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REVIEW

Reactive oxygen species serve as signals mediating glucose-stimulated somatostatin secretion from cultured rat gastric primary D-cells

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Abstract
Somatostatin plays an important role in glucose homeostasis. It is normally secreted in response to glucose and ATP generation is believed to be the key transduction signal of glucose-stimulated somatostatin secretion (GSSS). However, in the present study, in cultured rat gastric primary D-cells, GSSS was accompanied by increases in cellular reactive oxygen species (ROS). GSSS is dependent on the cellular ROS and independently of the ATP production linked to glucose metabolism. The antioxidant, α-lipoic acid or catalase inhibitor, 3-aminotriazole can influence the intracellular calcium concentration and abolish or further elevate GSSS. It is suggested that ROS production may serve as a signal modulating the necessary Ca^{2+} recruitment for GSSS. Since somatostatin is thought to exert broad regulatory functions on gastrointestinal physiology and nutrient intake, the interaction with ROS may lead to potential targets for mediating nutrition and energy homeostasis.

Keywords: *D-cells, somatostatin, ROS, glucose-stimulated somatostatin secretion*

Abbreviations: *3AT, 3-aminotriazole; AUC, area under curve; BSA, bovine serum albumin; CCCP, m-chlorophenylhydrazone; CICR, Ca^{2+}-induced Ca^{2+}-release; CL, chemiluminescence; DMEM, Dulbecco's modified Eagle's medium; EBSS, Earl's Balanced salt solution; ETC, mitochondrial electron transport chain; FCS, Foetal calf serum; F12, Ham's F-12 medium; GSH, glutathione; GSSG, glutathione disulphide; GSSS, glucose-stimulated somatostatin secretion; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; LA, α-lipoic acid; mROS, mitochondrial reactive oxygen species; MTT, Methylthiazolyldiphenyl-tetrazolium bromide; NAC, N-Acetyl-Cysteine; OPA, o-Phthalaldehyde; RGPC: rat gastric primary D-cells; ROS, reactive oxygen species.*

Introduction

Somatostatin is a powerful inhibitor of insulin and glucagon secretion and, thus, operates as an effective regulator of glucose homeostasis [1,2]. Although the importance of somatostatin in modulating gastrointestinal activity [3,4] and energy homeostasis [5] is recognized, when compared to hormones such as insulin, very little is known about the control of somatostatin secretion. Similar to the case of insulin secretion in β-cells, in the presence of glucose, closure of ATP-sensitive K^+-channels (K_{ATP}-channels) and a depolarization-evoked increase in cytoplasmic free Ca^{2+} concentration are believed to underlie the mechanism of glucose-stimulated somatostatin secretion (GSSS) from D-cells [6,7]. However, Zhang et al. [8] recently demonstrated that somatostatin secretion evoked by high glucose levels is K_{ATP} -channel-independent and is primarily dependent on Ca^{2+}-induced Ca^{2+}-release (CICR). Moreover, they found that actual metabolism of glucose is required for somatostatin secretion and speculated that metabolically generated ATP enhances intracellular Ca^{2+} stores, which can subsequently be released by CICR, but the precise mechanism was not identified. Although glucose metabolism leading to accelerated ATP generation was considered the key transduction signal for somatostatin secretion

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from D-cells, accumulating experimental clues now show that additional factors are involved in metabolism–secretion coupling. Braun et al. [9] recently found that glucose stimulation of somatostatin secretion involved both K_{ATP} channel-dependent and -independent processes. Additionally, several recent studies focusing on glucose stimulated insulin secretion from β-cells have shown that reactive oxygen species (ROS) derived from glucose metabolism were part of the signalling mechanism triggering glucose stimulated insulin secretion [10–12], although verification of this is still required.

It has been well documented that in the course of normal glycolytic and oxidative phosphorylation, ROS are also generated as a side product of ATP synthesis through the mitochondrial electron transport chain (ETC) and that the principal source of ROS in most mammalian tissues is the ETC itself [13–16]. ROS are thought of as the inevitable and undesirable by-products of aerobiosis and are believed to be generally hazardous for living organisms and damage all major cellular constituents. However, growing evidence now indicates that ROS play important roles in mediating diverse physiological responses such as cell proliferation, differentiation, exocytosis and apoptosis [17–19]. Importantly, ingestion of macronutrients including carbohydrate, fat and protein can bring about increased ROS production [20,21] and ROS can also modulate various aspects of gastrointestinal physiology and function [12,22,23]. Several study groups have found that the addition of glucose leads to a decrease in free cellular ADP concentration and a corresponding increase in ROS production [19,24]. Moreover, numerous research articles have demonstrated that ROS can increase cytosolic Ca^{2+} levels [25–28], one of the features necessary for somatostatin secretion. Many potential intermediates of exocytosis have also been recognized as downstream targets of ROS [29–31]. All of these features make ROS good candidates for sensitive regulation, directly or indirectly, of GSSS. The aim of the present study was therefore to investigate the potential role of glucose metabolism-induced ROS production as a signal for GSSS in cultured rat gastric primary D-cells.

