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Dapagliflozin modulates glucagon secretion in a SGLT2-independent manner in murine alpha-cells

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Short running title: Dapagliflozin and glucagon secretion

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Abstract

Aim SGLT2 inhibitors reduce renal glucose uptake with an insulin-independent mechanism. They also increase glucagon concentration; to which extent this is due to a direct effect on pancreatic alpha-cells remains unclear.

Methods In the present work, αTC-1 cells treated with the SGLT2 inhibitor Dapagliflozin (Dapa), were analyzed in terms of glucose transporters, molecular mediators of hormone secretion, glucagon and GLP-1 release, effects of somatostatin. Data were validated in murine and human pancreatic islets.

Results *Slc5a2* (SGLT2-encoding gene) was almost undetectable in αTC-1 cells, even by digital PCR technique with different probes. In contrast, *Slc5a1* (SGLT1-encoding gene), was constitutively abundant in αTC-1 and in islets, and was increased by Dapa; this was associated to a higher glucagon release, preceded by increased expression of Pre-proglucagon and Hepatocyte nuclear factor-4α. Looking at the candidate intracellular signaling pathway, reduced PASK and increased AMPKa2 expression was detected. GLUT1 and GLUT2 as well as regulators of glucagon release or of alpha-cell phenotype (Chromogranin-A, Paired Box-6, Proconvertase1/2, Synaptophysin) were unaffected by Dapa treatment, similarly to GLP-1 receptor expression or GLP-1 release. Low glucose did not influence the stimulatory effect of Dapa on glucagon release, which, instead, was almost fully reverted by *Slc5a1* silencing. The effect of Dapa on adenosine monophosphate-activated protein kinase (AMPK) and per-arnt-sim kinase (PASK), emerging regulators in lipid and glucose metabolism, was tested; an up-regulated AMPK-a2 seems to be the involved molecular signaling.

Conclusion We show here that in αTC-1 cells Dapa acutely upregulates SGLT1 expression and increases glucagon release with a SGLT1-dependent mechanism, with SGLT2 expression virtually undetectable. These results suggest an involvement of SGLT1 in modulating glucagon increase following SGLT2 inhibition.

Keywords SGLT2 inhibitors; SGLT1; glucagon; alpha-cells.

Introduction

SGLT2 inhibitors are novel drugs recently introduced in the market for the treatment of type 2 diabetes (T2DM). They induce glycosuria acting at the renal level by a fully insulin independent mechanism [1]; however, their administration determines several other relevant clinical effects, like a reduction of body weight due a true loss of calories, and a reduction in systolic blood pressure, mainly dependent upon a thiazide-like diuretic effect [2]. Besides these positive clinical actions, administration of SGLT2 inhibitors induces some potentially undesirable metabolic responses, like a raise in endogenous glucose production and increased glucagon levels. These observations, initially performed in two landmark clinical studies [3, 4] have casted doubts on the previously claimed almost exclusive presence of SGLT2 cotransporters in the kidney. In rodents and bovines, SGLT2 expression has been found in mammary gland [5, 6], while in humans SGLT2 has little or no detectable level in tissues other than renal cortex and medulla [7]. Recently, Bonner *et al* have documented the presence of SGLT2 in murine alpha-cells and in human pancreatic islets, where it appears responsible for an increased glucagon secretion [8]. However, glucagon secretion is an extremely complex issue, only partially known and certainly regulated by several mechanisms, ranging from paracrine effects of insulin and somatostatin to autonomic nervous system, from energy availability (changes in the ATP/ADP ratio) to intracellular ion fluxes [9-11]. Moreover, a role for SGLT1 cotransporters, in this scenario, cannot be ruled out: for example, SGLT2 inhibition by highly selective molecules, like those used for the treatment of T2DM, might induce a hyper-expression of SGLT1, likely responsible, in the kidney, for an enhanced glucose reabsorption in the distal tract of the proximal tubule, thereby limiting the therapeutic efficacy of these drugs [12]. SGLT1 seems also to be active in specific regions of the brain such as the hippocampus [13], thus contributing to glucose homeostasis, possibly through mechanisms other than the renal/gut glucose reabsorption; we may therefore hypothesize that above mentioned properties of SGLT1, as well as of other cotransporters, have yet undiscovered physiological significance.

In order to gain further insight into the mechanisms underlying the effect of selective SGLT2 inhibition on glucagon secretion, we designed the present study in a cellular model of alpha cells, trying to better define the role of SGLT2 and SGLT1 transporters in this crucial metabolic response.

Research Design and Methods

Cells and reagents - Murine αTC-1 (clone 6) cells were obtained from ATCC (ATCC® CRL-2934). Dapagliflozin (Dapa) was kindly provided by Astra Zeneca Int. Somatostatin was purchased by Hikma, Jordan.

Study protocol - αTC-1 cells (20^{th} passage) were cultured in Dulbecco's modified Eagle medium containing 25 mmol/l Glucose (Sigma-Aldrich, St Louis, MO, USA), 10% FBS (Gibco, Thermo-Fisher Scientific Waltham, MA, USA), 100 units/ml penicillin, and 100 μg/ml streptomycin, were maintained at 37°C/5% CO_2, changing media every three days; this high-glucose medium is commonly used to grow this cellular clone [14, 15]. Cells were seeded at equal density in six-well plates and incubated with 100 ng/ml Dapa for different time courses (30, 45, 60, 120, 240, 480 and 720min). Such Dapa concentration was chosen because corresponding to the mean therapeutic plasma concentration reached during treatment with this SGLT2 inhibitor in humans [16]. Media were collected and cells were lysed for mRNA and protein extraction. Some experiments were repeated after switching cells to starvation media (DMEM containing glucose 1.1 or 5.5 mmol/l, 1.5 g $NaHCO_3$, 0.25% BSA, and 15 mmol/l HEPES) and incubating them for 30, 45, 60, 120, 240 and 720min, to explore the effect of low glucose levels on hormone release.

