Oleylethanolamide impairs glucose tolerance and inhibits insulin-stimulated glucose uptake in rat adipocytes through p38 and JNK MAPK pathways

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Oleylethanolamide impairs glucose tolerance and inhibits insulin-stimulated glucose uptake in rat adipocytes through p38 and JNK MAPK pathways. Am J Physiol Endocrinol Metab 289: E923–E929, 2005. First published May 10, 2005; doi:10.1152/ajpendo.00555.2004.—Oleylethanolamide (OEA) is a lipid mediator that inhibits food intake and body weight gain and also exhibits hypolipidemic actions. OEA exerts its anorectic effects peripherally through the stimulation of C-fibers. OEA is synthesized in the intestine in response to feeding, increasing its levels in portal blood after the meal. Moreover, OEA is produced by adipose tissue, and a lipolytic effect has been found. In this work, we have examined the effect of OEA on glucose metabolism in rats in vivo and in isolated adipocytes. In vivo studies showed that acute administration (30 min and 6 h) of OEA produced glucose intolerance without decreasing insulin levels. Ex vivo, we found that 10 min of preincubation with OEA inhibited 30% insulin-stimulated glucose uptake in isolated adipocytes. Maximal effect was achieved at 1 μM OEA. The related compounds palmitolethanolamide and oleic acid had no effect, suggesting a specific mechanism. Insulin-stimulated GLUT4 translocation was not affected, but OEA promoted Ser/Thr phosphorylation of GLUT4, which may impair transport activity. This phosphorylation may be partly mediated by p38 and JNK kinases, since specific inhibitors (SB-203580 and SP-600125) partly reverted the inhibitory effect of OEA on insulin-stimulated glucose uptake. These results suggest that the lipid mediator OEA inhibits insulin action in the adipocyte, impairing glucose uptake via p38 and JNK kinases, and these effects may at least in part explain the glucose intolerance produced in rats in vivo. These effects of OEA may contribute to the anorectic effects induced by this mediator, and they might be also relevant for insulin resistance in adipose tissue.

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adipocytes, by a mechanism that involves PPARα receptors (20). The aim of the present study was to investigate the effect of OEA on glucose metabolism in adipose tissue by testing the in vivo influence on glucose tolerance test and the effect in vitro on glucose transport by rat adipocytes, as well as the mechanism whereby OEA may affect glucose metabolism.

MATERIALS AND METHODS

Materials. OEA and elaidylethanolamide (EEA) were synthesized in the laboratory as previously described (36). PEA was from Tocris Cookson (London, UK). Oleic acid (OA) and BSA (fraction V) were from Sigma Chemical (St. Louis, MO). 2-Deoxy-d-[3H]glucose (7 Ci/mmol) was from DuPont-NEN (Bad Homburg, Germany). JNK and p38 MAPK inhibitors (SP-600125 and SB-203580) were from Cell Signaling, New England Biolabs, and Sigma-Aldrich (Alcobendas, Madrid, Spain). Monoclonal antibodies anti-phosphoserine and threonine were from Sigma-Aldrich (Alcobendas). Monoclonal antibody anti-phospho-SAPK/JNK (T183/Y185) was from New England Biolabs, and monoclonal anti-phospho-p38 MAPK (T180/Y182) antibody was from BD Biosciences Pharmingen.

Glucose tolerance tests. Male Wistar rats weighing 180–220 g fed ad libitum were employed for glucose tolerance tests. Food was withdrawn in the early morning (4 h before the procedure). Awake rats were injected with OEA (5 mg/kg) 30 min, 6 h, or 24 h before glucose tolerance tests. This was carried out by injecting an intraperitoneal glucose load of 2 g/kg body wt. Blood samples were collected before (0 min) and 5, 10, 15, 30, 60, and 120 min after glucose administration. Glucose was determined using a standard glucose oxidase method.

