Oxytocin Increases Glucose Uptake in Neonatal Rat Cardiomyocytes

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We have recently shown that an entire oxytocin (OT) system, a peptide and its cognate receptors, is synthesized in the heart. In fetal and newborn hearts, OT exists in its extended three-amino acid form, OT-Gly-Lys-Arg (OT-GKR). OT translocates glucose transporter type 4 to the plasma membrane in human endothelial cells. Therefore, we hypothesized that the cardiac OT/OT-GKR system may be involved in the regulation of myocardial glucose uptake in physiological conditions and during metabolic stress such as hypoxia. Primary cultures of neonatal rat cardiomyocytes (CM) and cardiac progenitor cells expressing ATP-binding cassette efflux transporter G2 transporter (stem cell marker) were studied. OT (10 nM) increased basal glucose uptake in CM to 4.0 $\pm$ 0.2 fmol/mg protein, with OT-GKR (10 nM) elevating it to 5.3 $\pm$ 0.4 fmol/mg protein ($P < 0.001$) in comparison with 2.2 fmol/mg in control cells. OT had a moderate synergistic effect with 0.1 mM 2,4-dinitrophenol, augmenting basal glucose uptake to 5.5 $\pm$ 0.5 fmol/mg. OT-GKR (10 nM) was even more potent in combination with 2,4-dinitrophenol, increasing glucose uptake to 9.0 $\pm$ 1.0 fmol/mg. Wortmannin (0.1 μM), an inhibitor of phosphatidylinositol-3-kinase, significantly suppressed the effect of OT and insulin (10 nM) ($P < 0.001$), indicating common pathways. Our data suggest that OT and OT-GKR influence glucose uptake in neonatal rat CM and may thus play a role in the maintenance of cardiac function and cell survival during metabolic stress. **(*Endocrinology* 151: 482–491, 2010)**

Oxytocin (OT), originally considered to be a pregnancy hormone produced primarily in paraventricular and supraoptic nuclei of the hypothalamus, is also expressed in many organs, including insulin (INS)-sensitive tissues such as adipocytes, skeletal and cardiac muscles, the pancreas, and endothelial cells (1, 2). The OT system helps regulate responses to stress and injury, growth and development, nutrient absorption, energy metabolism, and hydromineral homeostasis (1). OT administration reduces feeding (3), and an obese phenotype is observed in male OT receptor (OTR) knockout (KO) mice (4). However, it has been also found that OT can increase feeding behavior (5). Furthermore, an OT deficit has been implicated in Prader-Willi syndrome, characterized by abnormal body composition and progressive obesity with increased cardiovascular morbidity and mortality (6). It has been previously observed that OT exerts some INS-like activity (7, 8). Experiments in dogs made diabetic with streptozotocin demonstrated that INS injections enhanced OT secretion, whereas elevated plasma glucose levels fell to control values (9). These findings indicated an OT link to INS-mediated glucose uptake in the body. Furthermore, it was recently shown that OT treatment increased glucose uptake in skeletal muscles precursor cell lines (10).

OT is primarily synthesized in magnocellular cells of the supraoptic nucleus and paraventricular nuclei of the hypothalamus (11). The initial translation product from the OT gene was identified as OT-Gly-Lys-Arg (OT-GKR)-neurophysin I. Neurophysin I is cleaved, and proteolytic cleavage of OT-GKR tripeptide by carboxypeptidase results in the release of OT-GK and OT-G. OT-G is converted by the α-amidating enzyme to mature amidated OT. The peptides OT-GKR (OTX), OT-GK, and OT-G are referred to as carboxy-extended forms of OT (12).

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Abbreviations: ABCG2, ATP-binding cassette efflux transporter G2; AMPK, AMP-activated protein kinase; AVP, vasopressin; Ca-CAMKK, calcium-calmodulin kinase kinase; CM, cardiomyocyte; DNP, 2,4-dinitrophenol; FBS, fetal bovine serum; INS, insulin; KO, knockout; OT, oxytocin; OTA, OT antagonist; OT-GKR (OTX), OT-Gly-Lys-Arg; OTR, OT receptor.

Besides several aberrations in social behaviors, OTR−/− mice display novel physiological defects, including obesity and dysfunction in body temperature (13). Compared with wild-type cohorts, conditional OTR KO mice (14) did not show increased sucrose consumption, which had been reported previously in OT KO mice (15). However, the OT system KO models cannot provide a complete explanation of the OT role in metabolic and cardiovascular functions, because vasopressin (AVP) may compensate for the lack of OT deficiency. There is ample literature showing that cross talk between these two hypophyseal systems exists *in vivo* (16, 17).

The heart preferentially oxidizes fatty acids in aerobic conditions. However, under metabolic stress triggered by hypoxia or ischemia, glucose utilization is increased, which protects cardiomyocyte (CM) viability and heart work (18). Consequently, a new therapeutic approach promoting glucose uptake by the heart exposed to metabolic stress is of great importance. Work from our lab has demonstrated OT and OTR expression in the rat and human heart. Furthermore, OT induces the secretion of cardioprotective factors involved in cardiac cell metabolism: atrial natriuretic peptide (19, 20) and nitric oxide (21). OT appears to be a cardiomyogenic factor (22). It stimulates Ca^{+2} mobilizations in differentiated CM produced from D3 embryonic stem cells (23). Interestingly, this effect is even greater in response to the extended form of OT, OT-GKR. The latter is the C-terminal amino acid extension of OT that is produced abundantly in the fetal hypothalamus (24) and rat uterus during parturition and early postpartum (25). In the present study, we investigated the hypothesis that OT evokes glucose uptake in neonatal rat CM under normal physiological conditions in the presence and absence of INS and, second, we assessed OT and OT-GKR actions in hypoxia induced by 2,4-dinitrophenol (DNP), a mitochondrial oxidative chain uncoupler that decreases adenosine-triphosphate synthesis (26). To understand the mechanism of OT-induced glucose uptake, we followed the pathway of INS signaling because it is known to act on glucose metabolism in parallel with OT (9).