Transfection and gene silencing - αTC-1 cells were plated at a density of $2x10^5$/well in 24-wells plate and transfected 24h later. *Slc5a1* silencing in αTC-1 cells was performed using Lipofectamine 3000 (1.5 µl/well) and Stealth siRNA oligonucleotide targeting *Slc5a1* gene (set of three: (MSS209159, MSS209160, MSS277112, from Applied Biosystems, Foster City, CA, USA) or scrambled siRNA oligonucleotide at a final concentration of 100nM following manufacturer's instructions. Transfection medium was changed after 24h and cells were maintained for further 48h before proceeding with Dapa treatment for 30min and 240min. Knockdown efficiency was confirmed by Real-Time quantitative PCR and by digital PCR using specific assays.

Laser capture microdissection (LCM) - Pancreatic murine tissue samples obtained from C57BL/6J mice, were frozen in O.C.T. compound (Sakura-Fintek, Torrance, CA, USA) and 8-µm sections were prepared. Sections were fixed in 70% ethanol for 30s, rinsed in RNAse-free water for 5seconds, stained with Mayer's hematoxylin solution and finally dehydrated. LCM was performed using Arcturus XT system (Arcturus Engineering, Mountain View, CA, USA) by melting thermoplastic films mounted on transparent LCM caps (HS-Caps) (Arcturus) on specific islet areas (islet core and islet periphery) as previously shown [17]. Captured cells were lysed and then stored at -80°C until RNA extraction.

Human pancreatic islets - Collagenase-isolated human pancreatic islets obtained from 3 non-diabetic multiorgan donors (BMI 26.3±2.5 Kg/m^2; age 59.7±12.7 years; 2M, 1F) were purchased from Lonza (Walkersville, MD, USA). Upon arrival, pancreatic islets were maintained in culture using CMRL-1040 supplemented with 10% FBS, L-Glutamine 1mmol/l and 1X Antibiotic/Antimycotic for 24h before being processed for experiments.

Gene and protein expression studies - Expression of *Slc5a1*, *Slc5a2, Slc2a1* and *Slc2a2* genes, encoding for SGLT1, SGLT2, GLUT1 and GLUT2 respectively, was quantified by real-time and digital PCR. Briefly, total RNA was extracted using RNeasy mini kit (QIAGEN GmbH, Hilden, Germany) or Picopure RNA isolation kit (for LCM captured cells). Extracted RNA from LCM captured cells were tested for RNA integrity using 2100 Bioanalyzer instrument (Agilent Technologies, Santa Clara, CA, USA) taking into consideration only those samples with a RIN (RNA Integrity Number) >5.0. cDNA was produced using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions, followed by a pre-amplification step as previously described [13]. Real-time PCR was performed in triplicate on an Eco-Real Time instrument (Illumina Inc., San Diego, CA, USA) and on a VIIA7 instrument (Applied Biosystems) following a standard protocol with specific TaqMan Gene Expression Assays. **Suppl. Table A** reports indications on all probes used in Real-Time PCR analyses. *Ct* values (cycle threshold) were used to calculate the amount of amplified PCR product relative to β-actin, and the relative amount of mRNA was calculated as $2^{-\Delta\Delta Ct}$. Results are expressed as fold change above reference sample.

The relative expression of *Slc5a2* gene was analyzed using different TaqMan assays (SGLT2-A, -B,-C,-D, Suppl. Table A) mapping along the gene, to avoid biases due to any potential spliced forms. For this gene, we also carried out an absolute gene expression quantification using digital PCR (ddPCR QX200, Bio-Rad, Hercules, CA, USA). Briefly, the same TaqMan Gene Expression assays (SGLT2-B and -D) were adapted to ddPCR, and experiments were performed as follows: 1 cycle at 95°C for 10min, 44 cycles at 95°C for 30s and 60°C for 1min, 1 cycle at 98°C for 10min, all at a ramp rate of 2°C/s (a conventional thermal cycler was used for the PCR step). The positive droplets were quantified using the Bio-Rad QuantaSoft software and the number of target molecules in each sample was estimated according to Poisson distribution.

Western Blot analyses - 20μg of total protein extracts were diluted in SDS-PAGE buffer and heated at 100°C for 5min. Samples and molecular weight markers were electrophoresed on Any kD Mini-Protean TGX gels and transferred to PVDF membrane. After a blocking step using BSA 3% in TTBS (TBS and Tween-20 0.05%) for 1h at room temperature, blots were repeatedly washed in TTBS and incubated overnight at 4°C with the following primary antibodies diluted 1:100: SGLT1 (sc-98974), SGLT2 (sc-393350) PASK (sc-74812), AMPK-a2 (sc-19129), pAMPK-a2 (2535 S), β-actin (sc-47778) and GAPDH (CAB932Hu22), purchased from Santa Cruz Biotechnology, Dallas, TX, USA; Cell Signaling Technology, Danvers, MA, USA; Cloud-Clone Corp, Houston TX, USA). To confirm the band specificity, anti-SGLT2 antibody (cat. 3690-100, BioVision Incorporated, Milpitas, CA, USA) diluted 1:200 was blocked by incubation with an equal volume of control peptide (cat. 3690BP, BioVision) for 30min at 37°C. Bands were detected by incubating the blot with species-specific secondary antibodies, followed by enzymatic chemiluminescence, and quantified by densitometric analysis using Image J software (NIH, Bethesda, MR, USA).

Glucagon secretion and its modulation - Aliquots of αTC-1 cell culture supernatants were collected and assayed for glucagon release; cellular glucagon content was also determined from cellular lysates in 0.1 mmol/l HCl. Hormone concentrations were measured by radioimmunoassay (Millipore Corp., Billerica, MA, USA). Release was measured as absolute value (pg/ml/µg protein) and referred to intracellular content [(surnatant/surnatant+content). 100; expressed as %]. Expression of alpha-cell genes of interest (Chromogranin A, Paired Box 6 [PAX6], Proconvertase1/2, Synaptophysin, ARX) was quantified by Real-Time PCR as described above; primers and probes are reported in **Suppl. Table A.**

Glucagon-like peptide 1 (GLP-1) receptor expression and GLP-1 release - GLP-1 receptor expression was analyzed by real-time PCR in αTC-1 cells, before and after treatment with Dapa 100 ng/ml for 30, 45, 60 and 720min. GLP-1 release was assessed using a RIA kit (Millipore Corp.).