For insulin measurements, awake rats were injected with OEA (20 mg/kg) or vehicle 30 min before glucose overload. This was carried out by injecting an intraperitoneal glucose load of 2 g/kg body wt. Animals were killed by decapitation 10 (n = 12), 30 (n = 12), and 60 (n = 11) min after the glucose load. Indeed, a naive group (n = 5) was killed to obtain the basal level of insulin. Blood was collected, and the plasma fraction was separated from it. Insulin levels were measured using a commercial rat insulin ELISA kit (Mercodia, Sweden).

Adipocyte isolation. Adipocytes were prepared from the epididymal fat pads of ad libitum-fed 100- to 160-g male Wistar rats, according to the method described by Rodbell (35) with minor modifications. Fat pads were minced and then digested with collagenase at 37°C for 1 h in KRb (in mM: 113 NaCl, 2 CaCl2, 5 KCl, 10 NaH2CO3, 1.18 KH2PO4, and 1.18 MgCl2), pH 7.4, supplemented with 20 mM HEPES, 6 mM glucose, and 1% BSA. Aggregated material was removed by filtration through a mesh cloth. Isolated adipocytes were washed three times, and the packed cells were subsequently suspended in the final volume of the same buffer for metabolic experiments (105 cells/ml).

Glucose transport. Glucose transport was assayed as uptake of the nonmetabolizable glucose analog 2-deoxy-d-[2,6,3H]glucose (7 Ci/mm mol), as previously described (37, 38). Adipocytes were incubated in the buffer described above without glucose at 37°C for 20 min in the presence or absence of insulin. When OEA was included in the experiment, it was added 10 min before insulin stimulation. When the chemical inhibitors of p38 (SB-203580, 10 μM) and JNK (SP-600125, 3 μM) kinases were used, they were added 5 min before the addition of OEA. Next, 0.5 μCi 2-deoxy-d-[2,6,3H]glucose was added (0.1 mM 2-deoxy-d-glucose), and the adipocytes were incubated for a further 10 min. The assay was terminated by two rapid washes with iced PBS buffer. Cells were finally solubilized with NaOH, and radioactivity was measured by scintillation counting.

GLUT4 translocation. Adipocytes were incubated at 37°C in the same buffer described above. Cells were treated for 20 min with insulin, after which the presence of GLUT4 in the plasma membrane was assessed by Western blotting using a specific rabbit antiserum (OSCR6, a gift from Dr. A. Zorzano, University of Barcelona, Barcelona, Spain; see Ref. 13). Plasma and microsomal membrane fractions were prepared as previously described (9). Plasma membrane-enriched fractions were separated by SDS-PAGE and transferred to nitrocellulose membranes for detection by immunoblotting.

Immunoprecipitation and Western blotting analysis. To assess GLUT4 phosphorylation in plasma membranes, this fraction was solubilized, and protein concentration was determined by the Bradford method using BSA as standard. Protein (0.5 mg) was precleared with 50 μl protein A-Sepharose (Pharmacia, Uppsala, Sweden) for 2 h at 4°C by end-over-end rotation. The precleared cellular lysates were incubated with appropriate antibodies for 3 h at 4°C (21). Next, 50 μl protein A-Sepharose were added to immune complexes, and incubation was continued for 2 h at 4°C. The immunoprecipitates were washed three times with lysis buffer. We added 40 μl of SDS-stop buffer containing 100 mmol/l dithiothreitol to the immunoprecipitates and boiled for 5 min. The soluble supernatants were then resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (40). The membranes were blocked with Tris-buffered saline 0.05% Tween 20 (TBST) containing 5% nonfat dry milk for 1 h at 23°C. The blots were then incubated with primary antibody for 1 h, washed in TBST, and further incubated with secondary antibodies linked to horseradish peroxidase. Bound horseradish peroxidase was visualized by a high-sensitive chemiluminescence system (SuperSignal; Pierce, Rockford, IL; see Ref. 19). The bands obtained in the Western blots were scanned and analyzed by the PCBS2.0 program. The amount of GLUT4 immunoprecipitated was controlled by specific immunoblot with anti-GLUT4 antibodies.