Materials and Methods

Cell culture media and supplements were purchased from Invitrogen, Canada Inc. (Burlington, Ontario, Canada), unless otherwise stated. Fetal bovine serum (FBS; 080450) was obtained from Wisent Inc. (Quebec, Canada). OT (O6379), STO-609 acetic acid (S1318), Wortmannin (W1628), DNP (D198501), and Hoechst dye (bisBenzimide H 33342 trihydrochloride, no. B2261) were obtained from Sigma (St. Louis, MO) or Supelco (Bellefonte, PA) (40057). OT-GKR (Gly^{10}, Lys^{11}, Arg^{12}, 43232_1) was synthesized by GenScript Corp. (Piscataway, NJ). Human recombinant zinc INS (12585-014) was purchased from Invitrogen. ($d(CH_2)_5{}^1$, $Tyr(Me)^2$, Thr^4, Orn^8, Tyr-$NH_2{}^9$)-Vasotocin β-Mercapto-β,β-cyclopentamethylene-propionyl-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Tyr-NH_2 (H-9405) (OT antagonist, OTA), AVP receptor antagonists: V2R-antagonist (1-Adamantaneacetyl^1, D-$Tyr(Et)^2$, Val^4, Abu^6, $Arg^{8,9}$)-AVP (H-7705), and V1R-antagonist ($d(CH_2)_5{}^1$, $Tyr(Me)^2$, Arg^8)-AVP (Manning compound) (H-5350) were obtained from Bachem (Peninsula Labs, Torrance, CA). InSolution AMP-activated protein kinase (AMPK) inhibitor Compound C (171261) was obtained from Calbiochem (La Jolla, CA) and 2-deoxy-D-[1-^{3}H] glucose 1 mCi/1.0 ml (TRK383) from Amersham (Buckinghamshire, UK). Protease inhibitor cocktail tablets (11 836 153 001) were obtained from Roche (Laval, Quebec, Canada) and MACS separation columns (130-042-401) from Miltenyi Biotec (Auburn, CA).

The antibody for cell magnetic separation, ATP-binding cassette efflux transporter G2 (ABCG2) D-20 (sc-25156) goat polyclonal IgG, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and R-Phycoerythrin-conjugated AffiniPure $F(ab')_2$ fragment rabbit antigoat IgG (305-006-006) was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Primary antibodies for Western blotting were from Cell Signaling Technology (Danvers, MA), and included antiphospho-phosphatidylinositol-3-kinase (PI3K) p85 (Tyr458)/p55 (Tyr199) (no. 4228), and anti-PI3K p85 (19H8) rabbit mAb (no. 4257). Monoclonal antiactin clone AC-40 (A4700) was obtained from Sigma. Positive control lysate 3T3/Src was a generous gift from New England Biolabs (Pickering, Ontario, Canada). Secondary antibodies, goat antirabbit IgG (no. 111-035-144) and goat antimouse IgG (no. 115-035-146), were obtained from Jackson ImmunoResearch Laboratories. Protein molecular weight SeeBlue Plus2 Pre-Stained Standard (LC5925) was obtained from Invitrogen. The Amersham enhanced chemiluminescence plus Western blotting detection system (RPN2132) was purchased from GE Healthcare (Sunnyvale, CA) and Progene autoradiography films (no. 39-20810) were obtained from Ultident Scientific (St. Laurent, Quebec, Canada).

Primary goat OTR antibodies for immunostaining (C-20) (sc-8102) and flow cytometry staining (N-19) (sc-8103), as well as normal goat IgG (sc-2028), were obtained from Santa Cruz Biotechnology. AlexaFluor594 donkey antigoat IgG (H+L) (A-11058) was obtained from Molecular Probes (Eugene, OR). The cardiac cell marker mouse prediluted monoclonal cardiac Troponin-T [SPM476] (ab27217) was obtained from Abcam (Cambridge, MA), and antimouse IgG (I-2000) from Vector Laboratories Inc. (Burlingame, CA). Finally, donkey antimouse AlexaFluor594 (A21203) was obtained from Molecular Probes.

Primary cultures of neonatal rat CM

All experiments conformed to guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Centre Hospitalier de l'Université de Montréal. We used the Neonatal Cardiomyocyte Isolation System (no. LK003300; Worthington, Lakewood, NJ). Primary CM cultures were prepared from ventricles of 1- to 4-d-old Sprague Dawley rats from two litters to ensure heterogeneity of the cell population. The pups were killed by decapitation with sterile scissors, and their beating hearts surgically removed and immediately placed in ice-cold Hanks' balanced salt solution. In two 60-mm Petri dishes, the hearts were minced into small pieces with a pair of razor blades and digested overnight by trypsinization at 4 C. The next morning, the tissue preparation was exposed at 37 C to the trypsin inhibitor for 10 min, then digested for 45 min with collagenase reconstituted in Leibovitz L-15 medium under