Effect of somatostatin - αTC-1 cells grown in standard conditions were pre-incubated with somatostatin 1 µg/ml, alone or in combination with Dapa, for 240 and 720min, to assess the effect of somatostatin on the expression of its receptor (measured by Real-Time PCR) and on glucagon release.

Intracellular signaling - PASK, total and phosphorylated AMPK-a2, PKCα and SNAP25 were assessed by Real-time PCR and western blot analysis as described above.

Statistical analysis Data are reported as means ± SD. Group differences were analyzed by two-way ANOVA for repeated measures and post hoc Bonferroni-Dunn test, with medium glucose concentration (5.5 or 1.1 and 22 mM) as main factor. A P value <0.05 was considered statistically significant.

Results

Expression of glucose transporters - Murine αTC-1 (clone-6) cells constitutively expressed *Slc5a1*, *Slc2a1* and *Slc2a2* genes, encoding respectively for SGLT1, GLUT1 and GLUT2 transporters (**Fig. 1A**). T**reatment of αTC-1 cells with Dapa did not influence either *Slc2a1* or *Slc2a2* gene expression, while *Slc5a1* (encoding for SGLT1) was promptly and significantly upregulated, ranging from a 90% increase after 30min to 35% after 60min.**

Unexpectedly *Slc5a2* (encoding for SGLT2) was only, and minimally, detectable using digital-PCR technique (**Fig. 1B**). The extremely low expression of this gene was even more evident when compared to murine kidney, and was confirmed by Western Blot analysis: even when a large amount of total protein (100 µg) was loaded for alpha-cells, the specific SGLT2 band was only barely detectable, despite the use of a highly specific antibody (demonstrated by the lack of signal when a control peptide was co-incubated, **Fig. 1C**).

In pancreatic murine samples, *Slc5a1* was differentially expressed in specific islet areas (islet periphery *vs* core) isolated by LCM: while *Slc5a2* was almost undetectable in both areas, *Slc5a1* expression was increased in the peripheral cell population (enriched in alpha-cells) respect to core cell population (enriched in beta-cells) (**Fig. 2A)**; the different composition of these two islet areas was confirmed by the preferential expression of insulin and pre-proglucagon genes in the core or in the periphery, respectively (**Fig. 2A**). The much higher expression of *Slc5a1* vs *Slc5a2* was also shown in human pancreatic islets from non-diabetic individuals (**Fig. 2B**); we were also able to confirm the increased SGLT1 protein expression, while SGLT2 protein was almost undetectable in human islets (**Fig. 2B**), despite the use of two different antibodies. **Fig. 2C** shows an example of islet area before and after laser capture microdissection.

Glucagon release - Dapa stimulated glucagon release; such increment, significant for short-term incubations, reached a mean 40% increase when we evaluated a more prolonged time course (**Fig. 3**). ~~after 720 min glucagon release was massive in both unstimulated and Dapa-treated cells.~~

Moreover, Dapa was able to increase the expression of pre-proglucagon as well as of HNF4α, a transcription factor, which modulates glucose transporters expression in human alpha-cells. Conversely, the expression of a series of genes (Chromogranin A, Pax6, Pcsk1; Pcsk2, Synaptophysin, ARX) modulating alpha-cell phenotype or glucagon release was not influenced by Dapa treatment (**Suppl. Table B)**.

Effects of different glucose concentrations - Low glucose (5.5 mM) *per se* did not significantly influence glucose transporters gene expression, even after 4 hours of exposition (**Suppl. Table C**). However, in αTC-1 cells, the stimulatory effect of Dapa on *Slc5a1* expression was maintained, while no effect on *Slc2a1* or *Slc2a2* was observed (**Suppl. Table C**). This was paralleled by the same effect on glucagon release observed in high glucose, *i.e.* an enhanced release induced by Dapa at 1.1 mM and 5.5 mM glucose concentrations after 45-60min incubation (**Fig. 4**); noteworthy, glucagon absolute levels were slightly higher in the presence of 1.1 mM glucose.

GLP-1 axis evaluation - Pre-proglucagon is a common precursor not only of mature glucagon, but also of other secretory products of alpha-cells, like GLP-1; therefore, we tested GLP-1 receptor gene expression and GLP-1 release in αTC-1 cells, observing that neither the former (slightly expressed in these cells) nor the latter were influenced by treatment with Dapa (**Suppl. Figure 1**).

Effect of somatostatin on αTC-1 cells - Our next step was to test whether somatostatin might be somehow involved in modulating the secretory response upon Dapa treatment. Data are reported in **Suppl. Figure 2.** Upon 240 min treatment, somatostatin alone was unable to significantly affect glucagon release; after 720 min, glucagon release was increased by 20% by Dapa and reduced by 20% by somatostatin (**Suppl. Fig. 2A**); however, when combined with somatostin, Dapa was unable to reverse or partially counteract somatostatin effect. Dapa did not exert any effect on somatostatin receptor expression (**Suppl. Fig. 2B**).

Slc5A1 gene silencing - To confirm that Dapa-induced glucagon release involves the activity of SGLT1, we silenced its encoding gene by siRNA transfection followed by measurement of glucagon release upon Dapa treatment. As shown in **Figure 5**, an effective *Slc5a1* gene silencing was documented both by Real-Time and digital PCR (**Fig. 5A and 5B**). As expected, an increase of pre-proglucagon gene expression upon Dapa treatment was observed; such increase was abolished upon *Slc5a1* silencing and, when silenced cells were challenged for 30 min with Dapa, a reduced pre-proglucagon gene expression was evident (**Fig. 5C**). Accordingly, glucagon release, increased by Dapa, did not vary in silenced cells, being not influenced by the concomitant presence of Dapa for 60min (**Fig. 5D**). This early phenomenon was also confirmed in silenced cells treated with Dapa for 240min.