JNK and p38 MAPK activity. To directly investigate the activation of JNK and p38 MAPK pathways, cells were incubated for 10 min with 1 μM OEA or vehicle. Cells were then lysed and solubilized. Protein concentration was normalized, and samples were denatured by adding SDS-stop buffer containing 100 mmol/l dithiothreitol and boiled for 5 min. Samples were then subjected to SDS-PAGE and analyzed by specific immunoblot with anti-phospho-JNK and anti-phospho-p38 MAPK. The amount of protein loaded in each lane was controlled by immunoblot with anti-β-actin antibody.

RESULTS

OEA impairs glucose tolerance in awake rats. To study the effect of OEA on glucose metabolism, we checked the effects of OEA in the management of plasma glucose in vivo. Thus we tested the in vivo effect on glucose uptake by performing glucose tolerance tests. First, we administered OEA (5 mg/kg ip) or vehicle 30 min before the glucose administration. As shown in Fig. 1A, OEA-treated animals had significantly higher plasma glucose after 30 min of glucose load, reaching glycemia levels >200 mg/100 ml. However, there were no significant differences in any other time point, suggesting an impairing effect on glucose tolerance rather than a diabetogenic effect. Only higher OEA doses (20 mg/kg) produced significantly higher glucose levels at 60 min postglucose, but always returned to basal levels after 120 min postglucose (data not shown).

As shown in Fig. 1B, this effect of OEA impairing glucose tolerance was dependent on the dose, and administration of lower amounts of OEA (1 mg/kg) did not increase significantly glycemic values at 30 min after the glucose load. When the rats were treated with 5 mg/kg OEA for a longer time (6 and 24 h), there were significantly higher glycemic values not only at 30 min but also at 10 and 15 min after glucose loading (Fig. 2). However, again, at this longer OEA exposition, no significant
differences were found in plasma glucose levels after 60 and 120 min postglucose loading.

On the other hand, acute administration of OEA (5 mg/kg) had no effect on basal glucose levels in 120 min of glucose monitoring (at 5, 10, 15, 30, 60, and 120 min) in normal rats (data not shown).

To check whether the increase in glucose levels by OEA in glucose tolerance test was mediated by an inhibition in insulin secretion, insulin levels were measured at 10, 30, and 60 min after glucose load. As shown in Fig. 3, OEA did not inhibit glucose-stimulated insulin secretion. Conversely, a significant increase in insulin levels was observed after 60 min of glucose load.

**OEA inhibits glucose uptake in isolated rat adipocytes.** Because we had found that OEA impairs glucose tolerance in the rat without inhibiting insulin secretion, we moved to the cellular level to study the possible mechanisms of OEA action.

Thus we investigated the effect of OEA on adipocyte metabolism by studying glucose uptake. To study the effect of OEA on basal glucose transport in rat adipocytes, we measured 2-deoxyglucose uptake. As shown in Fig. 4, top, 1 μM OEA partially inhibited basal glucose transport (~10%). A similar effect was observed with 1 μM EEA, whereas 1 μM OA had no effect, and 1 μM PEA had no significant effect. These results suggest the structural specificity of the effects of OEA on glucose uptake, and they parallel the effects on inhibition of feeding (36).

To study the effect of OEA on insulin-stimulated glucose transport in rat adipocytes, we measured 2-deoxyglucose uptake in the presence or absence of OEA and the related compounds EEA and PEA. Insulin stimulates glucose uptake about threefold over basal levels. As shown in Fig. 4, middle, OEA (1 μM) blunted the effect of insulin on glucose transport ~30%. Similar effect was obtained with 1 μM EEA, whereas no effect was obtained with PEA (only 5%, but not significant; Fig. 1).
This effect of OEA on insulin-stimulated glucose uptake was dependent on the dose (Fig. 4, middle). Thus OEA at 0.1 μM significantly inhibited insulin-mediated glucose uptake (~20%), and maximal effect was achieved at 1 μM OEA, which impaired insulin action by 30%.

