AMPK activation by glucagon-like peptide-1 prevents NADPH oxidase activation induced by hyperglycemia in adult cardiomyocytes

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Submitted 31 March 2014; accepted in final form 30 July 2014

Exposure of cardiomyocytes to high glucose concentrations (HG) stimulates reactive oxygen species (ROS) production by NADPH oxidase (NOX2). NOX2 activation is triggered by enhanced glucose transport through a sodium-glucose cotransporter (SGLT) but not by a stimulation of glucose metabolism. The aim of this work was to identify potential therapeutic approaches to counteract this glucotoxicity. In cultured adult rat cardiomyocytes incubated with 21 mM glucose (HG), AMP-activated protein kinase (AMPK) activation by A769662 or phenformin nearly suppressed ROS production. Interestingly, glucagon-like peptide 1 (GLP-1), a new antidiabetic drug, concomitantly induced AMPK activation and prevented the HG-mediated ROS production (maximal effect at 100 nM). α2-AMPK, the major isoform expressed in cardiomyocytes (but not α1-AMPK), was activated in response to GLP-1. Anti-ROS properties of AMPK activators were not related to changes in glucose uptake or glycolysis. Using in situ proximity ligation assay, we demonstrated that AMPK activation prevented the HG-induced p47phox translocation to caveolae, whatever the AMPK activators used. NOX2 activation by either α-methyl-D-glucopyranoside, a glucose analog transported through SGLT, or angiotensin II was also counteracted by GLP-1. The crucial role of AMPK in limiting HG-mediated NOX2 activation was demonstrated by overexpressing a constitutively active form of α2-AMPK using adenoviral infection. This overexpression prevented NOX2 activation in response to HG, whereas GLP-1 lost its protective action in α2-AMPK-deficient mouse cardiomyocytes. Under HG, the GLP-1/AMPK pathway inhibited PKC-β2 phosphorylation, a key element mediating p47phox translocation. In conclusion, GLP-1 induces α2-AMPK activation and blocks HG-induced p47phox translocation to the plasma membrane, thereby preventing glucotoxicity.

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There are seven AMPK genes encoding two α (1 and 2), two β (1 and 2), and three γ (1, 2, and 3) isoforms, respectively. AMPK is activated by phosphorylation of Thr172 in the catalytic domain by at least two upstream kinases. The first mechanism senses energy and involves AMP and liver kinase B1, whereas the second implies changes in intracellular calcium ion concentration and is mediated by calmodulin-dependent kinase kinase β (CaMKK-β) (18). AMPK acts as an energy sensor regulating energy homeostasis although its role extends well beyond metabolism (19).

In this work, we demonstrate that GLP-1 protects cardiomyocytes against HG-induced NOX2 activation through an AMPK-mediated mechanism.

MATeRIALS AND MEtHODS

Animals and materials. Animal handling was approved by the Animal Research Committee of the Université catholique de Louvain (2012/UCL/MD/003) and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All biochemicals were from Sigma, unless otherwise stated. SAMS and Extinum 9–39 peptides were kindly provided by V. Stroobant (Ludwig Institute, Brussels, Belgium). The antibodies used were: α-1 and α-2-AMPK (Kinostat); total-α-AMPK, phosphoAMPK (T172), acetyl-CoA carboxylase (ACC), eukaryotic elongation factor 2 (eEF2), c-myc-tag, phosphoPKC (pan) (B2 S660), GAPDH, hemaglutinin (HA) (Cell Signaling); phosphoACC (S79) (Pierce); caveolin-3 (Santa Cruz Biotechnology); and p47phox and Glut-4 (Millipore).

Isolation and culture of adult rat cardiomyocytes. Adult male Wistar fed rats were anesthetized with pentobarbital 50 mg/kg ip and killed by cervical dislocation before removal of the heart. The cardiomyocytes were isolated by perfusion with collagenase type II (1 mg/ml, Worthington) and cultured as previously described (6).

Briefly, cells were plated on laminin-coated dishes and incubated for 24 h in fresh medium 199 (Invitrogen, 5.5 mM d-glucose) containing 100 mg/ml L-glutamine, 5 mM creatine, 5 mM taurine, 0.1 mM T3, and 0.2% (wt/vol) BSA (fatty acid free) in the presence of 100 U/ml penicillin and 100 µg/ml streptomycin. Primary cultures of cardiomyocytes were incubated with the different concentrations of d-glucose and other additions, for the indicated periods of time and as detailed in the figure legends.