Effects of Dapa on intracellular signaling - In order to unveil putative molecular mechanism(s) potentially mediating the effect of Dapa on glucagon release, the following genes involved in signaling pathways of interest were explored: PAS Domain Containing Serine/Threonine Kinase (PASK) and total and phosphorylated AMP-activated protein kinase-2 (AMPK-a2); the former is expressed in murine pancreatic beta- and alpha-cells and its overexpression in αTC-1 cells and in human Langerhans islets inhibits glucagon release [18], while the latter has been recently demonstrated to stimulate glucagon release [19]. Untreated cells similarly express the two kinases; a short-term (30 min) incubation with Dapa significantly up-regulated AMPK-a2, while PASK was unchanged. When *Slc5a1* was silenced, AMPK-a2 was down-regulated by approximately 50%, although not influenced by Dapa (**Fig. 6A**); PASK did not significantly vary, confirming the lack of effect of Dapa on this pathway. Data at protein level, obtained in cells treated with Dapa for 4 h, confirmed the results of gene expression (**Fig. 6B**); more in detail, Dapa increased **total and, even more, phosphorylated AMPK-a2,** and *Slc5a1* silencing partially blunted this response, in terms of a **strong reduction of the total isoform** and no appreciable variation of the phosphorylated one. When cells were exposed to low glucose concentrations, AMPK-a2 was significantly ($p<0.05$) upregulated and PASK was down-regulated ($p<0.01$); Dapa did not further enhance this effect (**Fig. 6C**).

As mediators of intracellular signaling and of glucagon release mechanism, *Prkca* and *Snap25* expression was also evaluated upon 30 and 45min of Dapa treatment, with or without *Slc5a1* silencing; however, no significant change in the expression of these kinases was observed (**Suppl. Fig. 3**).

Discussion

The main novelty of the present study is the evidence for a relevant role of SGLT1 cotransporter in mediating glucagon release induced by SGLT2 inhibition.

The cellular model we have used somehow turned out as ideal to identify a potential role of SGLT1, because these cells do not express SGLT2 at any physiologically valuable level, while SGLT1 is constitutively well represented. The recently published paper by Bonner and colleagues [8] demonstrated that both SGLT1 and SGLT2 are expressed in human alpha-cells, while both are scarcely represented or absent in beta-cells. Here, we additionally showed that SGLT1 is predominantly expressed over SGLT2 not only in αTC-1 cell line, but also in laser-capture microdissected mouse primary alpha-cells and in human native pancreatic islets. A more relevant presence of SGLT2-encoding gene in the paper by Bonner could be likely due to technical differences (a pre-amplification of nucleic acids magnifying the detection of scarcely expressed molecules, while we worked on constitutive expression of tissues and cells) or, less likely, to the use of a different αTC-1 clone. In support of our data, a recently deposited complete dataset describing transcriptome analysis of human alpha- and beta-cells sorted from pancreatic islets of non-diabetic donors [20], reported: *a)* the expression enrichment of SGLT1 in alpha-cells vs beta-cells; *b)* the predominant expression of SGLT1 over SGLT2 (expression ranking based on signal intensity from microarray: SGLT1 3779/40030; SGLT2 24718/40030) in alpha-cells, further demonstrating the prevalence of SGLT1 expression in alpha-cells respect to SGLT2.

Another reflection is that the effect of Dapagliflozin in increasing SGLT1 expression is selective, both in terms of gene modulation and of secretory products: as a matter of fact, the expression of

GLUT1, *i.e*. the main glucose transporter of alpha-cells (where glucose transport is indeed much lower than in beta-cells) [21] and GLUT2, a key beta-cell specific transporter, usually scarcely represented in alpha-cells [22], does not vary following Dapagliflozin treatment.

The increased expression of SGLT1 is paralleled by a raise in glucagon release that rapidly occurs and remains stable along quite a long time frame; it is quantitatively relevant and may well explain the increase reported in *in vivo* studies [3, 4]. Hormone release and its intracellular content are both increased along the whole time-frame of the experiment, suggesting an early increase in newly-synthesized hormone production; a role for an enhanced release of cellular glucagon depots is plausible at the end of the observed time-course, and could be tentatively ascribed to glucoxicity itself, as recently suggested [23]. This is confirmed by the observation that the effect of Dapagliflozin on glucagon release was preceded by an early and significant upregulation of pre-proglucagon gene, a prohormone that, after selective enzymatic cleavage, also encodes GLP-1, GLP-2, oxyntomodulin and glicentin [24]. However, Dapagliflozin does not modulate either GLP-1 release or the expression of GLP-1 receptor (a G-protein linked to cAMP signaling); this observation is intriguing, representing a further and indirect confirmation of the specificity and selectivity of the drug effect on glucagon, and not on the whole secretory pattern of the alpha-cell. A fully active GLP-1 system, mainly localized in alpha-cells, has been recently described in human islets [25]; in this view, such selective effect and the lack of interference with the GLP-1 axis is welcome under the clinical viewpoint.

Another transcription factor upregulated by Dapagliflozin in this experimental model is HNF4α, highly expressed in murine [8] and human alpha-cells, downregulated by hyperglycemia, and promoter of the trans-differentiation of alpha into beta-cells [26]. In this scenario, by a pure speculation, beside the reduction of glucose toxicity, Dapagliflozin might indirectly contribute to maintain an adequate beta-cell number and function through the increased HNF4α expression. On the other hand, the lack of variation in the expression of other transcription factors, such as Chromogranin A (that affects islet composition and increases alpha-cell function), PAX6 (able to influence acute glucagon secretion in response to glucose and palmitate), Proconvertase 1/2 (endoproteolytic enzymes that regulate alpha-cell secretory pathway) or Synaptophysin (a marker of differentiated islet endocrine cells) [27] in Dapagliflozin-treated cells supports the hypothesis of a mere effect on the release pattern, rather than on the whole alpha-cell machinery. Even ARX, a gene essential for alpha-cell differentiation, whose functional inactivation promotes the reprogramming of alpha to beta-cells was not influenced by Dapagliflozin [28, 29].