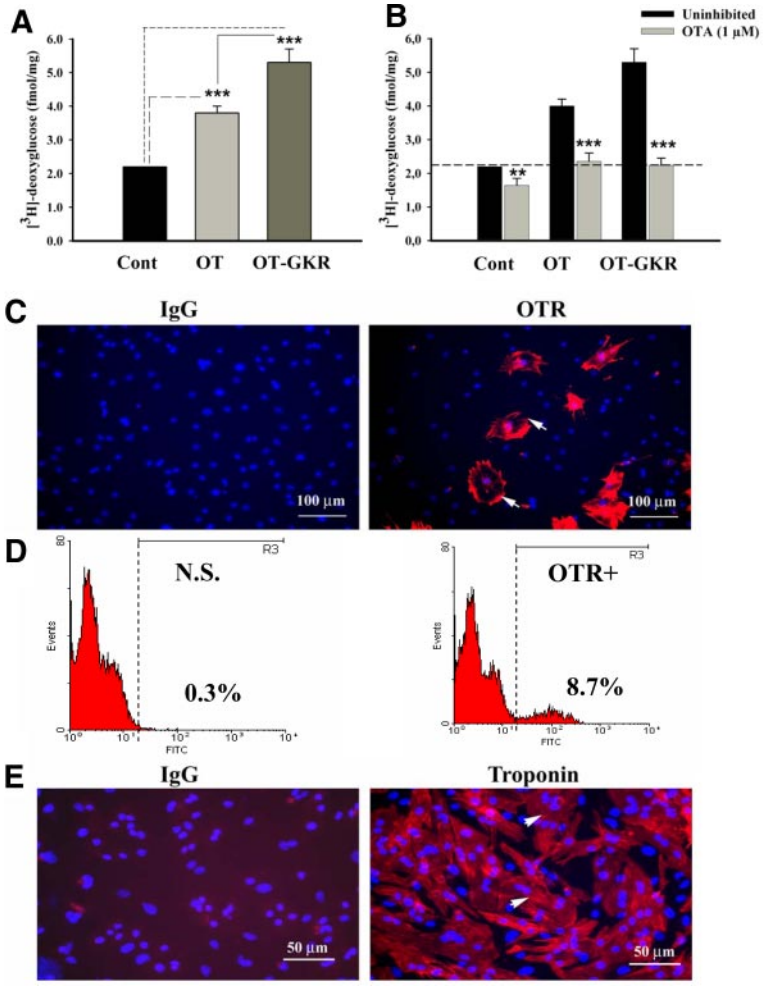

FIG. 1. OT and OT-GKR increase glucose uptake in CM through OTR. Subconfluent primary cultures of neonatal rat cardiac cells were stimulated for 30 min in a milieu without glucose by OT (10 nM) or its elongated form OT-GKR (10 nM) (A), or were pretreated for 30 min with 1 μM of an OTA (B), and were then exposed for the next 30 min to the uptake of radio-labeled glucose (**, $P < 0.01$; ***, $P < 0.001$). Cells were stained for the expression of cell surface OTR by immunofluorescence with primary goat anti-OTR antibody (1:30) (*arrowheads*, *right panel*), or with goat IgG (1:60) for negative controls and donkey antigoat (1:200) secondary antibody emitting red fluorescence. Hoechst dye was used to stain nuclei (*blue dots*) (C). The quantitative representation by flow cytometry is shown in panel D. Single cell suspension was stained for OTR (*right panel*) or with normal IgG as a negative control (*left panel*) and analyzed by flow cytometry. The cells were stained for the cardiac marker Troponin-T (*arrowheads*, *right panel*) or mouse IgG (1:1500) emitting red fluorescence for negative control (E).

slow shaking at 3 rpm at 37 C. Cells were then dispersed by trituration, filtered through a cell strainer, sedimented, centrifuged at 1,000 rpm for 5 min, and the supernatant removed. They were resuspended in DMEM-low-glucose culture medium with L-glutamine (no. 11885-084) containing penicillin-G (100 U/ml), streptomycin sulfate (100 μg/ml), amphotericin B (Fungizone) (0.25 μg/ml) (no. 15240-062), and 10% FBS before plating in 150-mm Petri dishes for 30-min incubation, which allowed selective attachment of nonmyocytes, mostly fibroblasts. Neonatal rat CM remained in the supernatant and separated from attached fibroblasts, which were used in other experiments or discarded. This step was repeated twice. The CM-enriched suspension was removed from the culture dishes, cells were counted, and viability was assessed by the Trypan blue dye exclusion method. This procedure allows pure cultures of CM to be obtained by fractionation. A portion of the cells was plated for further culturing and another portion used for sorting. The purity of cardiac cells was assessed by fluorescence staining with antibodies specific for the CM marker Troponin-T (Fig. 1E).

Glucose uptake

Cells were seeded in 12-well plates at 1.25×10^5 cells/well ($\sim 3.3 \times 10^4$ cells/cm^2) in DMEM-low glucose medium with 10% FBS and 1% antibiotic-antimycotic and cultured at 37 C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed after 24 h, then every 48 h. Beating cells appeared after 2 d in culture. After reaching subconfluency on d 5–6, cells were starved overnight in the same medium but containing only 0.5% FBS. On the day of the experiment, they were rinsed twice with serum-free DMEM-no glucose (no. 11966-025) and incubated at 37 C in 0.9 ml of medium for 30 min. Then, drugs were added and the cells incubated at 37 C for 30 min. The administration of inhibitors or antagonists preceded treatment with agonists by 15–30 min. 0.1 ml of radio-labeled glucose (0.5 μCi/well) was added per well and cells were further incubated at 37 C for 30 min. Glucose uptake was quenched by washing cells three times with ice-cold PBS. Cells were lysed in 1.0 ml of 0.5 M NaOH solution for 1 h at room temperature. Lysates were collected in scintillation vials in 4.0 ml of scintillation liquid, and each vial counted in a β-counter for 5 min. Glucose uptake was recorded as [^{3}H] counts/min and calculated as fmol/mg protein; for the experiment with ABCG2-labeled cells, the final results were expressed as the number of femtomoles/cell (GraphPad calculator; GraphPad, San Diego, CA).

Activation of PI3K phosphorylation

Freshly isolated primary CM were seeded in six-well plates at 3.0×10^5 cells/well ($\sim 3.1 \times 10^4$ cells/cm^2) in DMEM-low glucose medium with 10% of FBS and 1% antibiotic-antimycotic. The medium was changed after 24 h, then every 48 h. Beating cells appeared after 2 d in culture. After reaching subconfluency on d 5–6, the cells were starved overnight in the same medium containing only 0.5% FBS. On the day of the experiment, cells were incubated without serum for up to 2 h in DMEM-no glucose, then switched to serum-free DMEM-high glucose (no. 11960) with designated stimulants and incubated for 45 min. Stimulation was terminated by washing adherent cells once with ice-cold PBS and lysing in 100 μl of lysis buffer [containing: HEPES 50 mM, sodium chloride 150 mM, sodium fluoride 100 mM, sodium pyrophosphate 10 mM, EDTA 5 mM, 1% Triton X-100, 0.25% sodium deoxycholate (pH 7.5), protease inhibitor cocktail tablet, and activated sodium orthovanadate 1 mM]. The cells were scraped on ice and kept frozen. Protein in the supernatants was quantified by the Bradford method.

