaction was terminated by the addition of cold (0°C) phosphate-buffered saline also containing 20 mM D-glucose. Cells were washed three times with the same buffer, lyzed with 2% SDS and counted for radioactivity.

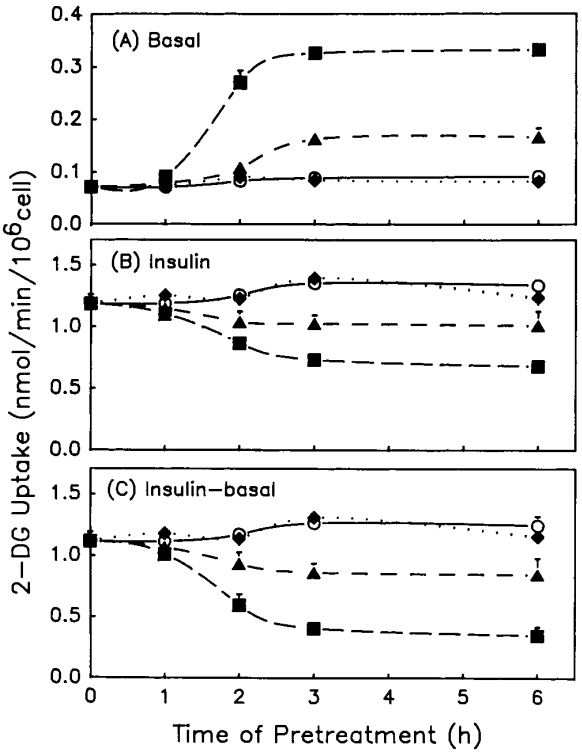

FIG. 1. Time-dependent effect of Br-C4 pretreatment on basal and insulin-stimulated 2-deoxyglucose (2-DG) uptake. 3T3-L1 adipocytes were pretreated with vehicle (○-○), 100 μM Br-C16 (●-●), 25 μM Br-C4 (▲-▲) or 50 μM Br-C4 (■-■) for various times and measured for basal and 10^{-7} M insulin-stimulated 2-DG uptake. Values shown are means ± S.E. (n=3).

Assay for lactate. Lactate concentration was determined spectrophotometrically using an NAD-lactate dehydrogenase coupled assay (20). Statistical differences were determined by Student's t-test.

RESULTS AND DISCUSSION

Both Br-C4 and Br-C16 inhibited the rate of palmitate oxidation with similar time courses, reaching a plateau after 3 h (12). When the effects of these inhibitors on glucose transport were examined, however, only pretreatment of cells with Br-C4 was found to augment basal while inhibit insulin-stimulated 2-deoxyglucose uptake in both a dose- and a time-dependent manner (Fig. 1). The time courses of inhibiting palmitate oxidation and influencing glucose uptake by Br-C4, nevertheless, do not correlate. Although both effects seem to reach a maximum after 3-h pretreatment, 50 μM Br-C4 inhibited palmitate oxidation by 40% at 1 h (12), albeit had no significant effect on glucose uptake at this time point (Fig.1). The reason for a lag phase in Br-C4-dependent effect on glucose transport is unknown, but it does not seem to involve *de novo* protein synthesis, since the effect of Br-C4 on glucose transport was not affected by co-incubation in the presence of cycloheximide, anisomycin or puromycin (data not

TABLE 1

Effect of Br-C4 and Br-C16 on Lactate Release by 3T3-L1 Adipocytes

Time of incubation (h)	Lactate release (nmol/10^6 cell)		
	Control	Br-C4	Br-C16
1	133 ± 13	134 ± 10	147 ± 8
2	198 ± 22	146 ± 15	188 ± 19
3	242 ± 31	219 ± 28	244 ± 35
6	347 ± 51	425 ± 47	481 ± 90

3T3-L1 adipocytes were incubated in the absence(control) or presence of 50 μM Br-C4 or 100 μM Br-C16 for various times and measured for lactate release in the incubation medium. Values are means ± S.E. (n = 4)

shown). Thus it seems that the effect of Br-C4 on glucose transport is not attributed to its inhibitory action on fatty acid oxidation. This conclusion is substantiated by the observation that inhibition of fatty acid oxidation by Br-C16 had no effect on glucose transport. The finding that lactate release from 3T3-L1 adipocytes were not altered by incubation in the presence of Br-C4 or Br-C16 for up to 6 h (Table 1) indicates that inhibition of fatty acid oxidation by either Br-C4 or Br-16 probably does not have any significant effect on the oxidative status of mitochondria. This is consistent with the notion that fatty acid oxidation plays only a minor role in energy production in adipocytes.

In a separate report, we have demonstrated the inhibition of lipolysis by Br-C4 in 3T3-L1 adipocytes (12). The inhibitory effect of Br-C4 may be attenuated by preloading cells with long chain fatty acids. The longer chain length was loaded, the greater attenuation was observed. We hypothesized that this attenuating effect of long chain fatty acids was due to their competition with Br-C4 for intramitochondrial conversion by enzymes involved in β-oxidation. Using the same approach, we tested if the effect of Br-C4 on 2-deoxyglucose uptake was influenced by prior treatment of cells with long chain fatty acids. As shown in Table 2, instead of attenuation, pretreatment with palmitate may actually potentiate the inhibition caused by Br-C4 on insulin-stimulated glucose transport. The implication of this observation is not clear, but may suggest that β-oxidation of Br-C4 is not a prerequisite for its action on glucose uptake in response to insulin. In agreement with this, the effect of Br-C4 on glucose transport was not altered by L-carnitine (data not shown).