Materials and methods

Materials

Animals were treated in accordance with the institution's guideline for the care and use of laboratory animals. Male Sprague-Dawley rats (250 ± 25 g) were obtained from Shanghai Laboratory Animal Center (Chinese Academy Sciences). Rotenone, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 3-aminotriazole (3AT), bovine serum albumin (BSA), Triton X-100, methylthiazolyldiphenyl-tetrazolium bromide (MTT) and glutathione (GSH) were purchased from Sigma-Aldrich Co. (Shanghai, China) α-lipoic acid (LA) and *N*-acetyl-L-cysteine (NAC) were purchased from Fushilai Medicine & Chemical Co., Ltd. (Jiangsu, China) Foetal calf serum (FCS), HEPES, luminal and horseradish peroxidase (HRP) were purchased from Biodee Biotechnology Co., Ltd. (Beijing, China) Ham's F-12 medium (F12), Dulbecco's modified Eagle's medium (DMEM), glutamine, Earl's Balanced salt solution (EBSS), Hank's balanced salt solution (HBSS) and collagenase (type I) were from Invitrogen Co. (Shanghai, China)

Cell preparation and culture

Rats were anaesthetized and killed by cervical dislocation, then stomachs were immediately excised. D-cells were prepared from freshly obtained rat fundic mucosa by sequential exposure to collagenase (0.35 mg/ml) and EDTA (1 mM), as described previously [32], and were plated at a density of 5×10^5 cells/ml in 24-wells plates coated with rat tail collagen (type I, ShengYou, Jiangsu, China). Culture medium was composed of F12 and DMEM (F12/DMEM 50:50, vol/vol, containing 10% heat-inactivated FCS, 10 mM HEPES, 100 mg/l gentamicin, 100 mg/l streptomycin, 100 mg/l penicillin, 2 mM glutamine, 8 mg/l insulin and 1 mg/l hydrocortisone). Before secretion studies commenced, the plates were incubated in an atmosphere of 95% air and 5% CO_2 at 37°C for 40 h.

Somatostatin secretion

The medium was removed and the cells washed three times with EBSS (containing 10 mM HEPES, 1% BSA, 2 mM glucose). Cells were then pre-incubated with 1 ml of EBSS containing the different test agents [LA (0.5 mM); NAC (10 mM); 3AT (2 mM); H_2O_2, control (no addition)]. Cells were washed three times with EBSS, then release studies were performed by adding 1 ml of this medium containing different glucose concentrations and/or pharmacological agents: 2 μM rotenone or 1 μM CCCP. Rotenone is an inhibitor of mitochondrial electron transport and can increase $O_2^{\cdot-}$ formation which is converted to H_2O_2 by superoxide dismutase [33]. CCCP is a protonophore-type uncoupler of oxidative phosphorylation and, together with rotenone, has been widely used to study the role of the mitochondrial ROS [34–36]. The cells were incubated for a further 2 h. At the end of the release studies, the supernatants were stored at –70°C until assayed for somatostatin. Somatostatin content was determined using a radioimmunoassay kit (Biotechnology Research Institute Huaying, Beijing, China).

Measurement of ATP, ROS, glutathione, and glutathione disulfide

Cellular ATP levels were measured using an ATP Bioluminescent Assay kit (Biodee Biotechnology Co., Beijing, China). ROS levels were measured by chemiluminescence (CL) in the presence of luminal (0.5 mM) and HRP (12 U/ml) using a thermostatically (37°C) controlled luminometer (MPI-B multiparameter chemiluminescence analysis system, Xian Ruimai Analytical Instruments Co., Ltd, Xian, China), as described previously [21]. The area under curve (AUC) was integrated to give total CL response intensity. Results were expressed as AUC per 1×10^6 cells. Levels of GSH and glutathione disulphide (GSSG) were measured with the fluorescence probe *o*-Phthalaldehyde (OPA, Sinopharm, Shanghai, China), as described previously [37]. The assay is based on GSH-specific conjugation with OPA and resulting fluorescence. The specificity is acquired for GSSG by reduction to GSH by glutathione reductase. A standard of known concentrations of GSH was used to create a calibration curve.

Measurements of intracellular free calcium concentration

The cells were loaded for 40 min with 5 μM Fura-2/AM (Molecular Probes, Biodee Biotechnology Co., Beijing, China) at 37°C in EBSS buffer. After loading, the cells were incubated in different experimental conditions. Then, the loaded cells were washed twice and resuspended at 10^7cells/ml in HBSS buffer placed in a square quartz cuvette. Intracellular Fura-2/AM fluorescence (excitation 340 nm, emission 500 nm) was measured (LengGuang Technology Co., Shanghai, China) as described previously [38]. Intracellular calcium concentration ($[Ca^{2+}]i$) was calculated using the following equation, as described by Grynkiewicz et al. [39]: $[Ca^{2+}]i=K_d\times(F-F_{min})/(F_{max}-F)$ [K_d is the dissociation constant for Ca^{2+} binding to Fura-2/AM, 224 nM under our experimental conditions; F is the fluorescence of the intracellular indicator; F_{max} is the fluorescence at a saturated Ca^{2+} after addition of 0.1% Triton X-100; F_{min} is the fluorescence at virtually zero $[Ca^{2+}]i$ after addition of 5 mM EGTA (Sinopharm, Shanghai, China) to cells made permeable with Triton X-100]. Background fluorescence was subtracted from the corresponding measurements for the calculation of $[Ca^{2+}]i$.

Evaluation of damaging effects of pharmacological agents

For evaluation of damaging effects of pharmacological agents, somatostatin resecretion tests were performed after release studies. Cells were washed three times with EBSS and incubated with 1 ml of this medium for 2 h, and then cells were stimulated again with the same concentration of the same pharmacological agents. Somatostatin secretion into the supernatant was then measured. The effects of pharmacological agents on cell viability were evaluated by MTT assay, as described previously [40]. After exposure to pharmacological agents, MTT (final concentration of 0.5 mg/ml) was added to the medium and the cells were incubated at 37°C for 4 h. Dimethyl sulphoxide (DMSO, Sinopharm, Shanghai, China) was then added to solubilize the formazan formed. Light absorbance was measured at 570 nm. The cell viability was calculated as a percentage relative to the viability of basal controls.