Isolation and culture of adult mouse cardiomyocytes (wild-type and knockout mice for AMPK-α2 subunit). Adult male mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). Cardiomyocytes were isolated by perfusion with collagenase DH enzyme (0.625 mg/kg, Roche). Cells were then plated on laminin-coated dishes and incubated for 2 h in fresh medium 199 (Invitrogen, 5.5 mM d-glucose) containing 100 U/ml penicillin and 100 µg/ml streptomycin. Primary cultures of mouse cardiomyocytes were treated as adult rat cardiomyocytes.

Measurement of ROS. Intracellular ROS production was measured by evaluating oxidation of the cell-permeable fluorescent probe 2',7'-dihydrodichlorofluorescein diacetate (H2DCF-DA, Invitrogen), as previously described (2).

Metabolic measurements. Glucose uptake and glycolytic flux were measured by the rate of de-titration of [2-3H]glucose and [3-3H]glucose, respectively (43). Purine nucleotides were measured in neutralized perchloric acid extracts following their separation by high-performance liquid chromatography (26).

GLUT4 translocation to the plasma membrane. Adenovirus expressing HA-Glut4-green fluorescent protein (GFP) was kindly provided by Dr. Corley Mastick (Department of Biochemistry and Molecular Biology, University of Nevada) (27). Glut4 is labeled at the COOH-terminal part with GFP and at the NH2-terminal part with HA. When Glut4 is translocated to the plasma membrane, HA can be detected at the cell surface. Experiments were performed 48 h after infection (multiplicity of infection 10). After treatment, cardiomyocytes were fixed with paraformaldehyde (4% vol/vol) on ice and placed in a blocking solution (bovine serum albumin, 5% wt/vol). Cells were then incubated with a primary antibody (anti-HA) followed by a secondary fluorescent antibody (Alexa Fluor 594). Glut4 at the plasma membrane appears as red fluorescent dots. Nuclei were stained with DAPI. Protein staining was visualized using a Zeiss.imagine.Z1 microscope equipped with an ApoTome device. Red fluorescent dots (HA) were quantified related to green (GFP) by the software Photoshop.

Immunoblot analysis and AMPK activity measurement. Cells were lysed in cold buffer containing 50 mM Hapes (pH 7.5), 50 mM KF, 1 mM KP, 5 mM EDTA, 5 mM EGTA, 15 mM β-mercaptoethanol, 1 µg/ml leupeptin, 1 mM benzamidin, 1 mM phenylmethylsulfonyl fluoride, 1 mM vanadate, and 1% vol/vol Triton X-100. Protein content was measured with bovine serum albumin as a standard (9). Immunoblots were performed with total extracts separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with milk or bovine serum albumin (5% wt/vol) and then blotted with the corresponding antibodies. After incubation with the appropriate secondary antibody, proteins were visualized using electrochemical luminescence (Pierce) and quantified using Image J. Band intensities were normalized relative to those of loading on the same gel. The loading control used was eEF-2 except for PKC (GAPDH). Immunoblotting with respective anti-total antibodies was performed on a separate gel to verify the absence of any modification in protein expression.

Total AMPK activity was measured after polyethylene glycol (PEG) fractionation and α-1-AMPK and α-2-AMPK activities were measured on respective immunoprecipitates, as previously described (48).

Protein colocalization using in situ proximity ligation assay. This technique allows visualization of a colocalization of two proteins (24). After treatment, cardiomyocytes were fixed with paraformaldehyde (4% vol/vol), permeabilized with Triton X-100 (0.2% vol/vol in PBS), and placed in a blocking solution at 37°C. Cells were then incubated overnight at 4°C with two primary antibodies (from two different species) raised against the two proteins of interest. Secondary antibodies raised against the two different species and coupled with fluorescent oligonucleotides were then added. If the proteins are at less than 40 nm from each other, a ligase creates a circle-shaped structure (“concatemer”), which is amplified by a polymerase. This amplified product is finally detected with complementary fluorescently labeled oligonucleotides that hybridize to the concatemers. The result is observed as red fluorescent dots appearing when proteins colocalize. Nuclei were stained with DAPI. Protein staining was visualized using a Zeiss.imagine.Z1 microscope equipped with an ApoTome device. Red fluorescent dots were quantified (AxioVision Rel 4.8).