There are two isoforms of glucose transporter, namely, GLUT1 and GLUT4, found in 3T3-L1 adipocytes (21,22). GLUT1 and GLUT4 are believed to be the responsible for basal and insulin-stimulated glucose transport, respectively (23). Our findings that Br-C4 enhances basal but inhibits insulin-stimulated 2-deoxyglucose uptake thus imply the involvement of both GLUT1 and GLUT4 transporters in the action of Br-C4. While the intrinsic activity or the translocation of GLUT1 may be enhanced, insulin-promoted redistribution or intrinsic activity of GLUT4 seemed to be decreased by the same treatment. Nevertheless, we can not exclude the possibility that the amount of GLUT1 or GLUT4 may also be altered by Br-C4. In an attempt to differentiate the various possibilities mentioned above, we performed immunoblot analysis of GLUT1 and GLUT4 transporter after insulin challenge in Br-C4-pretreated cells. As shown in Fig 2, after 3-h exposure to Br-C4, the content of GLUT1 in plasma membranes was increased (175 ± 57% above control, $P<0.05$) while its amount in low-density microsome fraction was decreased (57 ± 13% below control, $P<0.02$), indicating that Br-C4 may induce the translocation of GLUT1 from microsome fraction to plasma membranes. Insulin alone also increased GLUT1 content in plasma membranes by 209 ± 78% above control ($P<0.05$) but was unable to further increase the amount of GLUT1 in Br-C4-pretreated cells. On the other hand, whereas Br-C4 was without effect, insulin induced the translocation of GLUT4 from the intracellular pool to plasma membranes as expected (249 ± 58% above control, $P<0.02$) and this translocation was greatly eliminated (80 ± 18% decrease, $P<0.02$) in Br-C4-pretreated cells. The total amount of neither transporter was affected by Br-C4. Thus it appears that the machinery regulating the translocation of both glucose transporters is affected by Br-C4 pretreatment.

In conclusion, the present study has demonstrated

TABLE 2

Effect of Br-C4 on Basal and Insulin-Stimulated 2-Deoxyglucose Uptake by 3T3-L1 Adipocytes Pretreated with Fatty Acids of Varying Chain Length

Pretreatment	% of control	
	Basal	Insulin-stimulated
Br-C4 only	371 ± 14	43 ± 4
C4 + Br-C4	353 ± 16	38 ± 2
C8 + Br-C4	346 ± 17	35 ± 5
C16 + Br-Cr	361 ± 25	27 ± 4[a]

After 3T3-L1 adipocytes were preincubated in the absence or presence of 0.2 mM each of crotonate(C4), octanoate(C8) and palmitate(C16) for 3 h, vehicle (control) or 50 μM Br-C4 was added and the incubation was continued for another 3 h. Cells were then washed and measured for basal and insulin-stimulated 2-deoxyglucose uptake. Percent of control was obtained by comparing basal or insulin-stimulated activity (after being corrected for basal activity) of Br-C4 pretreated cells to that of the corresponding control cells. The control values for cells preincubated in the absence (none) or presence of C4, C8 and C16 were: (basal) 0.082 ± 0.002, 0.086 ± 0.004, 0.084 ± 0.002, and 0.098 ± 0.015 nmol/min/10^6 cell, respectively, and (insulin-stimulated) 1.282 ± 0.024, 1.300 ± 0.012, 1.363 ± 0.063, and 1.469 ± 0.017 nmol/min/10^6 cell, respectively (means ± S.E., n = 3).

[a] $p < 0.05$ compared to the effect of Br-C4 alone.

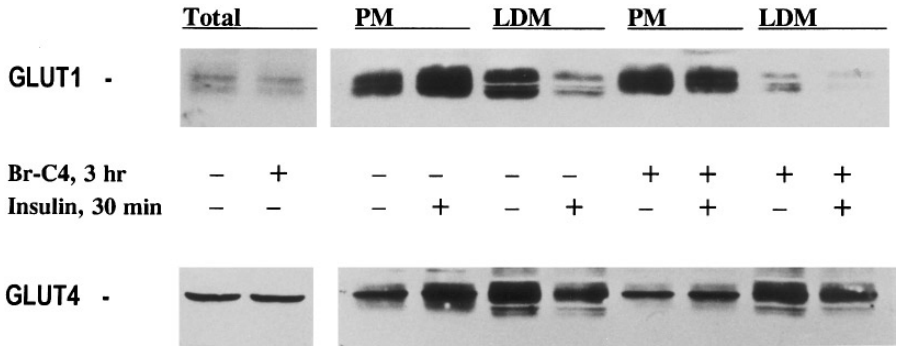

FIG. 2. Effect of Br-C4 pretreatment on the membrane content of GLUT1 and GLUT4 transporter protein in control or insulin-stimulated cells. After pretreatment of 3T3-L1 adipocytes without or with 50 μM Br-C4 for 3 h, cells were further incubated in the absence or presence of 10-7 M insulin for 30 min. Total cellular membranes, plasma membranes (PM) and low-density membranes (LDM) were then isolated, and were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using antiserum against GLUT1 or GLUT4 as described in "Experimental Procedures". Two other independent experiments gave similar results.

that Br-C4 has differential effects on basal and insulin-stimulated glucose transport. It enhances the former while inhibits the latter activity. The effect of this compound on glucose transport seems to be independent of its inhibitory action on β-oxidation. The translocation processes of both GLUT1 and GLUT4 transporters appear to be affected. Finally, the inhibition of insulin-stimulated glucose uptake by Br-C4 may provide a good model for studying insulin-resistance.

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