The nitric oxide-donating derivative of acetylsalicylic acid, NCX 4016, stimulates glucose transport and glucose transporters translocation in 3T3-L1 adipocytes

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Kaddai V, Gonzalez T, Bolla M, Le Marchand-Brustel Y, Cormont M. The nitric oxide-donating derivative of acetylsalicylic acid, NCX 4016, stimulates glucose transport and glucose transporters translocation in 3T3-L1 adipocytes. Am J Physiol Endocrinol Metab 295: E162–E169, 2008. First published May 20, 2008; doi:10.1152/ajpendo.00622.2007.—NCX 4016 is a nitric oxide (NO)-donating derivative of acetylsalicylic acid. NO and salicylic acid, in vivo metabolites of NCX 4016, were shown to be potential actors in controlling glucose homeostasis. In this study, we evaluated the action of NCX 4016 on the capacity of 3T3-L1 adipocytes to transport glucose in basal and insulin-stimulated conditions. NCX 4016 induced a twofold increase in glucose uptake in parallel with the translocation of the glucose transporters GLUT1 and GLUT4 to the plasma membrane, leaving unaffected their total expression levels. Importantly, NCX 4016 further increased glucose transport induced by a physiological concentration of insulin. The stimulatory effect of NCX 4016 on glucose uptake appears to be mediated by its NO moiety. Indeed, it is inhibited by a NO scavenger and treatment with acetylsalicylic or salicylic acid had no effect. Although NO is involved in the action of NCX 4016, it did not mainly depend on the soluble cGMP cyclase/protein kinase G pathway. Furthermore, NCX 4016-stimulated glucose transport did not involve the insulin-signaling cascade required to stimulate glucose transport. NCX 4016 induces a small activation of the mitogen-activated protein kinases p38 and c-Jun NH2-terminal kinase and no activation of other stress-activated signaling molecules, including extracellular signal-regulated kinase, inhibitory factor kβ, or AMP-activated kinases. Interestingly, NCX 4016 modified the content of S-nitrosylated proteins in adipocytes. Taken together, our results indicate that NCX 4016 induced glucose transport in adipocytes through a novel mechanism possibly involving S-nitrosylation. NCX 4016 thus possesses interesting characteristics to be considered as a candidate molecule for the treatment of patients suffering from metabolic syndrome and type 2 diabetes.

nitric oxide donation; adipocytes; glucose transporter translocation; S-nitrosylation; diabetes

Very high doses of salicylate are known to exert a glucose-lowering effect in patients suffering from Type 2 diabetes (3). This improved glycemic control is attributed to an improved insulin sensitivity and could be explained at least by two mechanisms. The first one results in the rescue of insulin signaling in the insulin-sensitive tissues. By inhibiting IKK (47) salicylate indeed decreases phosphorylation of insulin receptor substrate 1 (IRS1) on serine-307, a deleterious phosphorylation event for normal insulin signaling (18, 32). Second, because salicylate inhibits inflammatory responses through inhibition of IKK and cyclooxygenases (44, 47), it could inhibit the secretion of proinflammatory cytokines that alter insulin signaling (1).

The NO moiety can also have anti-inflammatory potential with benefit in insulin action because it was reported to directly inhibit IKKβ (35). NO could also be an important player in glucose and lipid homeostasis. NO may improve glucose disposal through its well-known vasodilatory activity (23) and may contribute to the control of glycemia through regulation of the IKKβ pathway (35). The phenotypic characterization of endothelial nitric oxide synthase (eNOS)-deficient and heterozygous mice clearly unmask an important role of NO in the control of glucose and lipid homeostasis, on top of its role in the control of arterial pressure (9, 11). Indeed, eNOS expression is required for full insulin-induced glucose transport in isolated muscle (11) and adipocyte (4).

In the present study, we explore whether NCX 4016 could control glucose transport in adipocytes, a cell type playing an important role in regulating body glucose homeostasis (21). The potential effect of NCX 4016 was determined in 3T3-L1 adipocytes in a comparative study with acetylsalicylic acid and sodium nitroprusside (SNP).

