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Decreased ADP-Ribosylation of the $G\alpha_{olf}$ and $G\alpha_s$ Subunits by High Glucose in Pancreatic B-Cells

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In HIT-T15 insulinoma B-cells incubated in presence of [^{32}P]NAD, we identified by autoradiography and immunoblotting ADP-ribosylation (ADP-R) of the trimeric G-protein $G\alpha_s$ and $G\alpha_{olf}$ subunits (45 kDa) induced by cholera toxin in M1 (120,000*g*) and M2 (70,000*g*) subcellular fractions containing plasma membranes, insulin granules, and mitochondria. This ADP-R indicates that these two fractions contain functionally competent $G\alpha$ subunits for adenylyl cyclase activation. Prolonged exposure of HIT-T15 cells to high glucose (25 mM instead of 6 mM) specifically reduced the ADP-R in $G\alpha_s$ and $G\alpha_{olf}$ subunits in the M1 fraction only, despite the clear increase of their accumulation in this compartment. A similar alteration in the ADP-R of the M1-associated $G\alpha_s$ and $G\alpha_{olf}$ subunits was observed in pancreatic islets isolated from fasted and fed rats. These results may explain, at least in part, the undesirable effects of sustained hyperglycemia on the cAMP-dependent process of insulin secretion in diabetes. © 2000 Academic Press

Key Words: **$G\alpha_{olf}$; $G\alpha_s$; ADP-ribosylation; glucose; insulin secretion; diabetes.**

The α subunits $G\alpha_s$ and $G\alpha_{olf}$ belong to the family of stimulatory heterotrimeric G proteins consisting of $G\alpha$, β, and γ, which transduce the effects of activated heptahelical receptors by specific external ligands (1, 2). Rat $G\alpha_{olf}$ displays an extensive aminoacid sequence homology (88%) with $G\alpha_s$ (3, 4), including the specific site of ADP-ribosylation (ADP-R) by cholera toxin (CT), thus making these subunits valid substrates for this bacterial toxin. The tissular distribution of $G\alpha_{olf}$ is more restricted than that of $G\alpha_s$, but was recently extended to rat brain, and peripheral tissues including Langerhans islets and the insulin-secreting B-cells (5–7).

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In Langerhans islets, heterotrimeric G proteins transduce signals regulating insulin and glucagon secretion (8–10). Most of the results published in the field of pancreatic islet G-proteins concern biochemical studies of $G\alpha_i$ and cAMP signaling pathways in B-cell lines (11–13). Little information is available about the ability of pancreatic B-cell $G\alpha_s$ and $G\alpha_{olf}$ regarding to undergo ADP-R by CT, a major cAMP-inducing agent that blocks the GTPase activity of $G\alpha$ subunits. The functional consequences of this CT-dependent ADP-R are the constitutive activation of the α subunit $G\alpha$ in the GTP-bound state, and constitutive elevation of intracellular cAMP levels by adenylate cyclase.

Since glucose is the major physiological stimulus of insulin secretion and synthesis in the pancreatic B-cell (14), and is detected at high concentrations in diabetic patients, we hypothesized that high glucose might interfere with the functional activation of the adenylyl cyclase (AC)-by $G\alpha_s$ and $G\alpha_{olf}$ in pancreatic B-cells. Accordingly, we carried out experiments to characterize three parameters: (1) $G\alpha_s$ and $G\alpha_{olf}$ expression in the clonal insulinoma B-cell lines HIT-15 and RINm5F and rodent pancreatic islets, (2) the function of these subunits, by showing that $G\alpha_{olf}$ and $G\alpha_s$ are ADP-ribosylated (ADP-R) by cholera toxin (CT), suggesting that these subunits are functional for AC activation, and (3) the modulation of their ADP-R levels *in vitro* by glucose, and in rats, by nutritional status.

MATERIALS AND METHODS

Cell culture. Two insulin-secreting B-cell lines were used: (1) hamster HIT-T15 at passages 70–76, derived from hamster islet cells transformed by simian virus 40 (15), and (2) rat RINm5F cells at passages 81–83, established from a transplantable rat islet cell tumor (16). HIT-T15 cells were grown in Ham's F-10 medium (Gibco BRL, France) supplemented with 15% horse serum 2.5% fetal calf serum (FCS), both from Boehringer (France), 2 mM glutamine and antibiotics. Between passages 70 and 76, the glucose concentration was ajusted to either 6 or 25 mM.