Statistical analysis

Data are expressed as means ± SD. For comparisons between groups, a Student's *t*-test was performed. A one-way ANOVA followed by a Tukey's test was used to compare all groups or selected groups. Pearson's correlation was used to determine the relation of ROS production to somatostatin secretion. A difference of $p < 0.05$ was considered statistically significant. Analysis was done with SPSS 13.0 (SPSS, Inc., Chicago, IL).

Results

Glucose induces ROS production and somatostatin secretion

Rat gastric primary D-cells (RGPC) were incubated with different concentrations of glucose for 2 h and the amount of somatostatin secreted into the media was measured. As shown in Figure 1A, glucose induced an increase of somatostatin secretion from RGPC in a concentration-dependent manner. Somatostatin in the 22 mM glucose group was increased 2.6- and 1.7-fold, respectively, compared with the control and 5.5 mM glucose group. This robust GSSS was accompanied by a significant increase in cellular ROS levels (Figure 1B) and decreased GSH/GSSG ratios (Figure 1C). The ratio of GSH/GSSG has been considered an important indicator of oxidative stress and redox signalling [41,42]. Thus, the observed shift in the redox state is consistent with the generation of ROS in response to glucose stimulation. We next investigated a possible causal link between ROS and GSSS. RGPC were pre-treated with LA (0.5 mM, 2 h), an antioxidant that can effectively quench ROS [43], or with the catalase inhibitor 3AT (2 mM, 2 h), washed and then stimulated with glucose in the same conditions. LA reduced ROS production accompanied by a complete blunting of GSSS. In contrast, 3AT increased endogenous ROS and resulted in a pronounced enhancement of GSSS (Figures 1D and E). Moreover, in these conditions, a significant positive correlation between ROS pro-