Western blot analysis

Equal concentrations of proteins were resolved by 8% SDS-PAGE. Separated proteins were transferred to nitrocellulose

membranes, blocked with 5%-milk-TBST [Tris base 10 mM, NaCl 100 mM, and Tween 0.1% (pH 7.5)], and probed overnight with primary antibodies and peroxidase-conjugated secondary antibodies. Blots were incubated with enhanced chemiluminescence reagent and visualized on film.

ABCG2 cell magnetic separation

ABCG2 cells were isolated from the total CM population with magnetic beads. Briefly, freshly prepared CM (~100 million cells) were centrifuged at 3,000 rpm for 5 min, resuspended in 1,000 μl of MACS buffer, stained with 100 μl of the first ABCG2 antibody, and incubated on ice for 1 h. Cells were washed and centrifuged twice with 20 ml of buffer to remove unbound primary antibody. Then, they were resuspended in buffer with the secondary R-Phycoerythrin-conjugated antibody AffiniPure and incubated on ice in the dark for 14 min. The cells were then washed with 20 ml of buffer and spun twice. Filtered cells were separated magnetically by placing columns in the magnetic field. The columns were washed three times with 3 ml of buffer, and the effluent was collected as $ABCG2^-$ unlabeled cell fractions and kept on ice. ABCG2-positive cells were collected in 5 ml of buffer and immediately flushed by the plunger supplied with the column. The 8×10^4 cells ($\sim 4 \times 10^4$ cells/cm^2) were plated in 24-well plates for primary cells, then cultured in DMEM-low glucose supplemented with 10% FBS and 1% antibiotic-antimycotic. They were cultured for 8–9 d. ABCG2+ cells did not show signs of beating at the end of this period.

Statistical analysis

Values are presented as means ± SD. The results are representative of a minimum of two to three biological experiments of four replicates each. For each replicate, separate wells were exposed to the same treatment during testing. "N" represents the total number of replicates for all experiments. The statistical significance of differences between experimental conditions was identified by one- or two-way ANOVA and finalized by all pair-wise multiple comparison procedures (Student-Newman-Keuls method). Differences were considered to be significant at $P < 0.05$.

Results

OT and OT-GKR stimulate glucose uptake in CM

OT increased basal glucose uptake in CM from 2.2 to 4.0 ± 0.2 fmol/mg protein ($P < 0.001$). Interestingly, glucose uptake in response to OT-GKR treatment (5.3 ± 0.4 fmol/mg) increased more effectively (by 35%) than by OT ($P < 0.001$) (Fig. 1A). A highly selective OTA significantly decreased glucose uptake stimulated by both molecular forms of OT (Fig. 1B), indicating that OTRs are involved in this process. It is noteworthy that the OTA significantly reduced basal glucose uptake in CM from 2.2 to 1.64 ± 0.21 fmol/mg ($P < 0.01$), suggesting a physiological role of the endogenous OT system in cardiac glucose uptake. Because OT can cross-react through AVP receptors, we used AVP receptor V1R and V2R antagonists (100 nM) to examine their involvement in the glucose uptake induced by OT or OT-GKR. We noted a lack of effect of both antagonists on glucose uptake induced by OT. However, the OT-GKR effect was inhibited in part by V1R antagonist (~60%).

The presence of specific OTR on the CM surface provides additional evidence of the potential of OT induction on glucose uptake by these cells. To address the presence of OTR in CM, two sets of antibodies were used depending on the particular experiment. To detect OTR by immunocytochemistry and Western blot analysis, we used a specific antibody against the epitope mapping at the C terminus of OTR of human origin (C-20). Western blot analysis of this OTR antibody in cardiac and uterine rat tissue showed one band of molecular mass approximately 66 kDa. Experimental references for this antibody have been published previously (22, 27). No reaction was obtained with AVP receptor (V1a) recombinant protein, and no reaction was observed with AVP receptor V2. For FACS analysis of OTR in living cells, we used an OTR-specific antibody raised against a peptide sequence mapped near the N terminus of OTR. This antibody was used to detect the OTR in the rat brain medial preoptic area (28). As presented in Fig. 1C, 14.4 ± 1.3 of cells per field of the total population of 150.8 ± 8.5 cells were positively stained with OTR-specific antibodies (Fig. 1C, *right panel*). Flow cytometry (Fig. 1D) shows that about 5–10% cells expressed OTR on the cell surface, 8.7% in representative experiments compared with 0.3% by nonspecific (N.S.) fluorescence. Almost all cells in the culture were stained with antibodies specific for the CM marker Troponin-T (Fig. 1E). Given that only about 10% of CM were positively stained by OTR antibody, our data indicate the high potency of OT in stimulation of glucose uptake in these cells.