Pancreatic islet isolation and subcellular fractionation. Rat pancreatic islets were isolated by collagenase (Boehringer, France) from newborn (3-day-old) and adult Wistar rats (weighing 200–250 g), as

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previously described. Briefly, the pancreas was digested at 37°C for 20 min, and islets were picked out manually under a stereo microscope and washed once with cold Hank's solution before fractionation experiments. Pancreatic islets, clonal B-cells HIT-T15 and pancreatic tissue were homogenized and submitted to differential centrifugation as follows (17): (i) 900*g* for 10 min, giving the nuclear (N) fraction containing cell debris; (ii) 7,000*g* for 3 min giving the M2 fraction containing heavy mitochondria, and (iii) 120,000*g* for 60 min giving the M1 fraction containing plasma membranes, mitochondria, microsomes and other organelles. The final supernatant was the cytosol (C). The pellets were resuspended in a lysis buffer containing 50 mM Tris-HCl (pH 7.2), 120 mM NaCl, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.5% NP10, 100 mg/ml PMSF and 0.1 IU/ml aprotinin.

Cholera toxin-catalyzed ADP-ribosylation. Cholera toxin (CT, 0.5 mg/ml) was activated at 37°C for 30 min in 50 mM NaH_2PO_4 buffer (pH 7.4) containing 10 mM DTT (18). Aliquots (20–240 μg protein) were incubated at 37°C for 2 h in a microfuge containing 100 mM NaH_2PO_4 (pH 7.4), 1 mM ATP, 1 mM GTP, 10 mM $MgCl_2$, 10 mM thymidine, 50 μg/ml activated CT, and 10 μM [^{32}P]NAD (30 Ci/mmole, Du Pont New England Nuclear), in a final volume of 100 μl. The reaction was stopped by adding 100 μl of ice-cold 20% trichloracetic acid (TCA). The microfuge was maintained at 4°C for 30 min and centrifuged at 14,000*g* for 15 min. The pellet was then washed with 200 μl of cold diethylether, centrifuged, dried under vacuum, and were then resuspended in Laemmli buffer for SDS–PAGE analysis.

Immunoprecipitation and Western blots. To distinguish the respective ADP-R levels of $G\alpha_{olf}$ and $G\alpha_s$ in membrane fractions, [^{32}P]ADP-ribosylated (ADP-R) proteins were immunoprecipitated using the pAbs against $G\alpha_{olf}$ (K-19) and $G\alpha_s$ (K-20). The C-18 pAb raised against the C-terminal 377–394 aminoacid sequence common to both $G\alpha_{olf}$ and $G\alpha_s$ was also used for the Western blots of islet homogenates, clonal HIT-T15 cells or RINm5F cells. The specificity of these three pAbs (Santa Cruz, CA) for immunodetection of the 45 kDa $G\alpha_s$ and $G\alpha_{olf}$ subunits (dilution 1:500) was validated using rat brain striatum homogenates as a positive control tissue for $G\alpha_{olf}$ expression (19), and by competition with the respective immunogen peptides (not shown). ADP-R was stopped by 100 μl of cold buffer containing 100 mM PO_4H_2Na, 50 mM HEPES (pH 7.4), 1% Triton X-100, 10 mM EDTA, 10 mM NaF, 10 mM $NaVO_4$ and 0.1 IU/ml aprotinin. After incubation for 30 min at 4°C, samples were centrifuged at 14,000*g* for 10 min. Aliquots of the resulting supernatant (100 μl) were incubated for 15 min at room temperature with 4 μl of each pAb K-19 or K-20, or overnight at 4°C with gentle rotatory shaking. Twenty μl of protein A-agarose (TEBU) was then added and the preparation was allowed to stand at room temperature for 2 h. The [^{32}P]-labeled immunoprecipitates were separated by a 12% polyacrylamide gel SDS–PAGE and transferred onto nitrocellulose membranes (Hybond) for autoradiography and chemiluminescence Western detection (ECL, Amersham, France).