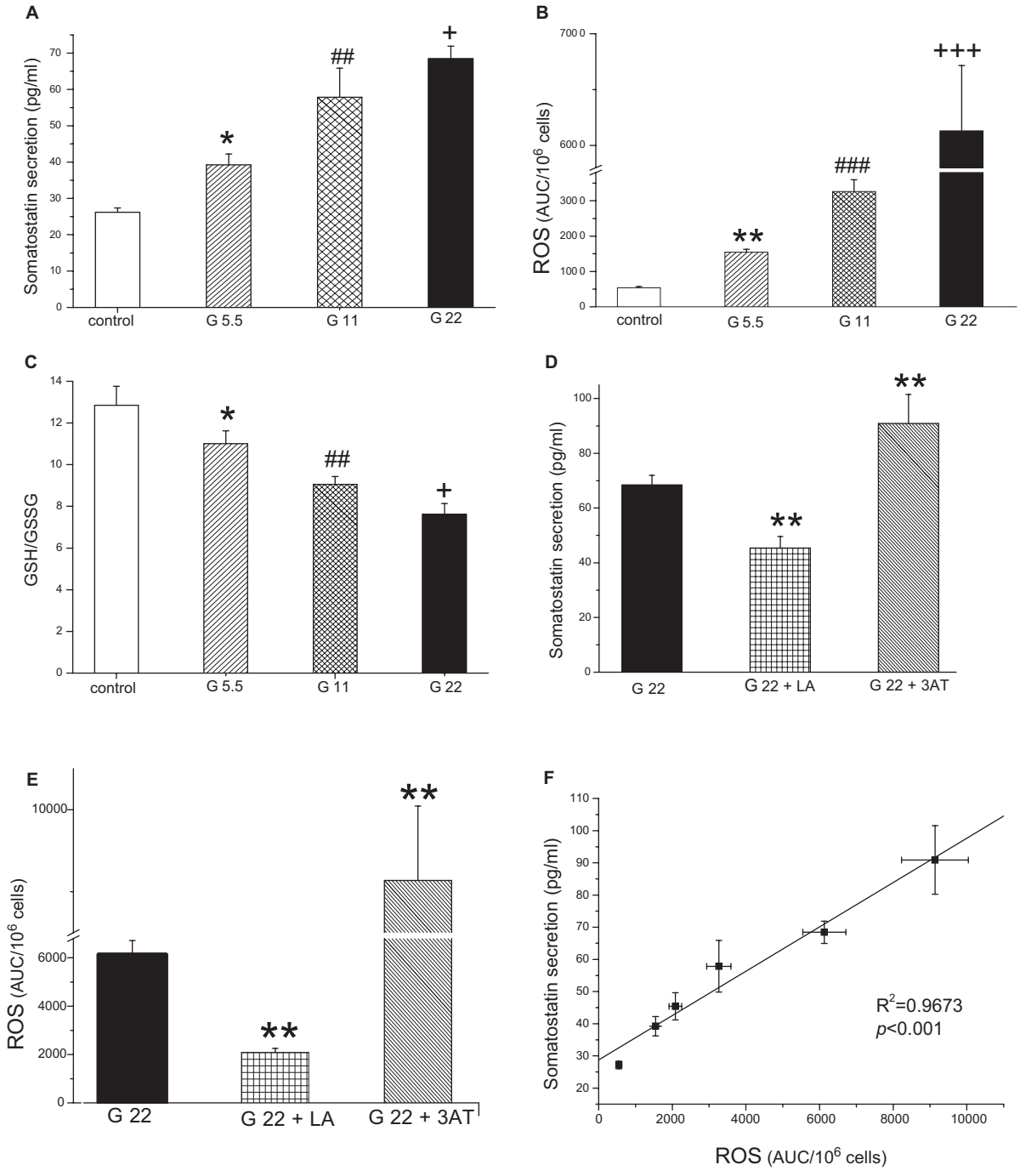

Figure 1. Glucose-induced ROS production and somatostatin secretion from the rat gastric primary D-cells (RGPC). (A) Glucose induced the secretion of somatostatin from RGPC, in a concentration-dependent manner. (B) Somatostatin release was accompanied by ROS production. (C) Effect of glucose on GSH-to-GSSG ratio. (A, B and C) Three independent experiments, $n=4$–9 per group; $^{*}p < 0.05$ or $^{**}p < 0.01$, 5.5 mM glucose (5.5 G) vs control; $^{\#\#}p < 0.01$ or $^{\#\#\#}p < 0.001$, 11 mM glucose (11 G) vs 5.5 mM glucose; $^{+}p < 0.05$, $^{+++}p < 0.001$, 22 mM glucose (22 G) vs 11 mM glucose. (D and E) Modulation of glucose-induced ROS production and glucose stimulation somatostatin secretion (GSSS) by antioxidant LA and catalase inhibitor 3AT. RGPC were pre-treated with LA (0.5 mM, 2 h) or with 3AT (2 mM, 2 h), washed and then stimulated with glucose in the same conditions. Three independent experiments, $n=4$–9 per group; $^{**}p < 0.01$ vs 22 mM glucose alone. (F) In these conditions, a significant positive correlation between ROS production and somatostatin secretion was found ($R^2=0.9673$, $p < 0.001$). Squares indicate the values of somatostatin ± SD, paired with ROS for each group.

duction and somatostatin secretion was found ($R^2=0.9673$, $p < 0.001$; Figure 1F). These results suggested that ROS production stimulated by glucose was required for GSSS and that ROS could be a potential signal for GSSS.

Exogenous H_2O_2 mimics GSSS

To confirm the link between glucose-stimulated ROS production and GSSS, we tested whether exogenous ROS have the ability to mimic GSSS. RGPC were exposed to H_2O_2 for 10 min and then incubated for

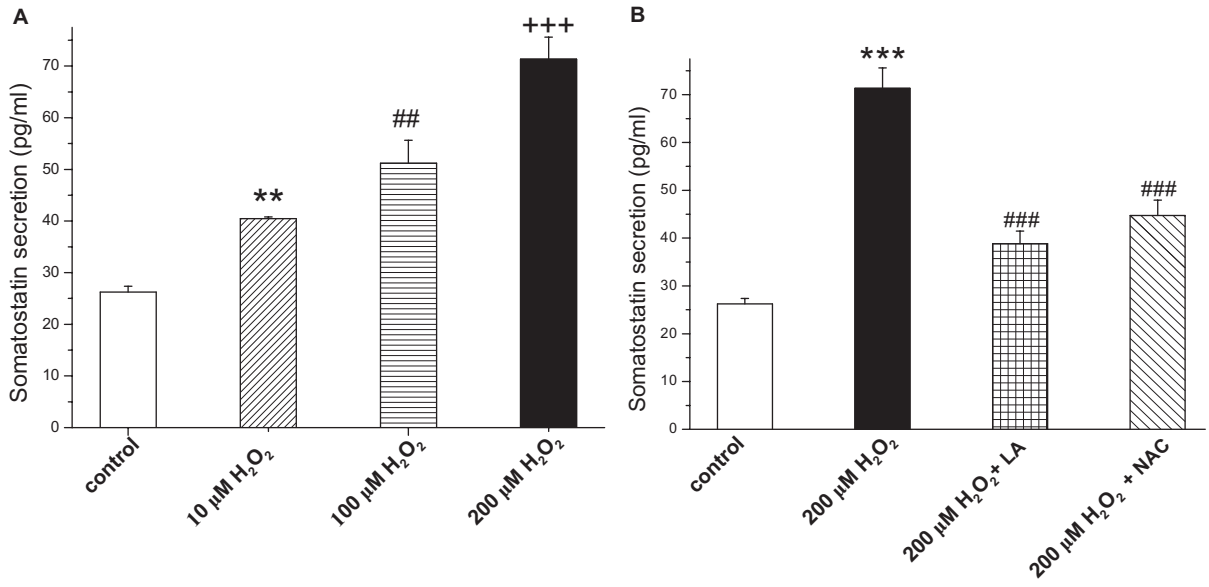

Figure 2. Effect of exogenous H_2O_2 on somatostatin secretion. (A) Effects of H_2O_2 on somatostatin secretion in RGPC. RGPC were exposed to H_2O_2 for 10 min and then incubated for 2 h in basal 2.0 mM glucose. Somatostatin content was determined using a radioimmunoassay kit. Three independent experiments, $n=4$–9 per group; $^{**}p < 0.01$, 10 μM H_2O_2 vs control; $^{\#\#}p < 0.01$, 100 μM H_2O_2 vs 10 μM H_2O_2; $^{+++}p < 0.001$, 200 μM H_2O_2 vs 100 mM H_2O_2. (B) Modulation of GSSS by antioxidant LA or NAC. RGPC were pre-treated with antioxidant LA (0.5 mM, 2 h) or with NAC (10 mM, 2 h), washed. Cells were exposed to 200 μM H_2O_2 for 10 min and then incubated for 2 h in basal 2.0 mM glucose. Pre-incubation with antioxidants reduced the H_2O_2-stimulated somatostatin secretion. Three independent experiments, $n=4$–9 per group; $^{***}p < 0.001$, control vs 200 mM H_2O_2; $^{\#\#\#}p < 0.001$, 200 mM H_2O_2, vs 200 mM H_2O_2+0.5 mM LA or 200 mM H_2O_2+10 mM NAC.