OT and OT-GKR increase glucose uptake by CM during metabolic stress induced by the hypoxic agent DNP

To mimic hypoxia in CM cultures, we used 0.1 mM DNP. As shown in Fig. 2A, glucose uptake by CM almost doubled after DNP stimulation compared with the untreated controls, from 2.2 to 4.0 ± 0.3 fmol/mg protein. In the presence of OT, glucose uptake by DNP-stimulated CM was further increased by 39.5% to 5.5 ± 0.5 fmol/mg ($P < 0.001$). OT-GKR was even more efficient in stimulating glucose uptake than DNP treatment (5.3 ± 0.4 *vs.* 4.0 ± 0.3 fmol/mg, respectively; $P < 0.01$) (Fig. 2B). We also found that OT-GKR increased glucose uptake in hypoxic conditions by 127.7% up to 9.0 ± 1.0 fmol/mg,

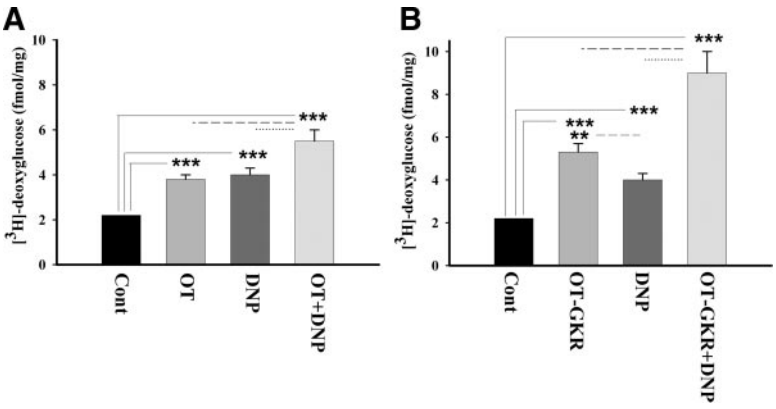

FIG. 2. Hypoxia-induced increase of glucose uptake in newborn rat CM is potentiated by OT and particularly by OT-GKR. DNP (0.1 mM), a hypoxia-inducing agent, enhances glucose uptake in newborn rat CM. This effect is potentiated by OT (10 nM) (A) and even more by OT-GKR (10 nM) (B) (**, $P < 0.01$; ***, $P < 0.001$).

compared with glucose uptake in hypoxic conditions alone.

OT mediates the increase of glucose uptake in CM via the INS signaling pathway

Figure 3 compares the OT effect with that of INS (10^{-8} M). INS alone elevated glucose uptake more potently in CM to 5.5 ± 0.4 compared with 3.8 ± 0.2 fmol/mg, which was 42% above that induced by OT ($P < 0.001$). There was no additive effect of OT in cultures cotreated with INS. In several cell phenotypes, PI3K signaling is associated with the critical pathways involved in INS-mediated glucose uptake (29–32).

To determine whether OT stimulates glucose uptake by this pathway, we blocked PI3K by a specific inhibitor, Wortmannin. Figure 3 shows that Wortmannin significantly suppressed glucose uptake stimulated by both OT and INS. Wortmannin also completely abrogated glucose uptake stimulated by OT-GKR (5.54 ± 0.51 *vs.* 2.00 ± 0.19 fmol/mg). However, glucose uptake increased by DNP was almost unaffected by Wortmannin treatment (6.31 ± 0.82 *vs.* 5.03 ± 1.17 fmol/mg) (data not shown). Thus, these findings suggest that OT heightens glucose uptake via an INS-like pathway.

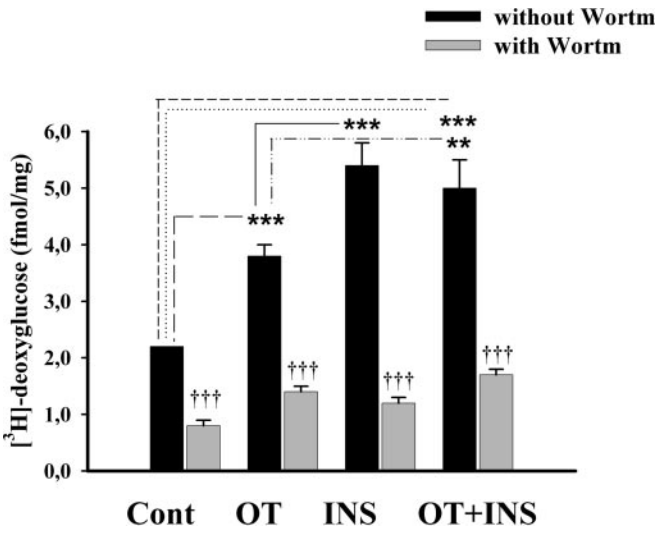

FIG. 3. OT mediates the increase in glucose uptake in newborn rat CM via an INS-like signaling pathway. Cells treated with OT (10 nM) or INS (10 nM) were assayed by radiolabeled glucose uptake. The PI3K-specific inhibitor Wortmannin (100 nM) was added 30 min before the agonists (two-way ANOVA; **, $P < 0.01$; ***, $P < 0.001$ or †††, $P < 0.001$).

PI3K is known to be involved in the mechanism of glucose transport (33) and glucose uptake through the INS receptor β phosphorylation (34). The question was whether the increase in glucose uptake by OT and OT-GKR would translate into PI3K phosphorylation. As seen in Fig. 4, after exposure to high glucose, both OT and OT-GKR triggered PI3K phosphorylation. Because INS acts via PI3K phosphorylation, it supports the existence of a common signaling pathway leading to increased glucose uptake in CM.

It has been shown that stimulators of AMPK, such as biguanides and oligomycin, enhance glucose uptake in INS-resistant CM and phosphorylate protein kinase B, a downstream kinase of PI3K. Furthermore, this process is blocked by Wortmannin (35). Thus, we next asked