RESULTS

ADP-Ribosylation of $G\alpha_{olf}$ and $G\alpha_s$ by Cholera Toxin

To study the CT-catalyzed ADP-R of $G\alpha_{olf}$ and $G\alpha_s$ in Langerhans islets, four subcellular fractions (M1, M2, N, and C) were prepared from newborn rats and assayed for [^{32}P]NAD incorporation. These $G\alpha$ subunits were then revealed by immunoblotting, using the specific pAbs K-19 and C-18. SDS–PAGE revealed a major 45-kDa [^{32}P]-labeled radioactive band in M1 (Fig. 1, lane 1) corresponding to the $G\alpha_{olf}$ and $G\alpha_s$ detected by pAbs K-19 and C-18, respectively.

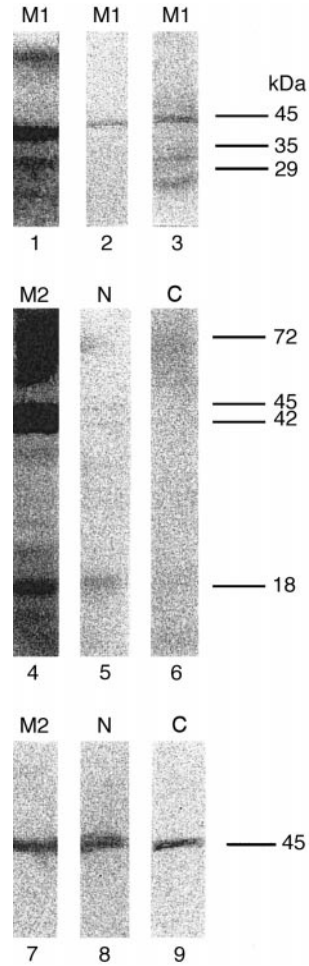

FIG. 1. ADP-ribosylation and immunoblot analysis of $G\alpha_{olf}$ and $G\alpha_s$ subunits in subcellular fractions of Langerhans islets prepared from newborn rat pancreas. Proteins from fractions M1 (28 μg), M2 (80 μg), N (250 μg), and C (80 μg) underwent ADP-ribosylation for 2 h at 37°C in the presence of activated CT and [^{32}P]NAD). The reaction was ended by precipitation with cold TCA. Top: The precipitated proteins, as well as two 28-μg aliquots of M1 native proteins, were separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. The M1 fraction containing [^{32}P]ADP-ribosylated proteins were autoradiographed (lane 1) using a Bio Max MR-1 film (Kodak), and the M1 native proteins were analyzed by immunoblotting, using pAbs K-19 against $G\alpha_{olf}$ (lane 2) or C-18 against $G\alpha_{s/olf}$ (lane 3), and an ECL kit (Amersham). Middle: Autoradiography of the [^{32}P]ADP-ribosylated proteins in the subcellular fractions M2, N and C (lanes 4–6), as described above. Bottom: After 4 weeks of [^{32}P] decay, these M2, N and C proteins were immunoblotted using pAb K-19 (lanes 7–9). Data are representative of 2 separate experiments.

The same $G\alpha_{olf}$ and $G\alpha_s$ 45-kDa bands were also observed by direct Western blot, using, in both cases, 28 μg of native M1 proteins (lanes 2 and 3). In the M2 fraction, two [^{32}P]-labeled bands of 42- and 45-kDa respectively were found (Fig. 1, lane 4), and $G\alpha_{olf}$ was identified by pAb K-19 as a radioactive 45-kDa band (lane 7). In contrast, no [^{32}P]NAD incorporation was observed in the nuclear (N) or cytosolic (C) fractions (lanes 5 and 6), although they