The main physiologic stimulus for glucagon release is represented by low glucose levels; therefore, we tested whether or not pulses of low glucose would modify the secretory response to Dapagliflozin. Overall, the response is preserved, both in terms of expression of SGLT1 encoding gene and of glucagon release, and αTC-1 cells confirm the capacity to adjust their secretory response according to environmental glucose. *Slc5a1*gene silencing confirms our hypothesis of a key role of this transporter in mediating this selective hormonal response. As expected, GLUT2 expression is slightly but significantly higher in the presence of low glucose respect to that found at 25 mmol/l glucose; this response, in an integrated biological system, can be regarded as a potentially defensive action toward hypoglycemia.

Paracrine mechanisms regulating glucagon secretion involve the release of inhibitory factors like somatostatin and, accordingly, blockade of alpha-cell somatostatin receptors increases glucagon secretion [30]. We here show that this regulatory mechanism is not affected by SGLT2 inhibition, neither somatostatin receptor expression varied upon stimulation with Dapagliflozin, adding knowledge to the global metabolic effect of Dapagliflozin, also considering that in the *in vivo* study documenting a raise of glucagon following its administration, somatostatin levels were not measured [3].

Lastly, in an attempt to explore intracellular pathways mediating the enhanced glucagon release induced by Dapagliflozin, we found an interesting effect of this drug on the reciprocal balance between PASK and AMPK-a2. In standard conditions, the effect of Dapagliflozin on glucagon secretion seems to take place *via* an activation of the pro-release kinase (i.e. AMPK-a2, with a prevalent upregulation of its phosphorylated isoform), rather than a down-regulation of the anti-release one (*i.e.* PASK). This hypothesis fits with the effect of *Slc5a1* silencing on both these kinases, at mRNA as well as at protein level: Dapagliflozin mainly increases phosphorylated AMPK-a2 while, in cells silenced for *Slc5a*, **it strongly reduces the total isoform with no appreciable effect on the phosphorylated isoform (likely for a different half-life of the two isoforms),** confirming the key role of the kinase as **main** mediator of this effect.

Noteworthy, this effect is quite different to that induced by low glucose, the most powerful stimulus of glucagon production and release in these cells (usually grown in high glucose concentrations): in such experimental setting, low glucose down-regulates PASK and, in parallel, stimulates AMPK-a2 up-regulation, while the effect of Dapagliflozin on both kinases is blunted. This suggests that SGLT2 inhibitors, even participating in the regulation of glucagon secretion, cannot overcome its main driver, *i.e.* hypoglycemia, mimicked by low glucose culture media in this experimental setting. The lack of effect of Dapagliflozin on Prkca and on Snap25, key molecules in mediating vesicular trafficking and exocytosis and fast Ca^{2+}-triggered release of hormones and neurotransmitters [31, 32] somehow validates the PASK/AMPK-a2 pathway as major candidates for the pro-secretory effects of Dapagliflozin.

Major strengths of our study reside in having identified for the first time a role for SGLT1 in modulating glucagon secretion induced by Dapagliflozin, providing a detailed analysis of other hormone patters normally regulating glucagon secretion. However, we acknowledge the limitation of using a murine cell model, even though some observations have been replicated in murine and in human islets.

Conclusions

In a murine model of alpha-cells largely used for studying the physiology and pharmacology of glucagon secretion, we have unveiled a role for SGLT1 in modulating the promoting effect of Dapagliflozin, a highly selective SGLT2-inhibitor, on glucagon release. This effect does not seem to involve other secretory activities of alpha-cells. Therefore, we might hypothesize that, even in the presence of a high renal SGLT2 selectivity, Dapagliflozin is still able to exert a physiologically relevant action on SGLT1 in other tissues, confirming a more complex and fine participation of these Na-glucose cotransporters in glucose homeostasis, and remarking the need of further studies addressing the extra-renal mechanisms of action of these fascinating compounds.

Acknowledgements

We thank Dr. Brenno D. Astiarraga for his helpful suggestions on glucagon determinations.

Funding

This work has been supported by an unrestricted grant from Astra Zeneca. The company did not have any role in designing the study, generating and analyzing data, writing the manuscript. FD received support from the Italian Ministry of Research (grant n. 2010JS3PMZ_008).

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Legend to Figures

Figure 1

A) Expression of *Slc5a1*, *Slc2a1* and *Slc2a2* genes, encoding for SGLT1, GLUT1 and GLUT2 respectively, in untreated αTC-1 cells (white bars) and after treatment with Dapagliflozin (Dapa) 100 ng/ml for a prolonged timeframe (different tones of gray bars). Data are reported as mean±SD of at least six experiments. * $p<0.01$ vs Untreated

B) *Slc5a2* digital PCR analysis: original imaging from digital PCR are shown, with an explicative comparison between the number of copies of detected mRNA from αTC-1cells, for the reference gene (β-actin) and in murine kidney.

C) A representative WB obtained by loading 100 µg of alpha-cells total protein shows the extremely scarce SGLT2 presence.

Figure 2

A) Real Time PCR analysis of Laser Captured microdissected alpha- and beta-cells for pre-proglucagon, insulin and *Slc5a1* and *Slca2*. Data are reported as fold change+SD respect to beta-cells. * $p<0.05$ vs beta-cells.

B) Real-Time PCR analysis of relative expression of *Slc5a2* and *Slc5a1* genes in human pancreatic islets. *$p<0.005$ vs core; §$p<0.001$ vs *Slc5a2*. Gene expression is confirmed by WB analysis, showing SGLT1 and SGLT2 protein in human pancreatic islets.

C) Representative image of an intact islet, its core and its periphery before and after Laser Capture microdissection.