2 h in basal 2.0 mM glucose. H_2O_2 elevated the somatostatin secretion in a dose-dependent manner in the basal glucose condition with no glucose challenge (Figure 2A). Somatostatin release in response to 200 μM H_2O_2 was similar in level to that obtained in response to 22 mM glucose stimulation. Pre-incubation with antioxidants LA (0.5 mM, 2 h) or NAC (10 mM, 2 h) reduced the H_2O_2-stimulated somatostatin secretion (Figure 2B). Taken together, these results further supported ROS as mediators of GSSS.

Effect of mitochondrial blocker or uncoupler on GSSS

Because ROS are 'by-products' generated by glycolytic and oxidative phosphorylation leading to ATP production, the possibility exists that ATP may be the mediator of the GSSS, rather than ROS, and that the observed changes in ROS might be only a concomitant phenomenon. We therefore performed a set of experiments to investigate the effect of the specific mitochondrial blocker, rotenone, and the uncoupler, CCCP, on GSSS. As shown in Figure 3A, rotenone (2 μM) significantly enhanced GSSS, when no change in ATP level occurred (5.5 G+rotenone vs 5.5 G) or even when ATP production was depressed (22 G+rotenone vs 22 G) (Figure 3B). In contrast, the shift in ROS was always consistent with somatostatin secretion (Figure 3C). Moreover, ATP production was not changed (slightly elevated, no statistically significant difference), but somatostatin secretion was completely abolished when cells were pre-treated with antioxidant LA (22 G+LA vs 22 G; Figures 3A and B). ROS enhancement was also completely eliminated at the same time (22 G+LA vs 22 G; Figure 3C). Furthermore, similar somatostatin secretion was seen at almost identical levels of ROS, whether these were generated by treatment with 22 G, 5.5 G+rotenone or 22 G+rotenone+LA, regardless of the cellular ATP levels (Figures 3A–C; marked by 'S'). Similar results were seen in response to CCCP. ATP was significantly reduced and yet somatostatin secretion was not modified in the 5.5 mM glucose group (Figures 3A–C). CCCP treatment completely abolished ROS generation and the GSSS induced by 22 mM glucose (Figures 3A and C; 22 G group). This uncoupler CCCP accelerates electron transit by dissipating the H^+ gradient, decreasing the probability of the electrons reacting with oxygen and thus leading to the abrogation of ROS production of mitochondrial origin. This indicated that no extra-mitochondrial ROS were involved and that it was ROS of mitochondrial origin that mediate the GSSS. Taken together, these complementary results strongly suggested that glucose metabolism-induced mitochondrial ROS (mROS) is a robust mediator of GSSS.

Effect of glucose concentration and pharmacological agents on intracellular calcium concentration

Because of the importance of Ca^{2+} to secretion events in cells [44,45], we tested the effect of glucose and pharmacological agents on intracellular calcium concentration ($[Ca^{2+}]i$). Elevated glucose con-