MATERIALS AND METHODS

Antibodies. Polyclonal antibodies against GLUT4 and GLUT1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against AS160 was from Abcam (ab5909; Cambridge, UK), and against phospho-Thr642-AS160 was from Biosource International (Camarillo, CA). Anti-phospho-Thr172-AMP-activated kinase (AMPK), phospho-Thr308-protein kinase B (PKB), phospho-Ser173-PKB, phospho-Thr180/Tyr182-p38, phospho-Thr202/Tyr204-extracellular signal-regulated kinase (ERK1/2, and phospho-Thr183/Tyr185-c-Jun NH2-terminal kinase (JNK) were from Cell Signaling Technology (Dan-
vers, MA). Anti-ERK1/2 was from Santa Cruz Biotechnology, whereas anti-PKB and p38 were from Cell Signaling Technology. Anti-JNK and anti cytochrome c were from BD Biosciences (San Jose, CA). Anti-actin was from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-coupled and horseradish peroxidase-coupled anti-antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Chemicals. NCX 4016 and acetyl salicylic acid were provided by NicOx (Sophia-Antipolis, France). Poly-L-lysine, the NO scavenger carboxy-2-phenyl-4,4,5,5-tetramethylidazoline-3-oxide-1oxyl (c-PTIO), the NO donor SNP, the inhibitor of NO-dependent guanylyl cyclase [1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), the cGMP-dependent protein kinase 1a (DT-2), Methylxanthine (250 nM), Dexamethasone (250 nM), and insulin (800 nM) did not induce any change in the amount of 2-DG uptake, whereas 200 μM sodium orthovanadate). Aliquots of homogenates were measured with the indicated specific antibodies.

RESULTS

NCX 4016 increases glucose uptake in 3T3-L1 adipocytes. We first looked at the effect of a short-term treatment with NCX 4016 on glucose uptake. Because the NCX 4016 is subjected to enzymatic metabolism to produce NO at slow rates, we treated adipocytes for 3 h to allow suitable intracellular delivery of NO (for review, see Ref. 6). 3T3-L1 adipocytes were treated with 200 μM of NCX 4016 or equimolar acetyl salicylic acid, the NO-donor SNP as the positive control, or a physiological concentration of insulin (0.5 nM) before measurement of 2-DG uptake as described in MATERIALS AND METHODS. Compared with control cells, NCX 4016 induced a significant increase in 2-DG uptake into adipocytes (Fig. 1A), which was lower with respect to insulin stimulation. Treatment with an equimolar concentration of acetyl salicylic acid (200 μM) did not induce any change in the amount of 2-DG uptake, whereas 200 μM of the fast NO donor SNP significantly increased 2-DG uptake. Because acetyl salicylic acid must be deacetylated to be active, we also tested the effect of salicylic
acid and found that 200 μM salicylic acid had no effect on 2-DG uptake (Fig. 1A). Expression levels of the two major glucose transporters expressed in adipocytes, GLUT4 and GLUT1 (28), were not modified by this short-term treatment with NCX 4016 or acetylsalicylic acid (Fig. 1B). The effect of NCX 4016 was dose dependent, with a detectable effect at 50 μM and a maximal effect at 200 μM, and time dependent because it increased by 25% glucose uptake after 1 h of incubation (data not shown).

NCX 4016 increases the plasma membrane levels of both GLUT1 and GLUT4 glucose transporters. Because expression levels of GLUT4 and GLUT1 were unchanged by NCX 4016, the increase in 2-DG uptake was likely to be due to an increase in the amount of glucose transporters at the plasma membrane. To measure the amount of GLUT4 and GLUT1 at the plasma membrane, 3T3-L1 adipocytes were treated for 0.5 h with insulin or for 3 h with 200 μM of NCX 4016, 200 μM acetylsalicylic acid (ASA), 200 μM sodium nitroprusside (SNP), or vehicle [0; 0.25% dimethyl sulfoxide (DMSO)]. Plasma membrane sheets immobilized on the glass cover slips were then prepared as indicated in MATERIALS AND METHODS before measuring GLUT4 (Fig. 2A) and GLUT1 (Fig. 2B) amount by quantitative immunofluorescence. NCX 4016 significantly increased the amount of GLUT4 at the plasma membrane, but moderately compared with insulin. By contrast, NCX 4016 was as efficient as 100 nM insulin to increase the amount of GLUT1 at the plasma membrane. Acetylsalicylic acid alone did not modify the amount of either GLUT1 or GLUT4 present at the plasma membrane, suggesting that the observed effect for NCX 4016 was dependent on the release of NO. This series of experiments indicates that NCX 4016 increases basal glucose uptake by inducing translocation of glucose transporters to the plasma membrane of 3T3-L1 adipocytes, with a more potent effect on GLUT1 than on GLUT4.