Figure 3

Glucagon secretion from murine αTC-1 cells, grown at 25 mmol/l glucose, in the basal state and after treatment with dapagliflozin (Dapa) 100 ng/ml for different time periods. Data are reported as mean±SD of at least four experiments. The % release (100 x supernatant)/(supernatant+content) is reported on the right *p<0.05 by ANOVA for repeated measures

Figure 4

Effect of two different glucose concentrations (very low: 1.1 mM and low: 5.5 mM) on glucagon release from untreated and Dapagliflozin-treated αTC-1 cells.

Data are reported as mean±SD of at least eight experiments.

* p<0.01 vs Untreated

Figure 5

Effect of *Slc5a1* gene silencing on *Slc5a1* expression (real time PCR, A); digital PCR, B); pre-proglucagon gene expression (C) and glucagon release at 45 min (D). Data are reported as mean±SD of at least three experiments.

*p<0.005 vs Control; §p<0.05 vs Dapa

Figure 6

A) PASK and AMPK-a2 gene expression in untreated αTC-1 cells (Control), cells treated with Dapagliflozin 100 ng/ml (Dapa), and cells where *Slc5a1* gene has been silenced, in the absence (siRNA) or presence of Dapa (siRNA+Dapa).

B) Protein expression **evaluated by Western Blot analysis** (PASK and total and phosphorylated AMPK-a2) in control cells and after 4 h of gene silencing is reported, together with the quantification of the bands. WB is representative of three experiments.

C) Effect of high (HG) and low (LG, 5.5 mM) glucose and/or Dapa on PASK and AMPK-a2 genes.

*p between 0.01 and 0.005 vs Control; §p<0.05 vs HG

Figure 1

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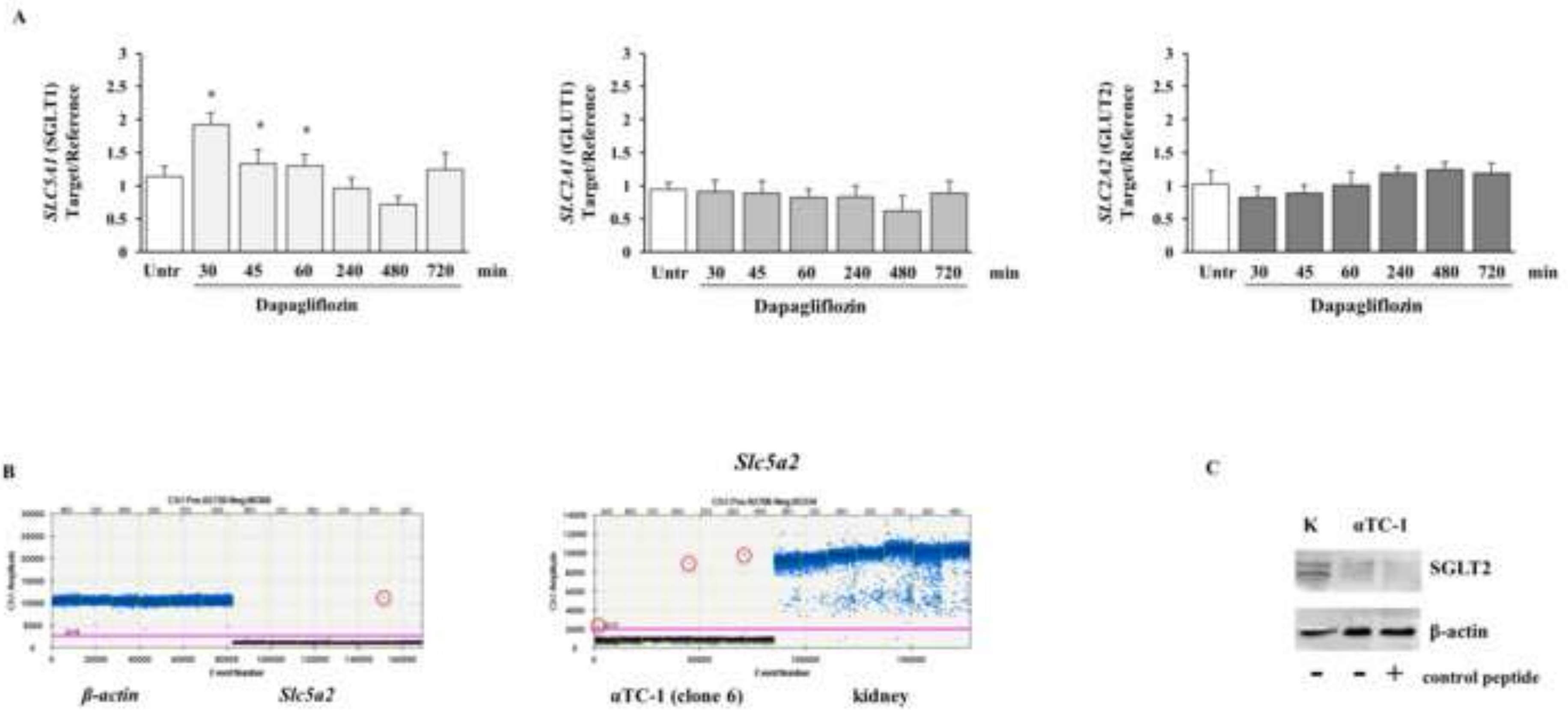