**Fig. 4.** Middle: rat adipocytes were incubated for 10 min in the absence (control) or presence of 1 μM OEA, elaidylethanolamide (EEA), palmitylethanolamide (PEA), or oleic acid (OA). Next, uptake of 2-deoxy-β-[3H]glucose for 10 min was measured as glucose transport activity. Middle: rat adipocytes were incubated for 10 min in the absence or presence of 1 mM OEA, EEA, or PEA. Next, 10 nM insulin was added, and cells were subsequently assayed for glucose transport. Data are means ± SE (n = 6 experiments run in triplicates). *P < 0.05 vs. control.

**Fig. 5.** A: OEA promotes GLUT4 phosphorylation but does not modify insulin-mediated GLUT4 translocation. Rat adipocytes were treated with or without OEA for 10 min. Next, insulin (10 nM) was added or not for 20 min, and cells were fractionated to obtain plasma membranes. Plasma membranes were separated by SDS-PAGE and immunoblotted for GLUT4. Plasma membranes were solubilized, and GLUT4 was immunoprecipitated (IP) and immunoblotted [Western blot (WB)] with anti-phospho-Ser/Thr antibodies. The amount of GLUT4 in the immunoprecipitates was assessed by anti-GLUT4 immunoblot. Immunoblots are representative of 3 different experiments.

**Fig. 5.** B: OEA increases the basal GLUT4 Ser/Thr phosphorylation level. The amount of immunoprecipitated GLUT4 was checked by specific immunoblot. When cells are stimulated with insulin after OEA pretreatment, the effect of OEA on the GLUT4 phosphorylation level is partially decreased, but does not revert to basal levels, suggesting that insulin promotes partial dephosphorylation of GLUT4.

**OEAA inhibition of insulin-stimulated glucose uptake is partially reverted by blocking p38 and JNK MAPK pathways.** Because OEA inhibits glucose uptake without modifying GLUT4 translocation to plasma membranes, but promoting its phosphorylation state, we looked for the possible mechanisms involved in this effect of OEA. One of the mechanisms reported for insulin resistance by phosphorylation is the activation of the p38 and JNK MAPK pathways.
transport activity. Data are means ± SE (n = 5 experiments run in triplicate).

*P < 0.05 vs. OEA.

Fig. 6. Effect of OEA inhibiting insulin-mediated glucose uptake is partially mediated by p38 and JNK kinase pathways. Glucose uptake was determined in isolated adipocytes as described in the legend to Fig. 3. When p38 MAPK inhibitor (SB-203580, 10 μM) or JNK MAPK inhibitor (SP-600125, 3 μM) were used, they were added 5 min before the addition of OEA (1 μM). Finally, the uptake of 2-deoxy-D-[3H]glucose for 10 min was measured as glucose transport activity. Data are means ± SE (n = 5 experiments run in triplicate).

Fig. 7. OEA stimulates p38 (B) and JNK (A) kinase pathways. Rat adipocytes were incubated for 10 min with 1 μM OEA or vehicle, solubilized, denatured, and analyzed by Western blot. JNK kinase activity was assessed by specific immunoblot using anti-phospho-JNK antibodies. A control experiment was included preincubating the cells with the pharmacological inhibitor SP-600125 (3 μM). p38 MAPK activity was assessed by specific immunoblot using anti-phospho-JNK antibodies. A control experiment was included preincubating the cells with the pharmacological inhibitor SB-203580 (10 μM).

DISCUSSION

This study describes for the first time a modulatory effect of the lipid mediator OEA on glucose metabolism in vivo, impairing glucose tolerance and in vitro inhibiting insulin-mediated glucose uptake in isolated rat adipocytes, and we have provided some clues about the possible mechanisms of this effect of OEA on glucose metabolism.