NCX 4016 increases 2-DG uptake after 24 h exposure in 3T3-L1 adipocytes through a NO-releasing dependent pathway. We next determined whether a longer incubation (24 h) with NCX 4016 could be more efficient in stimulating 2-DG uptake. 3T3-L1 adipocytes were treated for 24 h with 200 μM NCX 4016, acetylsalicylic acid, or vehicle as control and then stimulated or not with insulin. We observed that NCX 4016 induced a threefold increase in 2-DG uptake compared with control and acetylsalicylic acid-treated cells (Fig. 3A), whereas...
a 24-h treatment with 200 μM SNP increased 2-DG uptake by less than twofold (Fig. 3C). Also of importance, this effect was additive with the effect of a physiological concentration of insulin (0.5 nM) (Fig. 3A). Furthermore, the long-term exposure with NCX 4016 did not affect an acute effect of insulin at a maximally stimulating concentration. The increase in glucose transport activity of the adipocytes treated with NCX 4016 occurred without any change in the levels of expression of the transporters GLUT4 and GLUT1 (Fig. 3B). Because an equimolar amount of acetylsalicylic acid did not induce 2-DG uptake as does NCX 4016, the NO moiety of the compound was likely responsible for the observed effect. To demonstrate this, we used the specific NO scavenger c-PTIO, which abrogated in a concentration-dependent manner the effect of NCX 4016 (Fig. 3C). c-PTIO had no significant effect on basal glucose uptake (data not shown) but also inhibited the effect on 2-DG uptake induced by 24 h treatment with 200 μM SNP (Fig. 3C). Taken together, our results indicate that the NO moiety of NCX 4016 induces glucose uptake in 3T3-L1 adipocytes and that NCX 4016 did not alter insulin action on 2-DG uptake.

NCX 4016-induced 2-DG uptake is not efficiently affected by inhibitors of the soluble guanylyl cyclase pathway. Because NCX 4016 effect appears to be linked to the NO moiety, we tested whether the soluble guanylyl cyclase (the canonical physiological target of the NO signaling pathway) was involved in this effect. The incubation of adipocytes with ODQ or carnosine, inhibitors of the soluble form of the guanylyl cyclase, did not affect NCX 4016-induced 2-DG uptake, and ODQ also had no effect on basal glucose uptake (Fig. 4A). ODQ was effective at the concentration used. It indeed inhibited the induction by NCX 4016 of cytochrome c expression, a
NO-induced effect that was previously shown to be dependent on the soluble guanylyl cyclase (33) (Fig. 4B). Furthermore, the inhibition of the cGMP-dependent protein kinases, a downstream protein in the signaling cascade, by using KT-5823 slightly inhibited NCX 4016-induced 2-DG uptake without modifying basal glucose uptake, whereas the inhibition of GMP-dependent protein kinase-1a by the inhibitor DT-2 had no effect (Fig. 4C). The action of NCX 4016 is thus probably independent of the activation of the classical NO-activated signaling pathway.

NCX 4016 does not induce IRS phosphorylation and does not activate the PKB pathway. We investigated whether NCX 4016 could induce 2-DG uptake by activating insulin signaling molecules. We observed that NCX 4016 did not increase the tyrosine phosphorylation of the insulin receptor, nor of its substrates IRS, either after a short-term incubation (Fig. 5A) or a long-term incubation (Fig. 5B), the two first events after insulin binding required for insulin-induced glucose transport (45). Furthermore, it also did not induce the activation of insulin receptor downstream pathways, including the PKB pathway, which is involved in insulin-induced glucose uptake (20, 24). Indeed, NCX 4016 did not induce the phosphorylation of PKB and of the PKB substrate AS160 that is required for insulin-induced glucose transport (38) (Fig. 5).

Effect of NCX 4016 on stress-activated kinase pathways. Many cell types, including adipocyte and muscle, respond to multiple stresses such as inflammation, nutrient deprivation, or oxidative stress by increasing their capacity to transport glucose (see the following references for some examples: 2, 26, 30, 36, 39). We thus examined whether NCX 4016 is able to activate signaling pathways induced by these various stresses. Among the mitogen-activated protein kinases, NCX 4016 did not activate ERK1/2 at any time of the incubation period, whereas insulin did it transiently (Fig. 6, A and B). We observed an activation of p54 JNK and p38. This activation was detectable at all of the incubation periods, although it declined after 24 h, and it reached the level induced by insulin (Fig. 6, A and B). Thus the activation of these pathways is not robust compared with stress activators of these two kinases.