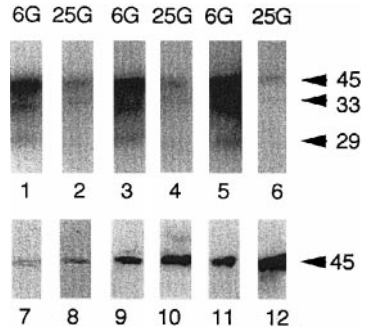

FIG. 2. ADP-ribosylation and immunoblot analysis of $G\alpha_s$ and $G\alpha_{olf}$ subunits in the M1 fraction prepared from HIT-T15 cells cultured for 2 weeks in the presence of high glucose. Upper: M1 [^{32}P]-labeled proteins from cells fed with 6 mM glucose (6G, odd number lanes) or 25 mM glucose (25G, even number lanes) were autoradiographed (lanes 1–6), using a Bio Max BMR-1 film (Kodak) and 96 h exposure at −80°C. Lower: After 4 weeks of radioactive decay, these membranes were then incubated with pAbs against $G\alpha_{olf}$ (lanes 7–8), $G\alpha_s$ (lanes 9–10) or both (lanes 11–12). Immune proteins were revealed using an ECL kit (Amersham) with 20-min exposure. Data are representative of 2 other experiments.

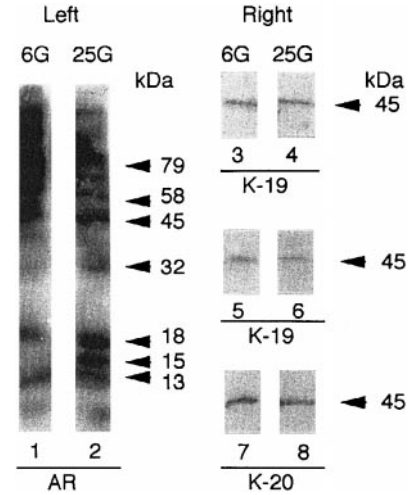

FIG. 3. ADP-ribosylation and immunoblot of $G\alpha_s$ and $G\alpha_{olf}$ subunits in the M2 fraction prepared from HIT-T15 cells cultured for 2 weeks in the presence of high glucose. Left: Autoradiography of [^{32}P]-ADP-ribosylated M2 proteins extracted from cells fed with 6 or 25 mM glucose (AR: lanes 1 and 2). Right: After 4 weeks of radioactive decay, these membranes were immunoblotted with pAb K-19 against $G\alpha_{olf}$ (lanes 3 and 4). As controls, M2 native proteins underwent direct Western blot with K-19 (lanes 5 and 6) and K-20 against $G\alpha_s$ (lanes 7 and 8).

contained the $G\alpha_{olf}$ protein identified by K-19 (lanes 8 and 9), and the ubiquitous $G\alpha_s$ protein.

ADP-Ribosylation of $G\alpha_{olf}$ and $G\alpha_s$ in HIT-T15 Cells Cultured in the Presence of High Glucose and Pancreatic Islets Isolated from Fasted and Fed Rats

As shown in Fig. 2 (lanes 1–6), when HIT-T15 B-cells were cultured in the presence of 25 mM glucose (25G), there was a dramatic drop in the ADP-R levels of the 45-kDa band in the M1 fraction, which did not occur in the B-cells fed with 6 mM glucose (6G). After a radioactive decay period, the same membranes were probed by Western blot, using the pAbs that detected $G\alpha_{olf}$ and $G\alpha_s$ (lanes 7–12). Positive 45-kDa immunoreactive bands identified $G\alpha_{olf}$ and $G\alpha_s$ proteins that were expressed more abundantly in the presence of 25 mM glucose, as probed with pAb K-19 ($G\alpha_{olf}$, lanes 7–8), K-20 ($G\alpha_s$, lanes 9–10), or C-18 pAbs ($G\alpha_{s/olf}$, lanes 11–12). This effect was specific for fraction M1, because in M2, the ADP-R (Fig. 3, Left: AR) and $G\alpha_s$ and $G\alpha_{olf}$ protein levels (Fig. 3, Right) were not dependent on glucose.