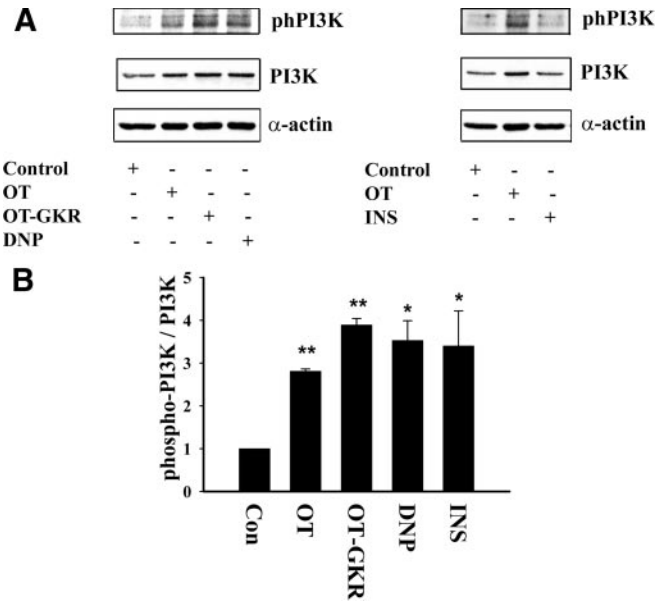

FIG. 4. OT and OT-GKR mediate the effects of increased glucose uptake in CM through PI3K phosphorylation. Short 45-min stimulation of CM with OT (10 nM), OT-GKR (10 nM), DNP (0.1 mM), or INS (10 nM) in the presence of high glucose specifically phosphorylated PI3K p85/p55 at tyrosine 458/tyrosine 199 (A, *top rows*). The endogenous level of total PI3K p85 protein on the same blots after stripping is shown in the *middle rows*. Blots were reprobed for α-actin (*bottom rows*) to verify equal loading. The *column graph* (B) reports optical density values related to those obtained from measurements of the band intensity of total PI3K (*, $P < 0.05$; **, $P < 0.01$).

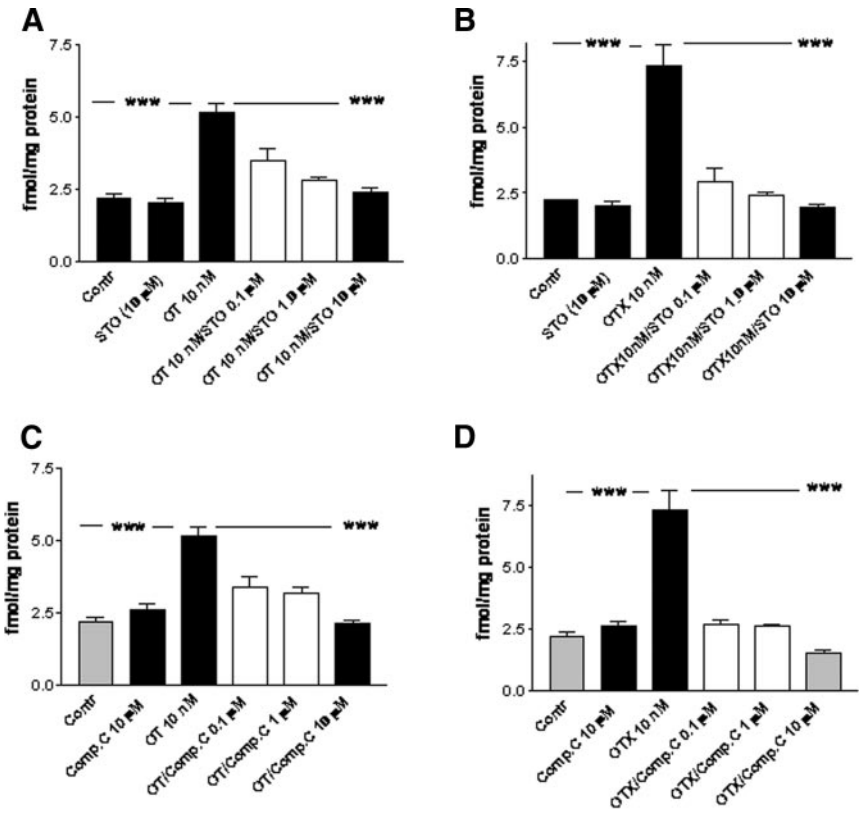

FIG. 5. Ca-CAMKK and AMPK pathways are implicated in OT/OT-GKR (OTX) system signaling. Starved CM were preincubated for 30 min with either the inhibitor STO-609 (0.1, 1.0, or 10 μM) (A and B) or the AMPK inhibitor Compound C (0.1, 1.0, or 10 μM) (C and D), and subsequently stimulated by OT (10 nM) or OT-GKR (OTX) (10 nM). Cells were assayed by glucose uptake (one-way ANOVA; ***, $P < 0.001$).

whether OT stimulation in newborn rat CM recruits the calcium-calmodulin kinase kinase (Ca-CAMKK)-AMPK pathway.

OT stimulates glucose uptake in CM through the Ca-CAMKK-AMPK pathway

To ascertain the roles of AMPK or Ca-CAMKK in OT/OT-GKR (OTX)-mediated signaling, we assessed the effects of two kinase inhibitors on glucose uptake. Pretreatment for 30 min with either STO-609 (Fig. 5, A and B) or Compound C (Fig. 5, C and D), an AMPK inhibitor, blocked OT-induced glucose uptake, confirming that both Ca-CAMKK and AMPK are involved in OT-induced glucose uptake.

OT and OT-GKR increase glucose uptake in purified ABCG2-positive CM

We examined putative cardiac stem cells from neonatal hearts to establish whether this exclusive fraction of CM expressing the ABCG2 transporter maintains the ability to elevate glucose uptake by the effects of OT and OT-GKR. Indeed, both OT and OT-GKR significantly increased ($P < 0.001$) basal glucose uptake by increasing the number of fmol/cell by $5.0 \times 10^5 \pm 9.1 \times 10^3$ and $4.23 \times 10^5 \pm 35.2 \times 10^3$, respectively, compared with untreated control cells, which corresponded to $2.26 \times 10^5 \pm 28.1 \times 10^3$ fmol/cell (Fig. 6). These cells vigorously resisted the metabolic stress induced by DNP and heightened glucose uptake by $7.83 \times 10^5 \pm 54.0 \times 10^3$ fmol/cell. The magnitude of the response to DNP in cells expressing ABCG2 marker represented a 3.5-fold increase, whereas the effect of DNP in whole CM represented 1.8-fold stimulation compared with their respective controls. We also noted that INS significantly enhanced basal glucose uptake in these cardiac stem cells from $2.26 \times 10^5 \pm 28.1 \times 10^3$ to $6.24 \times 10^5 \pm 14.5 \times 10^3$ fmol/cell ($P < 0.001$). OT-induced increases of glucose uptake in ABCG2 cells could be important for cell differentiation as well as cell regeneration.