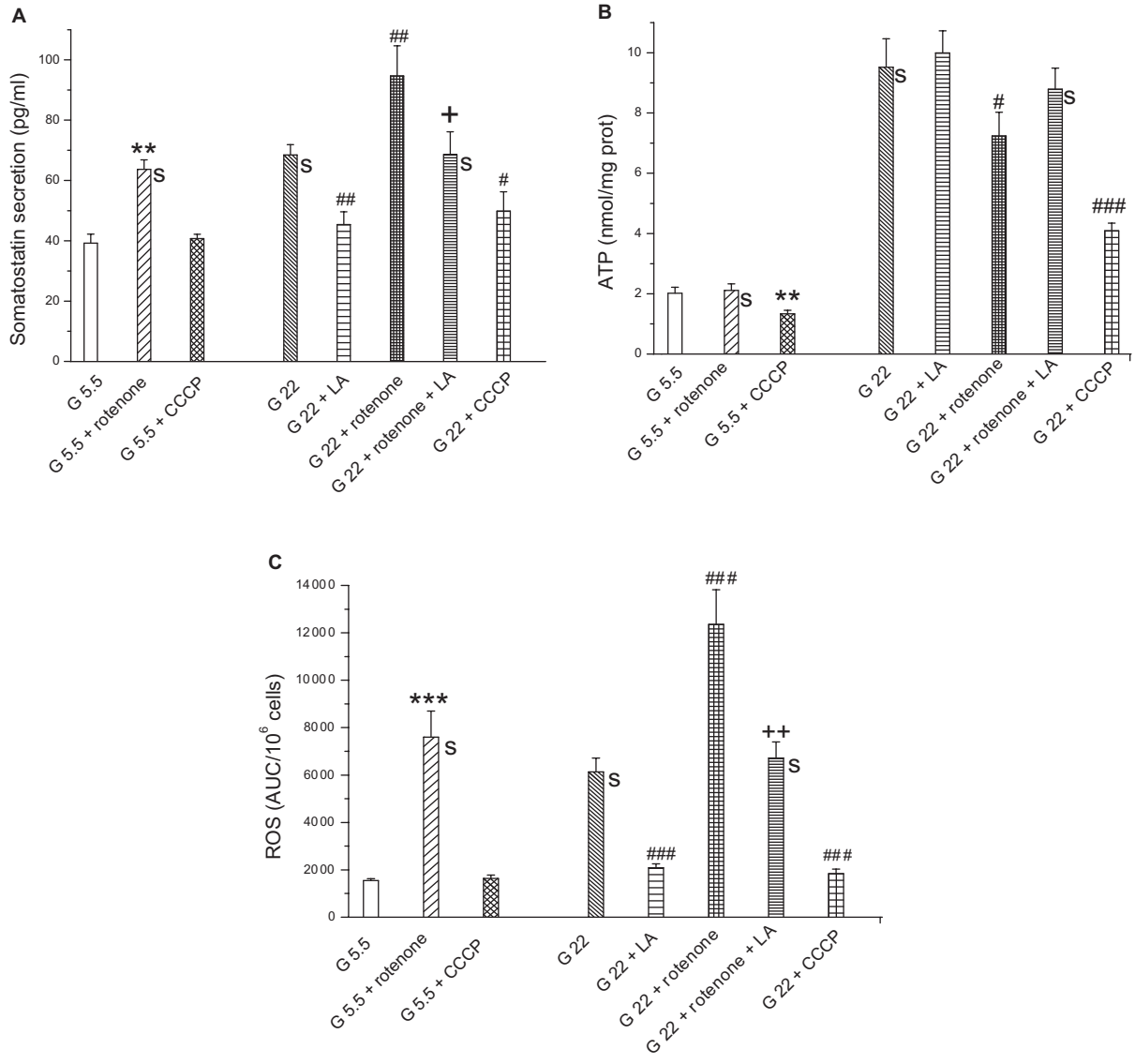

Figure 3. Effect of mitochondrial blocker and uncoupler CCCP on GSSS. Cells were pre-incubated with 1 ml EBSS or containing 0.5 mM LA. Cells were then washed three times with EBSS and release studies were performed in 1 ml of EBSS containing the different glucose concentrations and/or pharmacological agents [2 μM rotenone; 1 μM *m*-chlorophenylhydrazone (CCCP)]. (A) Effect of treatments on somatostatin secretion. (B and C) ATP content and ROS production in different treatment conditions. (A, B and C) Three independent experiments, $n = 4$–9 per group; $^{**}p < 0.01$ or $^{***}p < 0.001$, 5.5 mM glucose vs 5.5 mM glucose + 2 μM rotenone or 5.5 mM glucose + 1 μM CCCP; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, 22 mM glucose vs 22 mM glucose + 0.5 mM LA, 22 mM glucose + 2 μM rotenone, 22 mM glucose + 2 μM rotenone + 0.5 mM LA or 22 mM glucose + 1 μM CCCP; $^{+}p < 0.05$, $^{++}p < 0.01$, 22 mM glucose + 2 μM rotenone vs 22 mM glucose + 2 μM rotenone + 0.5 mM LA. Groups in which somatostatin secretion was achieved with almost identical levels of ROS produced, regardless of ATP level, are marked with the letter 'S' (A–C).

centration caused an increase of $[Ca^{2+}]i$ (Figure 4A) that could be abolished by pre-treatment with antioxidant LA and that could be further elevated by pre-treatment with 3AT (Figure 4B). $[Ca^{2+}]i$ mobilization caused by rotenone could also be completely abolished by pre-treatment with LA (Figure 4C). These results showed that glucose metabolism-induced mROS production plays a role as a signal for modulating the necessary Ca^{2+} recruitment for somatostatin secretion.

No damaging effects of pharmacological agents on RGPC

To verify that there were no damaging effects of the pharmacological agents used, MTT assays were performed on cells incubated in glucose and/or pharmacological agents, as described. As shown in Figure 5A, no differences were observed between control and pharmacologically-treated groups. For further confirmation, the RGPC were washed after treatment with pharmacological agents and somatostatin secretion was re-evaluated by stimulating the cells with the same pharmacological agents under the same experiment conditions. Results showed that somatostatin secretion was still maintained at the same original level (Figure 5B) and suggested that the differences in somatostatin release in previous experiments had not been due to degranulation or cellular loss. Taken together, these results excluded the possibility of