Adenoviral infection. Adult rat cardiomyocytes were infected (multiplicity of infection 100) as described (6) with two different adenoviral constructions, one expressing constitutively active form of p47phox and the other expressing GFP alone. Experiments were performed 48 h after infection.

Quantitative PCR. Total mRNA was extracted from cardiomyocytes using a chloroform/isopropanol procedure (TriPure, Roche). Reverse transcription was performed for 1 h at 37°C with 1 µg RNA using an iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR was performed on IQ5 (Bio-Rad) using the qPCR Core kit for Sybergreen (Eurogentec). The mRNA level for each gene in each sample was normalized to the housekeeping gene hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1), after ΔΔCt correction. The nucleotide sequences of primers used were as follows: pr7p70: 5'-TCACCGAGATCTACGAGTTC-3' (sense) and 5'-TCCCATAGGCGCTGT-TGAAAT-3' (antisense), Hprt1 5'-GTCACCGGTTCGTACGGT-ATG-3' (sense) and 5'-ACAGAGGCGCAATGTTGAT-3' (antisense).
Statistical analysis. All the values are expressed as means ± SE. Comparisons were performed using ANOVA followed by a Bonferroni post hoc correction. $P < 0.05$ was considered statistically significant.

RESULTS

AMPK activators and GLP-1 decrease the hyperglycemia-induced ROS production. As expected (2), incubation of adult rat cardiomyocytes with HG concentrations (HG, 21 mM glucose) increased ROS production (Fig. 1). This HG-induced ROS production was almost completely inhibited by A769662 (12.5 μM) and phenformin (0.5 mM), two well-known AMPK activators (Fig. 1, A and B). Interestingly, GLP-1 also inhibited HG-induced ROS production. The effect was dose dependent and maximal between 100 and 300 nM (Fig. 1 C). Preincubation of cardiomyocytes with Exendin 9–39, a GLP-1 receptor antagonist, blocked the protective effect of GLP-1 on ROS production, indicating that this effect of GLP-1 was mediated via its receptor (Fig. 1 D).

AMPK phosphorylation and activation in cardiomyocytes exposed to hyperglycemia. Increased ROS production has been suggested to activate AMPK in various cellular models (53). Here, AMPK phosphorylation was actually slightly decreased in HG medium, after 2 and 24 h of incubation (Fig. 2 A). Notwithstading, AMPK activation by A769662 or by phenformin persisted under hyperglycemic conditions (Fig. 2 B) and was obviously not affected by the oxidative stress.

AMPK activation by GLP-1. Incubation of cardiomyocytes at 5 mM glucose with GLP-1 increased AMPK phosphorylation in a dose-dependent manner (Fig. 3 A), which started between 5 and 15 min and was maintained for at least 3 h (Fig. 3 B). This increased phosphorylation corresponded to AMPK activation, measured after PEG fractionation (Fig. 3 C). Accordingly, the phosphorylation of ACC, the bona fide substrate of AMPK, was also increased (Fig. 3 D). Measurement of AMPK activity after specific immunoprecipitation of α1-AMPK and α2-AMPK, the two catalytic subunits of AMPK expressed in the heart (41), demonstrated that α2-AMPK, but not α1-AMPK, was activated by GLP-1 (Fig. 3 E and F). The AMP-to-ATP ratio was unaffected by GLP-1 (Fig. 3 G), suggesting that GLP-1 activates AMPK without affecting the adenine nucleotide content. Remarkably, the inhibition of HG-induced ROS production was inversely proportional to the increased AMPK phosphorylation.