We did not observe any phosphorylation of the AMPK by NCX 4016, a kinase activated by fuel deprivation and in muscle also by exercise (17, 29). NCX 4016 did not decrease either the amount of inhibitory factor κB, unlike interleukin-1β (Fig. 6C), indicating that it did not activate the inflammatory nuclear factor-κB pathway in 3T3-L1 adipocytes. Taken together, our results indicate that NCX 4016 are not strong activators of stress kinase pathways.

NCX 4016 induces protein S-nitrosylation. Because the canonic NO-induced signaling pathway does not seem to play the major role in the effect of NCX 4016 on glucose transport, we assessed whether it could modulate protein S-nitrosylation in adipocytes. 3T3-L1 adipocytes were incubated for 24 h with solvent, 200 μM SNP, or 200 μM NCX 4016. By using the biotin switch assay, we were able to detect significant amounts of S-nitrosylated proteins in adipocytes cultured in control conditions (Fig. 7). Indeed, protein profiles obtained in the presence or absence of MMTS were quantitatively and qualitatively different, an indication of the efficient blockade of the free thiols that could not react any more with biotin-BMCC. Thus the labeled proteins observed in the presence of MMTS correspond to S-nitrosylated proteins. Interestingly, NCX 4016 increased the amount of two S-nitrosylated proteins with calculated apparent molecular weight of 60 and 68 kDa. SNP also affected the S-nitrosylation of these two proteins but to a lower extent.

DISCUSSION

In the present study, we report that the NO-donating derivative of acetylsalicylic acid (NCX 4016) evokes deoxyglucose uptake in 3T3-L1 adipocytes. We propose that the NO moiety of NCX 4016 is the inducer of glucose uptake in adipocytes. Indeed, an equimolar amount of the acetylsalicylic or salicylic moiety did not show any activity. This is in accordance with previous reports that demonstrate that the NO donor SNP increases glucose uptake in 3T3-L1 adipocytes (42) and isolated muscle (12). Furthermore, we found that the NO scavenger c-PTIO inhibits the action of NCX 4016 as well as that of...
SNP. Interestingly, NCX 4016 was more effective after 24 h treatment compared with 3 h treatment. This result suggests that slow release of NO from NCX 4016 induces a sustained increase in basal glucose uptake.

The effect of NCX 4016 on glucose uptake occurs without any changes in the expression levels of the glucose transporters expressed in 3T3-L1 adipocyte GLUT1 and GLUT4. NCX 4016 could thus act either by promoting the activity of plasma membrane-inserted transporters or by increasing the amount of glucose transporters at the plasma membrane. We favor this latter hypothesis because more GLUT4 and GLUT1 in the plasma membranes of NCX 4016-treated adipocytes have been found. The fact that GLUT1 is increased to a similar extent by NCX 4016 and insulin, whereas insulin is more potent to increase GLUT4 content in plasma membranes, strongly suggests that NCX 4016 essentially acts on the endosomal pools of glucose transporters, rather than on the GLUT4-specific sequestration compartments (for review on GLUT4 trafficking in adipocytes see Refs. 7 and 34). Furthermore, it also suggests that the action of NCX 4016 on glucose uptake could be extended to other cells and tissues that all expressed GLUT1. In accordance, NCX 4016 was shown to increase glucose uptake without modifying the expression level of GLUT1 in CD3-CD28 costimulated lymphocytes (14). It is thus possible that NCX 4016 could be effective in vivo to increase glucose disposal in resting conditions, an attractive hypothesis that remains to be tested by in vivo administration of the compound to animals.

Although NO was involved in the action of NCX 4016, it seems that it does not mainly require active soluble guanylate cyclase/protein kinase G pathways. Indeed, the use of various inhibitors interfering with this pathway does not efficiently inhibit NCX 4016-induced glucose uptake. NCX 4016 does not trigger the activation of some of the insulin signaling molecules, such as IRS and PKB, involved in the stimulation of glucose uptake, in accordance with the previously mentioned SNP action in 3T3-L1 adipocytes (42).