Figure 1

Figure 2

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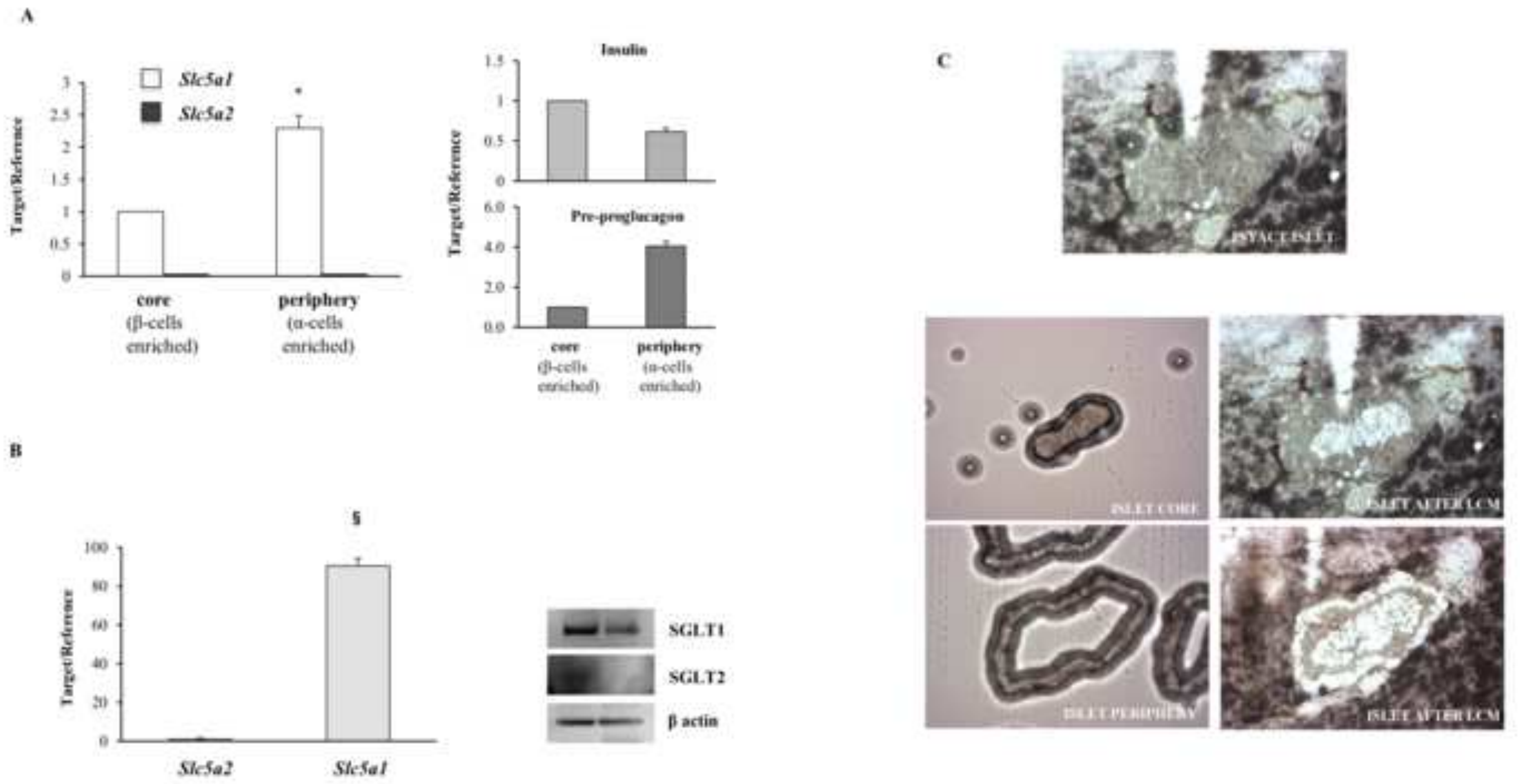

Figure 2

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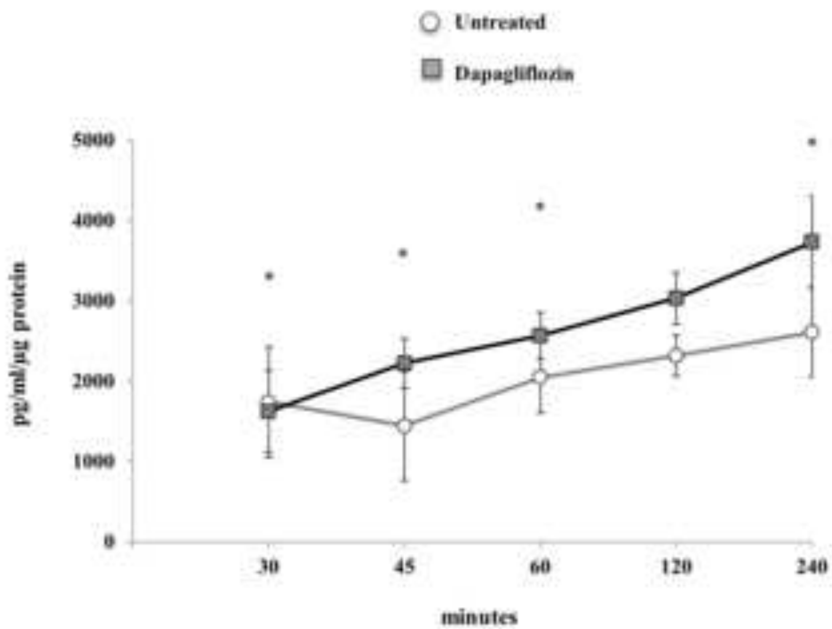

	% release	
	Control	Dapa
30 min	----	----
45 min	----	----
60 min	6.2±0.7	9.3±0.8*
120 min	6.5±0.8	10.2±0.8*
240 min	6.3±0.5	9.6±0.8*
480 min	11.4±0.8	14.6±0.9*