Fig. 1. Inhibition of hyperglycemia-induced reactive oxygen species (ROS) production by AMP kinase (AMPK) activators in adult rat cardiomyocytes (ARC). 12.5 μM A769662 (A) or 0.5 mM phenformin (Phen) (B) were added in the culture medium, 15 min before increasing glucose concentration (high-glucose, HG, 21 mM). ROS production was measured 2 h after HG. The data are means ± SE, n = 5. *Statistically different from normoglycemia. $Statistically different from the corresponding HG sample without treatment. C: correlation between increasing glucagon-like peptide (GLP)-1 concentration, AMPK activation (■), and inhibition of ROS production (□) under HG conditions. GLP-1 was added 15 min before HG. ROS production and AMPK phosphorylation on threonine 172 were measured 2 h after HG. The data are means ± SE, n = 4. *Statistically different from HG conditions without GLP-1. NG, normoglycemia. D: inhibition of GLP-1 effect by Exendin 9–39. 10 μM Exendin 9–39 and 100 nM GLP-1 were added 15 min before HG. ROS production was measured 2 h after HG. The data are means ± SE, n = 3. *Statistically different from normoglycemia. $Statistically different from the corresponding HG sample without treatment.
lation upon GLP-1 stimulation, indicating that both phenomena could be causally related (Fig. 1C).

As AMPK controls glucose metabolism in the heart, we wondered whether AMPK activators, i.e., A769662, phenformin, and GLP-1, could modify glucose uptake and glycolysis under normal and HG conditions. Only phenformin stimulated glucose uptake and glycolysis, whereas A769662 and GLP-1 remained without any effect in normoglycemia (Fig. 4, A and B). Phenformin was indeed the only AMPK activator able to induce Glut4 translocation to the plasma membrane by contrast with A769662 and GLP-1 (Fig. 4C). No modification in Glut4 content was observed after AMPK activation (Fig. 4D). As expected, incubation of cardiomyocytes under HG conditions stimulated glucose uptake and glycolysis. Here again, phenformin was able to further increase glucose uptake, whereas A769662 or GLP-1 had no effect (Fig. 4, A and B). This reinforced the idea that HG-induced ROS production is not entirely related to glucose metabolism.

**Crosstalk between NOX2 and AMPK signaling pathways.** Hyperglycemia triggers Rac1 activation and the translocation of p47phox to the plasma membrane, which leads to NOX2 activation and subsequent ROS production (2). Because caveolae seem to be required for the HG-induced PKC-β2 activation (22), we investigated the interplay between caveolae, NOX2 activation, and the subsequent ROS production. Disruption of the caveolar structure by methyl-β-cyclodextrin prevented HG-induced ROS production (Fig. 5A), thus suggesting that caveolae could be crucial for the HG-induced ROS production. Accordingly, using in situ proximity ligation assay (PLA), we demonstrated that p47phox subunit was translocated close to caveolin-3 (Cav3) in response to HG (Fig. 5B). These data strengthen the notion that an HG signalosome is located in caveolae. Moreover, α-methyl-d-glucopyranoside (AMG), a nonmetabolizable glucose analog that is transported by a SGLT-type of transporter and known to mimic HG (2), induced p47phox translocation as well (Fig. 7B). Although A769662, phenformin, and GLP-1 did not exert similar action on glucose uptake, they all blocked p47phox translocation close to Cav3 (Fig. 5, B–D). Similarly, GLP-1 treatment counteracted p47phox translocation in response to HG (Fig. 5, E and F). The inhibitory effect of AMPK activators could not be explained by any modification in p47phox transcription or protein content (Fig. 6, A and B). GLP-1 (Fig. 7, A–C) as well as A769662 and phenformin (data not shown) inhibited the HG-mimicking effect of AMG. It also blunted NOX2 activation by angiotensin II (Fig. 7, D–F). Taken together, the data indicate that AMPK activation, either by GLP-1 or other AMPK activators, blocks a key signaling element required for NOX2 activation in the caveolar structure, whatever the stimulus. Accordingly, phosphorylated form of AMPK could be detected close to Cav3 (data not shown).

To demonstrate that AMPK activation indeed inhibits HG-induced p47phox translocation, we expressed a constitutive active form of α2-AMPK (CA-AMPK) by adenoviral infection. Adenovirus-expressing GFP was used as a control. We verified that GFP did not interfere with the PLA signal and more importantly that adenovirus infection (100 multiplicities of infection, 48 h) did not affect HG-induced p47phox translocation (Fig. 8A). CA-AMPK is a truncated form of AMPK containing c-myc tag. It could be detected after a 48-h infection by immunoblotting using c-myc antibody as already described (Fig. 8B) (6). At this time point, CA-AMPK expression increased ACC phosphorylation and prevented HG-induced p47phox translocation (Fig. 8, B–D).