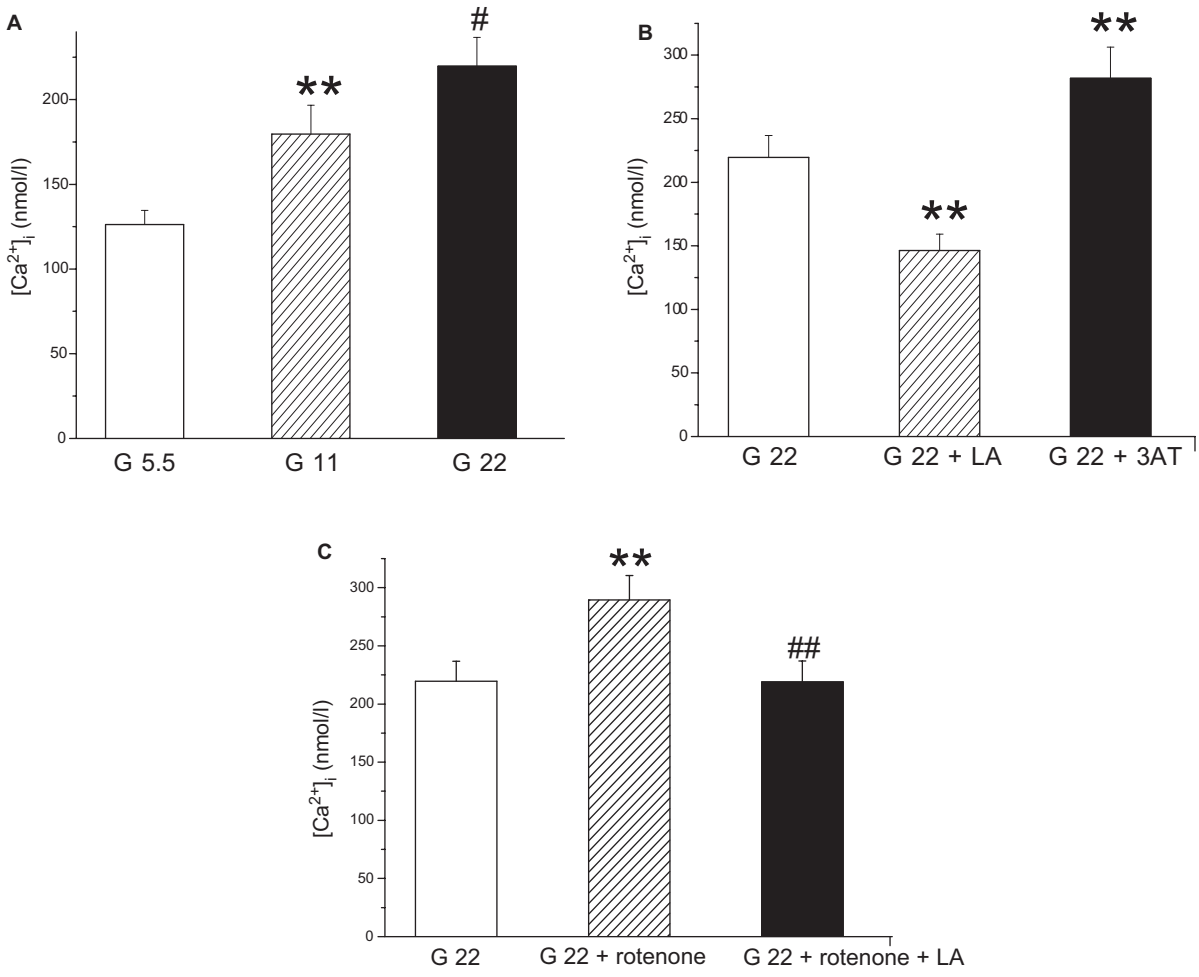

Figure 4. Effect of glucose concentration and pharmacological agents on intracellular calcium concentration ($[Ca^{2+}]i$). The cells were loaded for 40 min with 5 μM Fura-2/AM at 37°C in EBSS buffer. After loading, the cells were incubated in different experimental conditions. Then, the loaded cells were washed twice and resuspended at 10^7 cells/ml in HBSS buffer. Intracellular Fura-2/AM fluorescence was measured. (A) Elevated glucose concentration caused an increase of $[Ca^{2+}]i$. Three independent experiments, $n=4$–9 per group; $^{**}p < 0.01$, 11 mM glucose vs 5.5 mM glucose; $^{\#}p < 0.05$, 22 mM glucose vs 11 mM glucose. (B) Modulation of glucose-induced the increase of $[Ca^{2+}]i$ by antioxidant LA and catalase inhibitor 3AT. RGPC were pre-treated with antioxidant LA (0.5 mM, 2 h) or with catalase inhibitor 3AT (2 mM, 2 h) and then stimulated with 22 mM glucose. Three independent experiments, $n=4$–9 per group; $^{**}p < 0.01$ vs 22 mM glucose alone. (C) Effect of rotenone and antioxidant LA on glucose-induced the increase of $[Ca^{2+}]i$. Cells were pre-incubated with 1 ml EBSS or containing 0.5 mM LA, washed and then stimulated with 22 mM glucose and/or rotenone. Three independent experiments, $n=4$–9 per group; $^{**}p < 0.01$, 22 mM glucose vs 22 mM glucose + 2 μM rotenone or 22 mM glucose + 2 μM rotenone + 0.5 mM LA; $^{\#\#}p < 0.01$, 22 mM glucose + 2 μM rotenone vs 22 mM glucose + 2 μM rotenone + 0.5 mM LA.

damaging effects of pharmacological agents on RGPC.

Discussion

Through a combination of its hormonal and neuronal characteristics, somatostatin regulates gastrointestinal physiology and intake of nutrients. In return, available nutrients stimulate the secretion of somatostatin [3]. In this study, we found a novel regulation point between somatostatin and nutrients. Glucose-induced ROS proved to be a robust mediator of somatostatin secretion. Importantly, the ROS have been found to be of mitochondrial origin in the GSSS experiment and, thus, the mechanism of mediation between somatostatin and glucose might now be somewhat more clear. Our explanation for the observations reported here is that elevated glucose reinforces mitochondrial respiration, which in turn increases the production of mROS. The enhanced mROS then acts as a signal for somatostatin secretion. In return, elevated somatostatin inhibits glucose uptake, which then reduces the production of ROS. A similar study we conducted, *in vivo*, found that the administration of oil or protein by gavage increased ROS production and induced somatostatin secretion in mice (unpublished).