Because OEA is produced by adipose tissue and a lipolytic effect has been demonstrated in vivo (20), we wanted to check the effect of OEA on glucose metabolism in a physiological system, such as the glucose tolerance test in rats. We found that acute administration of OEA (30 min) produced a dose-dependent impairment in glucose tolerance, with significantly higher glycemic levels 30 min postglucose compared with controls. Plasma glucose levels returned to normal levels after 60 min and continued to be normal 120 min after glucose loading. The glucose intolerance was sustained until 60 min after glucose loading only when high doses of OEA (20 mg/kg) were employed. Under these conditions, a possible indirect effect of OEA inhibiting insulin secretion was ruled out by measuring plasma insulin levels. Rather, we observed an increase in insulin levels, probably because of the higher glucose concentration reached after the glucose loading. When rats were exposed for a longer time (6 and 24 h) with 5 mg/kg OEA, the glucose intolerance was also observed at earlier time points (10 and 15 min postglucose). These results suggested that OEA produces glucose intolerance in vivo, and this effect can be explained, at least in part, by the lipolytic effect of OEA. However, according to the results in vitro, the 30% inhibition of glucose uptake by the adipocytes in the presence of OEA may definitely contribute to the glucose intolerance. We have not checked the possible effect of OEA in muscle glucose transport, which accounts for most of the glucose uptake in the whole animal. Nevertheless, if there is an inhibitory effect on glucose uptake by muscle cells, it might be slight since there is only a mild glucose intolerance effect of OEA in rats in vivo. Besides, we have found that OEA slightly inhibits (~10%) basal glucose uptake in isolated rat adipocytes after a short-term preincubation (10 min). This rapid effect, as occurs with feeding inhibition that can be observed 30 min after OEA injection, should not be mediated by regulation of gene expression, even though OEA has been found to be a ligand of PPARα receptors (15). However, this mechanism may participate in the longer time effects of OEA. In any case, PPARα receptors are mainly expressed in hepatocytes, the intestine, and in very early stages of adipocyte differentiation (26), whereas PPARγ is the major receptor of the family, present in mature adipocytes and playing an important role in the expression of the glucose transporters, insulin receptor and insulin signaling, and therefore improving insulin action, including glucose transport (21, 26, 31, 41). However, even though PPARγ is an important regulator of glucose metabolism (21,
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26, 31, 41), OEA has no direct effect on PPARγ activation, as recently reported (15). Nevertheless, the effect of OEA on the adipocyte glucose uptake seems to be specific and structure dependent, because OA and PEA had no effect, whereas the isomer EEA was similar in potency to OEA. Therefore, different mechanisms may underlay the different effects of OEA controlling feeding and lipid metabolism on the one hand (15) and the control of glucose uptake by the adipocyte on the other. Additional targets for OEA in addition to PPARα receptors are guaranteed since in PPARα receptor knockout mice OEA, administration induces specific metabolic effects such as increments in triacylglycerol contents in adipocytes (20).

Because we found that OEA inhibited glucose uptake in isolated rat adipocytes, we wanted to study the possible effect of OEA on plasma glucose levels. No effect of OEA on basal plasma glucose levels was observed in a 120-min monitoring of normal rats. This result may be explained by the fact that only 10% inhibition in glucose uptake in isolated adipocytes may not be sufficient to produce hyperglycemia in the whole animal.

OEA significantly inhibits the insulin-stimulated glucose uptake in isolated rat adipocytes by 30%. The effect is also structurally selected, since the isomer EEA was similar in potency to OEA, whereas PEA was significantly less effective, in a similar way to that observed in the anorexic effect of OEA in rats. As discussed before for the OEA effect on basal glucose uptake, the acute OEA inhibition of insulin-mediated glucose uptake cannot be explained on the basis of a genomic effect mediated by the activation of PPARα or PPARγ; therefore, alternative pathways may mediate these effects of OEA in the adipocyte.