**α2-AMPK is required for the inhibition of ROS production by GLP-1.** To demonstrate whether AMPK was required for the inhibition of ROS production by GLP-1, we compared its effect in adult cardiomyocytes from wild-type or α2-AMPK knockout mice. Under HG conditions, mouse cardiomyocytes exhibited a p47phox translocation close to Cav3 (Fig. 9, A and B). The HG effect could be mimicked by AMG, as it is the case in rat adult cardiomyocytes (data not shown). Cardiomyocytes from α2-AMPK knockout mice were exposed to HG and compared with α2-AMPK wild-type cells. HG-induced

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**Fig. 2.** AMPK phosphorylation and activation under hyperglycemia in ARC. A: effect of short-term (2 h) and long-term (24 h) hyperglycemia on basal AMPK phosphorylation on threonine 172 (pThr172-AMPK). B: effect of long-term hyperglycemia on AMPK activation by 12.5 μM A769662 or 0.5 mM Phen. Cardiomyocytes were incubated for 24 h in HG medium before A769662 or Phen treatment. AMPK phosphorylation was evaluated 2 h after treatment. The data are means ± SE, n = 4. *Statistically different from the corresponding untreated sample.
AMPK-mediated inhibition of ROS production by GLP-1

DISCUSSION

The results reported here show that, in cardiomyocytes, the protective effects of GLP-1 against glucotoxicity are mediated by the α2-isofrom of AMPK and that AMPK activation, whatever the stimuli used, counteracts p47phox translocation to the plasma membrane. The protective effects of AMPK activators do not involve transcriptional regulation of NOX2 subunits. To our knowledge, this is the first demonstration that AMPK and, more specifically α2-AMPK, is a key element in the signaling pathway downstream of GLP-1 in adult cardiomyocytes. In this section, we discuss the beneficial effect of GLP-1, AMPK activation, and its interaction with NOX2 and ROS production.

p47phox translocation was similar in α2-AMPK−/− and α2AMPK+/* cardiomyocytes (Fig. 9, A–C). GLP-1 counteracted this translocation in α2-AMPK+/* cells (Fig. 9, A and B) but had no effect in α2-AMPK knockout cells (Fig. 9, A and C). These results definitively demonstrated that AMPK mediated GLP-1-protective properties against HG.

AMPK inhibits the PKC-β2-induced p47phox translocation. HG conditions are known to activate PKC-β2 in cardiac caveolae (22). In this last experimental approach, we studied whether PKC-β2 activation is responsible for p47phox translocation and NOX2 activation. Our data show that inhibition of PKCs by GF109203X counteracted the HG-induced ROS production in rat cardiomyocytes (Fig. 10A). LY379196, a specific PKC-β2 inhibitor (50), blocked the HG-induced ROS production (Fig. 10B) as well as PKC phosphorylation (Fig. 10C) and the subsequent p47phox translocation in response to HG (Fig. 10, D and E). This strongly suggests that PKC-β2 exerts a crucial role in the HG-induced p47phox translocation.

Finally, AMPK activation by GLP-1 could inhibit the glucose-induced activation of PKC-β2. Indeed, GLP-1 decreased the HG-induced phosphorylation of PKC in rat cardiomyocytes (Fig. 10F) as well as in mouse cardiomyocytes from α2-AMPK+/* heart (Fig. 9D). In agreement with our hypothesis, GLP-1 did not reduce PKC phosphorylation in α2-AMPK knockout cells (Fig. 9D).