After immunoprecipitation of the $G\alpha_{olf}$ and $G\alpha_s$ in the M1 fraction of HIT-T15 cells, using the K-19 and K-20 pAbs (Fig. 4, lanes 1–4), we obtained direct evidence that the ADP-R levels of these two 45-kDa proteins were lowered by long-term exposure to high glucose (25 mM, lanes 2 and 4). Similar results were observed for $G\alpha_s$ and $G\alpha_{olf}$ in the M1 proteins of Langerhans islets isolated from either 64 h- fasted rats (lanes 5 and 7) or fed rats (lanes 6 and 8). As control, we checked that the K-19 $G\alpha_{olf}$ pAb did not immunoprecipitate the $G\alpha_s$ protein in the M1 fraction prepared from fed rats (lane 8), because $G\alpha_s$ was still detected in the TCA precipitate of the corresponding supernatant (lane 9).

DISCUSSION

In pancreas, the $G\alpha_{olf}$ protein is expressed in the insulin-secreting B-cells, one the four types of endo-

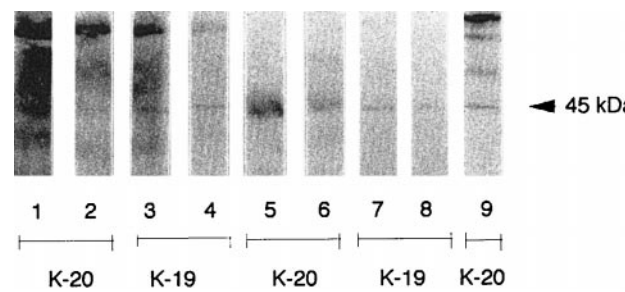

FIG. 4. Immunoprecipitation and autoradiography of the [^{32}P]ADP-ribosylated $G\alpha_s$ and $G\alpha_{olf}$ subunits in the M1 fraction of HIT-T15 cells and Langerhans islets prepared from adult rat pancreas. The [^{32}P]-labeled M1 proteins were immunoprecipitated and analyzed by SDS–PAGE and autoradiography, using either: (i) the pAb K-20 against $G\alpha_s$ for HIT-T15 cells fed with 6 or 25 mM glucose (lanes 1 and 2, respectively) and for fasted or fed rat islet cells (lanes 5 and 6); (ii) the pAb K-19 against $G\alpha_{olf}$ for HIT-T15 cells fed with 6G or 25G (lanes 3 and 4) and for fasted or fed rat islet cells (lanes 7 and 8). The [^{32}P]-labeled $G\alpha_{olf}$ subunit that was not immunoprecipitated by the $G\alpha_s$ pAb K-19 in fed rat islet cells (lane 8) was precipitated by TCA from the corresponding supernatant, and revealed, using the pAb K-20 (lane 9). Autoradiography was performed at −80°C for 2 days, using a BioMax BMR-1 film (Kodak) with an intensifying screen at −80°C.

crine cells identified in Langerhans islets (20). Its function is still unknown. However, $G\alpha_{olf}$ is believed to activate membrane-bound adenylate cyclase and cAMP production (3), a key signaling pathway for insulin release. We recently demonstrated that $G\alpha_{olf}$ is present in insulin secretion granules in pancreatic B-cells (20), suggesting that this G protein might be involved in regulating insulin release at the cytoplasmic and plasma membrane levels. These features led us to investigate the location and functional status of $G\alpha_{olf}$ and $G\alpha_s$ in subcellular fractions prepared from pancreatic B-cells in culture and pancreatic islets isolated from fasted or fed rats. The respective function of $G\alpha_{olf}$ and $G\alpha_s$ was then compared in these fractions, as reflected by the well known ability of $G\alpha_s$ to be ADP-ribosylated and activated by cholera toxin (CT). The site of CT-catalyzed ADP-R is the Arg201 residue, a critical site for the GTPase activity of $G\alpha_s$. Thus, ADP-ribosylated $G\alpha_s$ is unable to produce the hydrolysis of bound-GTP and constitutively activates adenylate cyclase.