The effect of insulin increasing glucose uptake is mainly because of the translocation of GLUT4 to the plasma membranes (3, 14, 46); however, we found no inhibitory effect of OEA on GLUT4 translocation upon insulin stimulation in rat adipocytes. Therefore, the mechanisms underlying the OEA inhibition of glucose transport may include the modulation of glucose transporter regulation. In fact, it has been proposed that intrinsic glucose transport activity of GLUT4 may be regulated (16, 43). For example, early studies showed that isoproterenol, other β-adrenergic agonists, and pharmacological agents that increase or mimic cAMP all inhibit insulin-stimulated glucose transport in muscle and adipose tissue, without changing the plasma membrane content of the transporter, thus suggesting the modulation of the intrinsic activity of GLUT4 (25, 27, 34, 39). Indeed, it was subsequently demonstrated that isoproterenol can stimulate phosphorylation of GLUT4 in vivo and that the cAMP-dependent protein kinase A can do it in vitro (24, 28, 34), suggesting that the observed attenuation of glucose transport activity may result from phosphorylation of GLUT4.

Because we have found that OEA can phosphorylate GLUT4 present in the plasma membrane, a possible interpretation is that this may be one of the mechanisms of OEA inhibition of insulin-stimulated glucose transport. We have also found that insulin impairs the GLUT4 phosphorylation upon OEA stimulation, although not enough to reach basal levels, which is consistent with the inhibition of insulin-stimulated glucose uptake by OEA. In this line, insulin has previously been found to reduce the amount of phosphorylated GLUT4 at the plasma membrane (24). We have not detected the insulin effect on basal phosphorylation level because our system was not sensitive enough to detect basal phosphorylation. On the other hand, other models that have been proposed to explain the effects of isoproterenol on glucose transport, such as the occlusion or incomplete fusion of GLUT4-containing vesicles at the plasma membrane, cannot be completely ruled out (42, 45).

Because the mechanism of OEA inhibition of GLUT4 seems to involve phosphorylation, we explored the possibility of OEA activation of kinase pathways known to mediate insulin resistance, such as the stress-activated kinases p38 and JNK kinases, which are activated by a variety of exogenous and endogenous stress, inducing stimuli, such as hyperglycemia, oxidative stress, osmotic stress, proinflammatory cytokines, heat shock, and ultraviolet irradiation (2, 12, 22, 30). Thus we employed chemical inhibitors of these pathways to check their contribution to the OEA inhibition of insulin-mediated glucose uptake. In this context, insulin-stimulated glucose transport impaired by oxidant stress has been previously found to be restored by a specific inhibitor of p38 MAPK (2, 10). We have found that both inhibitors of p38 and JNK kinases can partially restore the insulin-mediated glucose uptake in isolated adipocytes, suggesting the participation of these pathways in the mechanism underlying the inhibition of glucose transport by OEA. Besides, additional pathways may also participate in these molecular mechanisms, since the specific inhibition of each or both stress pathways is not sufficient to fully restore the insulin stimulation of glucose uptake. In any case, we assessed the OEA activation of these pathways by using the anti-phosphokinase strategy. The activation of p38 and JNK kinases by OEA stimulation was attenuated by pretreatment with the specific inhibitors SB-203580 and SP-600125, respectively.

In summary, these results suggest that the lipid mediator OEA inhibits insulin action in the adipocyte, impairing glucose uptake by a mechanism that seems to involve p38 and JNK kinase pathways, and these effects may be physiologically relevant, since OEA induces glucose intolerance in rats in vivo. These effects of OEA may contribute to the anorexigenic effects induced by this mediator, and to the blockade in weight gain because of the decremental availability of energetic resource to the adipocyte. They might be also relevant for insulin resistance in adipose tissue.

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