Discussion

The primary finding of this study was that OT increased glucose uptake in newborn rat CM cultures. Optimal stimulation of glucose uptake was observed at 10^{-9} M OT and was not enhanced by increased OT concentration. At the same molar concentration, glucose uptake by CM was even more potently stimulated by

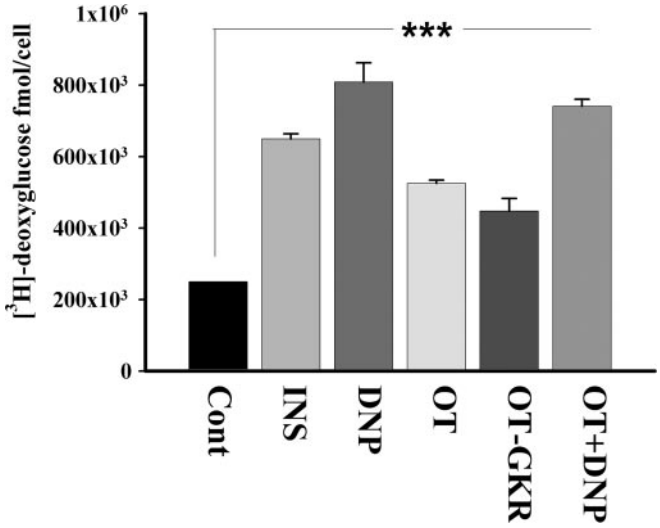

FIG. 6. Glucose uptake is stimulated in fractions of rat cardiac cells expressing the ABCG2 transporter. Glucose uptake by ABCG2-positive CM stimulated with OT (10 nM), INS (10 nM), DNP (0.1 mM), OT-GKR (10 nM), or OT+DNP was triggered by the addition of radiolabeled glucose (0.5 μCi/well) (two-way ANOVA; ***, $P < 0.001$).

the elongated precursor form of OT, OT-GKR. The effect was specific and blocked by an OTA but not by AVP antagonists, suggesting OTR involvement. Moreover, immunocytochemistry and flow cytometry analysis showed that OTRs are expressed on CM, although their presence was disclosed in only about 10% of the cells isolated from the heart. Because only a fraction of CM expressed immune OTR, we investigated whether OT stimulated glucose uptake in the CM subpopulation positive for the stem cell marker ABCG2. These cells responded to OT, INS, and hypoxic treatment with enhanced glucose uptake, compared with the total CM population. To gain an insight into the mechanisms of intracellular events, we demonstrated that PI3K, a well-established INS-signaling pathway, is involved in OT-stimulated glucose uptake in CM. Indeed, Wortmannin, the inhibitor of PI3K, reversed OT- and OT-GKR-induced glucose uptake. Furthermore, the results supported the involvement of the Ca-CAMKK and AMPK pathways, because the respective inhibitors of these enzymes, STO-609 and Compound C, blocked stimulated glucose uptake.

The OTA also inhibited glucose uptake at the basal level, suggesting a metabolic function of the cardiac OT system. The OT content in newborn rat CMs has been reported in studies from our laboratory (36, 37). Indeed, we have consistently demonstrated that OT content measured by RIA in CM culture to be approximately 228 ± 55 pg/ml, and that release of OT from CMs was linear over time at a rate approximately 1 pg/ml · min in a cell culture density of 1.25×10^5 cells/cm^2.

Although OTR in neonatal CM of ventricles are rare, OT/OT-GKR increased glucose uptake in CM with high potency. There are a few explanations for this observation. First, it is possible that only a CM subpopulation contains OTR and that this group includes ABCG2-positive cells and other cell subtypes involved in unknown functions. These cells may require the presence of OT or OT-GKR for increased glucose uptake in critical situations such as hypoxia. Another explanation is that immunoassay techniques are not sensitive enough to detect all functional OTR. Possibly, the localization of OTR in caveolae (38, 39) hampers antibody access to specific antigens. Furthermore, specialized regions, lipid rafts, and caveolae are found in plasma membranes enriched in cholesterol. Lipid rafts represent a membrane compartment in which the signaling of human OTR, a member of G protein-coupled receptors, may be specifically regulated (40, 41). It has been shown that cholesterol affects ligand binding to OTR in a highly specific manner. Two-fold-increased enrichment of OT-binding sites has been detected in cholesterol-rich, caveolin-containing domains of the plasma membrane (38). It is known that the membranes of caveolae invaginations remain continuous with the plasmalemma (42), thereby providing spatial continuity with the cell exterior; however, certain populations can detach to form discrete cytoplasmic caveolae vesicles of 80–100 nm in diameter (43, 44). Thus, OTR trapped in these vesicles cannot be detected by a specific antibody.

Our results indicate the potent ability of OT-GKR to stimulate glucose uptake in newborn rat CM. Hypothalamic posttranslational processing of the OT-neurophysin precursor involves the formation of C-terminally extended OT that serves as an intermediate prohormone. The initial translation product from the OT gene is OT-GKR-neurophysin I (23). During posttranslational processing, neurophysin I is cleaved, and proteolytic cleavage of OT-GKR tripeptide by carboxypeptidase results in the release of OT-GK and OT-G. Finally, OT-G is converted by α-amidating enzyme to mature amidated OT. Recent studies have suggested the potential physiological role of these intermediate forms. Elevated blood levels of extended OT molecules, but not of biologically-active, amidated OT, have been found in response to estrogen treatment and associated with blood pressure reduction (23). Interestingly, the concentration of these molecules in fetal sheep plasma was 35-fold higher than hormonally-active OT in the early stages of development (23). Similarly, the amidated OT form is not detectable in the fetal rat brain until embryonic d 21, despite abundant expression of the principal intermediate form, OT-GKR, during the same time period (23).