Fig. 3. AMPK activation by GLP-1 in ARC. A: dose response of GLP-1 (from 0 to 1 μM) on AMPK phosphorylation (pThr172-AMPK). GLP-1 was added 2 h before AMPK phosphorylation was measured. The data are means ± SE, n = 5. B: time course of pThr172-AMPK after stimulation with 100 nM GLP-1. The data are means ± SE, n = 3. C: effect of 100 nM GLP-1 on total AMPK activity, 2 h after treatment. The data are means ± SE, n = 5. D: acetyl-CoA carboxylase (ACC) phosphorylation on serine 79, 2 h after stimulation with 100 nM GLP-1. α1-AMPK (E) and α2-AMPK (F) activity measured 2 h after stimulation with 100 nM GLP-1. The data are means ± SE, n = 6. *Statistically different from the corresponding untreated sample. G: AMP to ATP ratio was measured 2 h after treatment with 100 nM GLP-1. The data are means ± SE, n = 6. 1 μM oligomycin was used as positive control for AMP/ATP ratio (not presented).
Fig. 4. Stimulation of glucose uptake (A), glycolysis (B), and Glut4 translocation to the plasma membrane (C) in response to AMPK activators in ARC. A and B: glucose uptake and glycolysis following incubations with 12.5 μM A769662, 0.5 mM Phen, or 100 nM GLP-1 in normoglycemia and hyperglycemia. Glucose uptake and glycolysis were measured 2 h after HG. AMPK activators were added 15 min before HG. The data are means ± SE, n = 4 (A), n = 6 (B). $Statistically different from untreated sample. *Statistically different from normoglycemia. Glut4 translocation to the plasma membrane (C) and Glut4 content (D) were evaluated under normoglycemia, 2 h after 12.5 μM A769662, 0.5 mM Phen, and 100 nM GLP-1. C: arrow indicates Glut4 translocated to the plasma membrane [detected by an anti-hemaglutinin (HA)-Tag antibody]. The data are means ± SE, n = 5. *Statistically different from control. D: immunoblot is representative of 3 independent experiments.
Fig. 5. Effect of AMPK activators on subcellular distribution of p47phox in response to hyperglycemia in ARC. A: caveolar structure disruption on HG-induced ROS production. Cardiomyocytes were treated with 100 μM methyl-β-cyclodextrin (MBCD) 15 min before HG. ROS was measured 2 h after HG. The data are means ± SE, n = 3. *Statistically different from normoglycemia. $Statistically different from the corresponding HG sample without MBCD.

B–F: effect of AMPK activators on p47phox and Cav3 colocalization in response to hyperglycemia. B: effect of 12.5 μM A769662 and 0.5 mM Phen on HG-induced p47phox colocalization with Cav-3, as detected by proximity ligation assay (PLA), indicating a translocation to the plasma membrane. Typical pictures of the effect of A769662 or of Phen are shown in C and D, respectively. Quantification (E) and an example (F) of GLP-1-induced inhibition of p47phox translocation in response to HG (100 nM GLP-1). A769662, Phen, and GLP-1 were added in the culture medium 15 min before HG. PLA was performed 90 min after HG. The data are means ± SE, n = 5. *Statistically different from normoglycemia. $Statistically different from the corresponding HG sample without treatment.
**Beneficial effect of GLP-1.** The metabolic effects of GLP-1 are well known. In pancreatic β-cells, GLP-1 rapidly increases cyclic AMP and intracellular calcium, which in turn promotes insulin exocytosis in a glucose-dependent manner, thus contributing to glucose homeostasis. During the last years, GLP-1 has been used to treat patients with type 2 diabetes because these patients exhibit a reduced postprandial GLP-1 level although GLP-1 action is maintained (12).

GLP-1 also exerts some beneficial effects in the heart, which extend beyond its systemic metabolic effects. For example, GLP-1 treatment, either GLP-1 agonist or DPP-4 inhibitors, limits ischemic injury in perfused hearts in intact animals (8, 34, 52) as well as in cardiomyocytes (33). The data suggest that the phosphoinositide 3-kinase-Akt pathway is involved in this protective effect of GLP-1. An indirect effect of GLP-1, favoring insulin release in intact animal, is unlikely because the effect is readily observed ex vivo, i.e., in cells in culture as well as in isolated hearts. Similarly in humans, GLP-1 significantly reduces the release of troponin I and the infarct size (25, 47). In addition, GLP-1 infusion results in a hemodynamic improvement in an animal model of nonischemic dilated cardiomyopathy (29) and reverses cardiac remodeling in high-fat diet mouse model (30).