We evidenced the ability of $G\alpha_{olf}$ and $G\alpha_s$ to be ADP-R by CT in the subcellular fractions M1 (120,000*g*) and M2 (7,000*g*) of pancreatic B-cells. These two M1 and M2 subcellular fractions are therefore the only subcellular compartments in which $G\alpha_{olf}$ and $G\alpha_s$ display this index of their potential ability for AC activation in the processes of B-cell signal transduction. Our previous work and other reports showed that the M1 fraction contained plasma membrane, cytoplasmic organelles, and insulin granules (17, 21, 22), whereas the M2 fraction mainly constited of heavy mitochondria. At the plasma membrane level, $G\alpha_{olf}$ and $G\alpha_s$ are subject to regulation by environmental factors, such as glucose levels and transport, other nutrients, hormones and metabolites which might alter their appropriate conformation or processing as signaling molecules or substrates for CT-catalyzed ADP-R. Conversly, in other $G\alpha$ subunits that do not activate AC, such as $G\alpha_i$ and $G\alpha_o$, ADP-ribosylation by CT only occurs after their coupling to agonist-activated receptors, although they harbor the conserved arginine site for ADP-R and are still associated with the plasma membrane (18, 23). The $G\alpha_i$ subunits are also associated with insulin-secreting granules, and in βTC-3 cells, are involved in insulin exocytosis (22). It is also known that the affinity of the G-protein receptor-associated G-protein for guanine nucleotides may also affect CT-catalyzed ADP-R reaction (24). Our data shed light on this field by demonstrating, for the first time, that prolonged exposure of B-cells to high glucose specifically reduced the level of the CT-catalyzed ADP-R of $G\alpha_{olf}$ and $G\alpha_s$ in the M1 subcellular fraction. Similar results attributable to the nutritional status of the rats are observed in pancreatic islets isolated from fasted or fed animals, indicating the pathophysiological relevance of our data, and suggesting that this diet-dependent process is reversible, from the fed to the fasting state. Of particular interest, is the evidence we found here that high glucose levels are associated with increased accumulation of $G\alpha_{olf}$ and $G\alpha_s$ proteins in the M1 fraction, the operating compartment for activating AC in the signal transduction process. A challenging question is how glucose may create M1 subcompartment in which $G\alpha_{olf}$ and $G\alpha_s$ signaling molecules are no longer functional, and accumulate. By these routes, high glucose might, in diabetic patients, significantly reduce B-cell insulin secretion in response to nutrients and insulinotropic metabolites and hormones. In support to this hypothesis, we observed that long-term culture of both insulinoma B-cells HIT-T15 and βTC-3 in the presence of high glucose for 2 weeks resulted in remarkable down-regulation of intracellular cAMP production in response to 0.1 μM truncated glucagon-like peptide-1, a major regulator of insulin secretion in man (data not shown). Other undesirable toxic effects of high glucose should be considered *in vivo*, including apoptosis (25). However, such form of cellular toxicity was not involved in our experimental conditions, because βTC-3 cells cultured for 2 weeks in the presence of 25 mM glucose exhibited increased cell growth, accompanied with reduced number of apoptotic cells, as measured by flow cytometry (6% apototic cells instead of 9% at 6 mM glucose, data not shown). Trimeric G-protein subunits are known to evolve in many conformational and functional steps for coupling to cell surface serpentine receptors and interaction with several signaling elements such as $\beta\gamma$ subunits, ADP-ribosylation factors, regulators of G protein signaling, such as RGS, AGS and RAMPS (26–29). The Gγ subunit isoforms are also subject to glucose-dependent, receptor-independent carboxyl methylation/demethylation cycle in insulin-secreting cells, with the consequent activation of phospholipases that release arachidonic acid (30). Similarly, transient Gβ phosphorylation at the histine residue to form Gβ-P was induced by a GTP-specific protein kinase, producing the active form Gα-GTP after phosphate transfer from Gβ-P to Gα-GDP in human and rodent B-cells (31).

In conclusion, our data demonstrate molecular and functional alterations of the $G\alpha_s$ and $G\alpha_{olf}$ proteins and their abnormal accumulation in subcellular compartments of pancreatic B-cells in response to long-term exposure to high glucose concentrations. Thus, high glucose might induce intracytoplasmic sequestration of $G\alpha_{olf}$ and $G\alpha_s$ in a nonfunctional state in insulin-secreting cells. This adverse effect, involving heterotrimeric G subunits and AC may therefore explain, at least in part, the general process of glucotoxicity in diabetic patients, particularly the B-cell defects of abnormal insulin secretion in response to various secretagogues and glucose.

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