Despite abundant expression of the entire functional OT system in the developing heart (27), the function of OT prohormones remains unknown. Recently, we reported that the OT precursor molecule OT-GKR has a profound cardiomyogenic action on the D3 stem cell line that could be of physiological importance in cardiac development (23). Presently, we are providing evidence that, besides OT/OT-GKR's differentiation ability, such molecules have a substantial effect on metabolism. Glucose, the principal energy substrate for the fetus and newborn, is essential for normal fetal metabolism and growth (45). Fetal glucose utilization is increased by INS produced in growing amounts by the developing fetal pancreas as gestation proceeds, which enhances glucose utilization among INS-sensitive tissues, such as the heart, which increase in mass and thus need glucose during late gestation (46). Our data suggest a possible role of OT in this process, because OT molecules have a synergistic effect on glucose uptake stimulated by INS. We have also demonstrated that OT enhances glucose uptake stimulated by hypoxia. This observation points to a potential role of OT in providing enhanced glucose supply to the fetal heart in conditions of impaired placental function (47) and the possible use in the development of therapeutic strategies for the heart disease.

OT is a known modulator of INS release (48). For this reason, we explored whether OT acts through INS-acti-

vated pathways or by an INS-independent mechanism. There is growing evidence that PI3K plays a central role in INS signaling to metabolic pathways. The role of PI3K in INS-stimulated glucose transport has been established conclusively by the use of the PI3K inhibitor Wortmannin (49). PI3K has a pivotal role in the metabolic and mitogenic actions of INS, and it has been shown in 3T3-L1-adipocytes that PI3K is involved in the signaling pathways by which INS stimulates glycogen synthesis (50). PI3K activity has been reported to be significantly lower in obese subjects with impaired INS receptor signaling compared with healthy controls (30). PI3K is part of the MAPK cascade, as observed in Chinese hamster ovary fibroblasts stimulated by gastric inhibitory polypeptide, a glucose-dependent vasoactive insulinotropic polypeptide (51). Wortmannin inhibited OT-stimulated MAPK activity in INS-1 cells, an INS-secreting cell line (52). We further added to these results by ascertaining the involvement of PI3K in OT-stimulated glucose uptake in CM, which could be important in diabetic cardiomyopathies.

Lee *et al.* (10) reported that OT mediates glucose uptake in C2C12 cells. C2C12 cells are a useful model for the study of the differentiation of nonmuscle cells to skeletal muscle cells. This is an important limitation of their study, because skeletal muscles metabolize large amounts of glucose to fuel contraction. Therefore, the conclusion drawn from their experiments that OT stimulates glucose uptake in skeletal muscles remains to be completed. Because OT has been reported to activate Ca-CAMKK-AMPK in C2C12 cells, we investigated whether OT and OT-GKR induce this pathway in newborn rat CM. Because STO-609 or Compound C, an AMPK inhibitor, blocked OT-induced glucose uptake in CM, Ca-CAMKK and AMPK are possibly involved in the process. AMPK is a key kinase controlling many cellular processes, particularly pathways maintaining cellular energy status (53). AMPK is activated during metabolic stress, and not only induces a number of energy-producing metabolic pathways, but also inhibits energy-consuming pathways (53). In skeletal muscle (54, 55) and newborn rabbit hearts (56), stimulation of AMPK by decreases in circulating INS increases glucose and fatty acid oxidation. On the other hand, activated AMPK inhibits fatty acid and cholesterol synthesis, thereby reducing ATP-consuming anabolic pathways (57). It has also been shown that activation of AMPK increases glucose uptake, particularly in INS-resistant CM (35). The ability of OT to stimulate the AMPK pathway during glucose uptake points to therapeutic approaches in INS resistance.

The finding that OT stimulates glucose uptake in hypoxic conditions is important and has therapeutic implications. For example, metformin, an antidiabetic drug, attenuated oxidative stress-induced CM apoptosis and prevented the progression of heart failure in dogs, along with activation of AMPK (58). The activation of AMPK in the heart after ischemia and reperfusion is recognized as cardioprotective, because AMPK activation promotes glucose uptake, glycolysis, and limits apoptosis and cell damage (59, 60). AMPK activation and Ca-CAMKK stimulate phosphorylation of various downstream kinases, including the eukaryotic elongation factor 2 kinase implicated in reduction of CM hypertrophy (61).

Previous studies have revealed OT's involvement in the differentiation of various stem cell types into functional CM. These include mouse embryonic stem cells (22, 27, 62) and somatic Sca+ c-kit− cells isolated from the mouse heart (63). OT also stimulates the differentiation of cells expressing ABCG2 transporter and isolated from the adult rat heart (64). ABCG2-positive cells contain stem cells (a side population), the phenotype recognized by the efflux of Hoechst 33342 dye (65–67). Cardiac tissue expresses ABCG2-positive cells (68) involved in the differentiation of cardiac stem cells, an important process in the damaged myocardium (69). Presently, we showed that in the CM population positive for the ABCG2 marker, OT and OT-GKR, similarly to INS and hypoxia, increased glucose uptake, which could be important for cell differentiation and survival in this early stage of development. Recently, it was found that myocardial tissue, presumably incapable of any self-repair in case of cardiac insult, is capable of limited regeneration through the activation of putative cardiac progenitor cells (70–73). Our observation that OT heightens glucose uptake in ABCG2-positive CM more significantly than in nonselected CM suggests the importance of OT in the metabolism of progenitor cells.

In summary, we established that OT and OT-GKR can increase glucose uptake in CM in normal physiology and during hypoxic conditions, which could improve cell survival and consequently heart performance.

Acknowledgments

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