Fig. 6. Effects of NCX 4016 on various stress-activated kinases. 3T3-L1 adipocytes were incubated during the indicated period of times with vehicle (0), 100 nM of insulin, 200 μM of ASA, or 200 μM of NCX 4016. In B, as a control of AMPK activation, 3T3-L1 adipocytes were treated for 24 h with 100 μM 5-aminimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR). Results for short-term incubations and longer incubations with NCX 4016 are shown in A and B, respectively. In C, the degradation of inhibitory factor αB (IαB) was induced by a treatment with 20 ng/ml interleukin (IL)-1β for 0.5 h. NS, non-specific band detected with the anti-IαB antibody. Total cell homogenates were then prepared, and 40 μg of proteins from each condition were separated by SDS-PAGE, transferred to PVDF membrane, and probed with the indicated antibodies.

Fig. 7. Effects of NCX 4016 on protein S-nitrosylation. 3T3-L1 adipocytes were treated for 24 h with solvent (0), 200 μM SNP, or 200 μM NCX 4016. Total cell lysates were subjected to the biotin switch assay as described in MATERIALS AND METHODS. S-nitrosylated proteins [conditions +S-methylmethanethiosulfonate (MMTS)] were detected with anti-biotin antibodies. The intensity profiles of S-nitrosylated proteins in control (0) and NCX 4016 conditions are shown. Chemiluminescence was detected by using the FUJIFILM Las-3000 apparatus, and the time of exposure is 10 times less for the condition −MMTS compared with that with MMTS. The Western blot is representative of 3 independent experiments.
We found that p38 is activated by NCX 4016. However, this NCX 4016-induced p38 activation is certainly not responsive of the effect of NCX 4016 on glucose uptake. Indeed, although p38 has been involved in insulin-induced glucose transport (41), it is not involved in the translocation of glucose transporters at the plasma membrane (41), whereas we demonstrate that NCX 4016 controls this process. Furthermore, it is not known whether activation of p38 would induce by itself glucose transport.

The increase in glucose uptake observed after a treatment with NCX 4016 was comparable with the effect of 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR; see Refs. 37 and 46). AICAR is a potent activator of AMPK, which has been involved in the activation of eNOS (10). It is thus possible that NCX 4016 and AICAR induce the same signaling pathway to induce glucose uptake. Because NCX 4016 is supposed to act downstream of AMPK, it is normal that NCX 4016 does not phosphorylate and activate AMPK (Fig. 6). However, AICAR has been described to inhibit insulin action on glucose uptake in 3T3-L1 adipocytes (37), whereas NCX 4016 does not. The various stresses known to increase basal glucose uptake in adipocytes (such as tumor necrosis factor-α or oxidative stress) are also known to be potent troublemakers of insulin metabolic action (5, 40). Therefore, NCX 4016 represents an interesting compound because, although it increases glucose uptake in adipocyte, it has no negative impact on insulin sensitivity, although it could activate the stress kinases p38 and JNK that are implicated in insulin resistance. It is possible that the level of activation of these kinases by NCX 4016 is too small to induce insulin resistance or that NCX 4016 counteracts the action of these deleterious pathways downstream of the activation of the stress kinases.

The action of the NO moiety of NCX 4016 would trigger a noncanonical signaling pathway possibly involving S-nitrosylation of target proteins (27) to promote glucose uptake. S-nitrosylation of N-ethylmaleimide-sensitive factor is involved in the redistribution to the plasma membrane of the postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid subtype of glutamate receptors elicited by N-methyl-d-aspartate receptor agonists (22). A similar mechanism could be envisaged for glucose transporter translocation. Interestingly, we found that NCX 4016 selectively increased the S-nitrosylation of two proteins with an apparent molecular weight of 60 and 68 in adipocytes. The future identification of these proteins could certainly help to understand how NCX 4016 induced glucose uptake in adipocytes.

In cardiovascular models, NCX 4016 showed a better efficacy than aspirin in counteracting thromboembolism, myocardial infarction, and vascular reactivity and remodeling (6, 19), features well related to the mechanism of action of NO donors. Furthermore, NCX 4016 appeared to have little or no gastric toxicity in various animal models (19) and in healthy human volunteers (13) and prevented induced apoptotic events on endothelial (15) or gastric chief cells (16). The ability of NCX 4016 to induce glucose uptake without negatively affecting insulin sensitivity in adipocytes could thus be of therapeutic help in better controlling glycemia in diabetic patients while maintaining a protective vascular effect through NO activity.

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DISCLOSURES

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