**AMPK activation by GLP-1.** Our data show that α2-AMPK is activated in response to GLP-1. AMPK activation has been described in intact heart after a long-term treatment with GLP-1 (30, 44). Here, we report that AMPK activation by GLP-1 occurs in cardiomyocytes within 1 h, i.e., in the short term. More importantly, genetic tools allowed us to demonstrate that AMPK is required and mediates the protective effects of GLP-1.

AMPK activation could be responsible for some GLP-1 effects described above, such as the protection against ischemic injury, because α2-AMPK is known to be cardioprotective (49). Glycolysis stimulation by AMPK is one of the mechanisms explaining cardioprotection against ischemic damage conferred by this protein kinase (35). AMPK activators such as metformin or phenformin stimulate glucose uptake in cardiomyocytes. Unexpectedly, GLP-1 did not stimulate glucose uptake although it did activate AMPK. The lack of GLP-1 effect on glucose uptake is in agreement with data reported for the isolated perfused heart (28) but disagrees with others (51). The discrepancy could result from either a systemic effect of GLP-1 or a potentially insulin-sensitizing effect of AMPK activators (6, 16). In any case, the protective effect of GLP-1 that we observed in cardiomyocytes is not explained by a stimulation of glucose uptake and glycolysis. Similar results were recently obtained with A769662, another AMPK activator (43), which activates AMPK without any effect on glucose metabolism.

As regards the mechanism of AMPK activation by GLP-1, this effect is not related to an increase in AMP-to-ATP ratio. GLP-1 binding to its receptor could trigger a Gq signaling event leading to AMPK activation. A Gq-coupled receptor has indeed already been reported (20, 31). We can speculate that an increase in calcium concentration after GLP-1 stimulates CaMKK-β, which, in turn, activates AMPK. Our preliminary results showed that AMPK activation by GLP-1 was decreased by STO609, an inhibitor of CaMKK-β (data not shown). However, these data should be confirmed and include an in-depth analysis of CaMKK-β in cardiomyocytes.

**Relationship between AMPK activation and ROS production.** In certain cells, increased ROS production could result in AMPK activation. Indeed, specific NOX2 overexpression in endothelial cells activates AMPK, inducing NO synthase activation (37). Similarly, in H9C2 cardiomyoblasts, mitochondrial ROS generation in response to nitrite exposure also activates AMPK (21). However, we did not observe such a ROS-dependent AMPK activation in cardiomyocytes incubated under HG conditions. Under these conditions, ROS production tended to decrease AMPK phosphorylation as already reported (17). By contrast, pharmacological activation of AMPK using A769662, phenformin, and GLP-1 counteracted the ROS-induced AMPK activation by avoiding permeability transition pore opening (32) or by promoting mitochondrial biosynthesis through proliferator-activated receptor-γ coactivator-1α. AMPK also inhibits ROS-induced mitochondrial fission and collapse of the mitochondrial membrane potential (7). Finally, AMPK promotes autophagy, minimizing oxidative stress-induced cellular damage by phosphorylating ULK-1 or inhibiting p70S6 kinase phosphorylation (38). NOX2 activation precedes and/or triggers mitochondrial ROS production and other oxidative damages in response to hyperglycemia (39). Here, we show that AMPK

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Fig. 6. Effect of AMPK activators on p47phox expression in ARC. Quantitative analysis of mRNA (A) and protein (B) expression of p47phox, 2 h after 12.5 µM A769662, 0.5 mM Phen, and 100 nM GLP-1. A: mRNA expression was normalized to hypoxanthine guanine phosphoribosyl transferase 1 after ΔΔCt correction. The data are means ± SE, n = 6. B: immunoblot is representative of 3 independent experiments.
Fig. 7. Inhibitory effect of GLP-1 on NADPH oxidase 2 (NOX2) activation induced by nonmetabolizable glucose analog or angiotensin II in ARC. 16 mM α-methyl-D-glucopyranoside (AMG) (A–C) or 1 μM angiotensin II (AgII, D–F) were used to activate NOX2 and mimic HG. NOX2 activation was evaluated by ROS production (A and D) and by p47phox translocation (B and E) after 2 h and 90 min of treatment, respectively. 100 nM GLP-1 was added in the culture medium 15 min before AMG or AgII. C and F: typical picture of p47phox translocation under AMG (C) or AgII (F). The data are means ± SE, n = 4. *Statistically different from untreated samples. $Statistically different from the corresponding sample in the absence of GLP-1.
activation by GLP-1 blocks an early event in the pathway that leads to glucotoxicity before any mitochondrial protection or transcription of antioxidant genes.