Our results are also supported by those of Arebi et al. [32], who demonstrated that nitric oxide (NO) regulated the release of somatostatin from cultured gastric rabbit primary D-cells. Arebi et al. also mentioned in their literature that neuronal nitric oxide

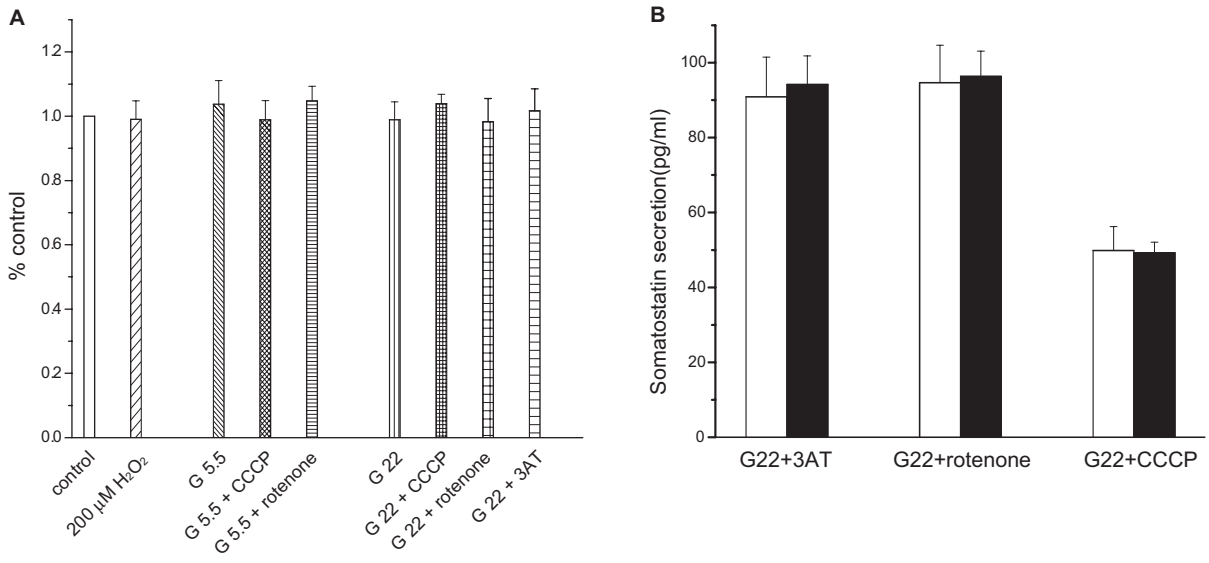

Figure 5. Effects of different pharmacological agents on RGPC. (A) MTT assays were performed simultaneously in glucose and/or pharmacologically treated groups. Three independent experiments, $n=4$–9 per group; No difference in these groups was observed. (B) Somatostatin resecretion tests were performed after release studies. The RGPC were washed three times with EBSS after treatment with pharmacological agents and cells were then stimulated again with the same pharmacological agents. The black column showed the respective ability of the same pharmacological agents to restimulate somatostatin secretion. Three independent experiments, $n=4$–9 per group; No difference in somatostatin secretion was observed in corresponding groups.

synthase (nNOS) and endothelial nitric oxide synthase (eNOS) are Ca^{2+}/calmodulin-dependent enzymes and produce NO in response to increased intracellular Ca^{2+} [46]. Thus, it might be that other signalling molecules that have the capacity to increase intracellular Ca^{2+} have then stimulated somatostatin secretion, accompanied with the production of NO. Numerous reports have demonstrated that ROS increase cytosolic Ca^{2+} levels [25–28]. In the present study, it is confirmed that mROS increased intracellular Ca^{2+} and mediated GSSS. We were interested in knowing whether a specific ROS was responsible for mediating GSSS (i.e. H_2O_2, $O_2^{\bullet-}$, etc.). We found that Diethyldithiocarbamate (DDC), an inhibitor of CuZn-SOD that can increase the endogenous cellular levels of $O_2^{\bullet-}$ [47], had no significant enhancement effect on GSSS (data not shown). In contrast, the catalase inhibitor 3AT significantly increased GSSS (Figure 1D). These results suggested that it may be H_2O_2 that is the active species that mediates GSSS and this might also explain why somatostatin secretion was very significantly increased by exogenous H_2O_2 (Figure 2A). However, H_2O_2 concentration was not directly detected in our experiments, as most of the probes used to detect H_2O_2, including the classical dichlorodihydrofluorescein ($DCFH_2$), have been found to also react with other peroxides to form products with identical spectral properties [48]. Consequently, we still cannot directly prove that the ROS mediating GSSS is exclusively H_2O_2.

In the current study, we found no evidence to suggest that ATP levels were directly involved in the regulation of GSSS. We found that ROS mediated GSSS independently of the ATP production linked to glucose metabolism (Figures 3A–C). This result was in contradiction with the accepted model for GSSS that involves K_{ATP}-channel closure in response to elevated ATP and a depolarization-evoked increase in cytoplasmic free Ca^{2+} concentration [6,7]. It is possible that K^+-channels themselves are regulated by ROS [31,49]. As well, both K_{ATP}-channel-dependent and -independent processes are involved in GSSS [9] and, in our study, the effect of ROS may have overwhelmed any K_{ATP}-channel action. However, it is confirmed that ROS serve as necessary signals for modulating the necessary Ca^{2+} recruitment for somatostatin secretion and this result is supported by these studies that ROS can increase cytosolic Ca^{2+} levels [25–28]. Indeed, the triggering action of Ca^{2+} does not seem to completely explain the stimulation of somatostatin secretion by glucose and the precise mechanism that couples glucose catabolism to somatostatin secretion is far from being identified.

In summary, our findings suggest that glucose-induced ROS serve as signals mediating GSSS. Taking into account the significance of somatostatin in regulation of nutrient intake [3] this finding advances a possible pathway whereby D-cells induct ROS/redox status to regulate nutrient intake. This might further clarify the complex relationships between ROS/redox status and nutrition, energy balance and health.

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