**Crosstalk between AMPK and NOX2.** Several lines of evidence support the notion that AMPK antagonizes NADPH oxidase activation. In endothelial cells, AMPK decreases the ability of NOX2 to be activated in the long term. It reduces the expression of activating subunits. Indeed, α2-AMPK deletion increases expression of p47phox, p67phox, and gp91phox in endothelial cells due to Ik-Bα degradation inducing NF-κB translocation, whereas AMPK activation by 5-aminoimidazole-4-carboxamide 1-β-n-ribofuranoside (AICAR) does the opposite (46). The absence of α1-AMPK drastically increases NOX2 expression in response to angiotensin II (36) whereas AMPK activation counteracts angiotensin II action, potentially related to NOX2 (42). At least in endothelial cells, both AMPK isoforms act as a brake against potential excessive ROS production through NADPH oxidase. Moreover, AMPK activation could inhibit NOX2 activation by reducing p47phox translocation to the plasma membrane although it has never been clearly investigated in the heart. For example, in human neutrophils, AMPK activator (AICAR) attenuates phorbol 12-myristate 13-acetate-induced p47phox phosphorylation and translocation to cell surface (1). In endothelial cells, AMPK activation using rosiglitazone counteracts hyperglycemia-induced p47phox translocation to the plasma membrane although this effect appears after 24 h of treatment (10).
The current hypothesis is that AMPK activation by GLP-1 blocks the hyperglycemia-induced PKC activation. This is supported by our results showing that the effect of GLP-1/AMPK on PKC is lost in α2-AMPK knockout cells. A recent study in endothelial cells reinforces this interpretation (3).

Finally, it has to be noted that GLP-1 could also affect NOX2 via a mechanism that is independent of AMPK. GLP-1 prevents increase in NOX2 observed in diabetes via a PKA-Rho-dependent pathway in microvascular endothelial cells (45).

**Conclusion.** In the heart, GLP-1 activates the α2-isoform of AMPK, which then inhibits the hyperglycemia-induced NOX2 activation by limiting PKC phosphorylation and p47phox translocation to the caveolae. Our results show that GLP-1 blocks early signaling events responsible for gluco-toxicity.

**ACKNOWLEDGMENTS**

We are grateful to N. Marquet and A. Ginion for technical assistance. We acknowledge Eli Lilly for providing LY379196 and Dr. Corley Mastick (Department of Biochemistry and Molecular Biology, University of Nevada) for providing adenovirus-expressing HA-Glut4-GFP protein.

**GRANTS**

This work was supported by grants from the Fonds National de la Recherche Scientifique (FNRS), Belgium, and by grants from ‘Action de Recherche
Fig. 10. Role of PKC-β2 in hyperglycemia-induced NOX2 activation in ARC. 10 μM GF109203X (nonspecific PKC inhibitor, A) and 10 nM LY379196 (PKC-β2 inhibitor, B) were added to the culture medium 15 min before HG. ROS production was measured 2 h after HG. The data are means ± SE, at least n = 5. *Statistically different from normoglycemia. $Statistically different from the corresponding HG sample without treatment. C: immunoblot confirming the inhibition by 10 nM LY379196 on HG-induced PKC phosphorylation. D and E: effect of 10 nM LY379196 (LY) on p47phox colocalization with Cav3, in response to HG. Typical pictures (D) and quantification (E) of the effect of LY379196 on p47phox translocation in response to HG. Data are means ± SE, n = 4. *Statistically different from normoglycemia. $Statistically different from the corresponding HG sample without treatment. F: cardiomyocytes were treated with 100 nM GLP-1 for 15 min before HG. PKC phosphorylation was evaluated 90 min after HG. The data are means ± SE, n = 4. *Statistically different from normoglycemia. Extracts from cardiomyocytes treated with PMA were used as a positive control (C+).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