Figure 3

Figure 4

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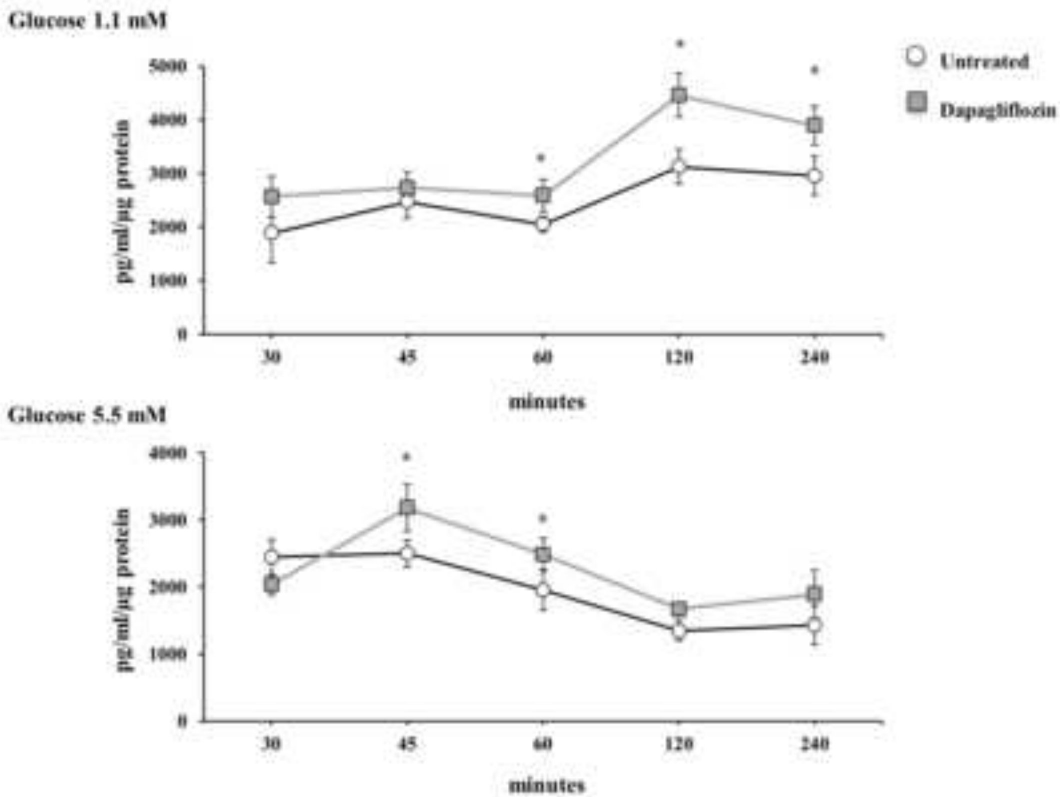

Figure 4

Figure 5
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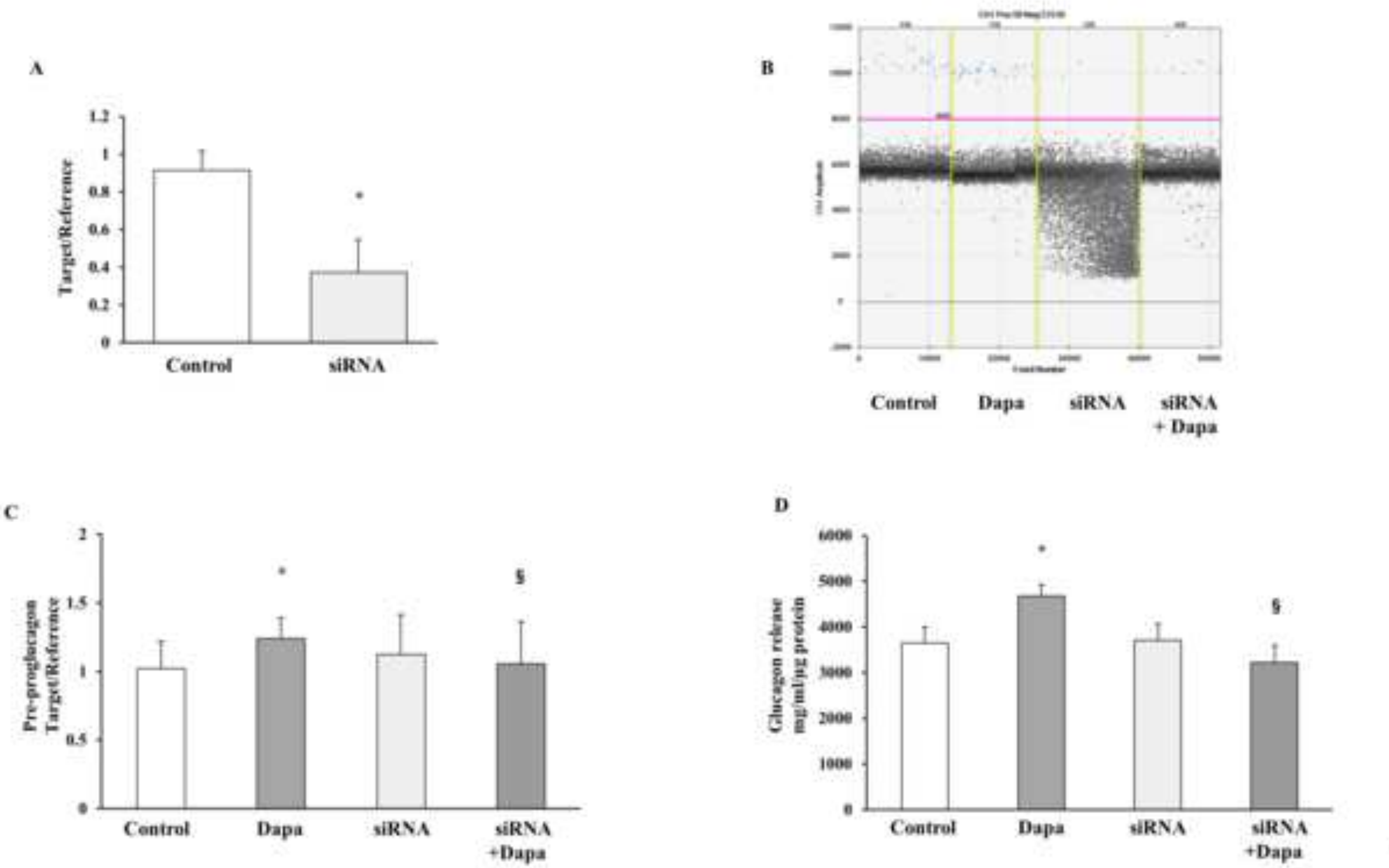

Figure 5

Figure 6
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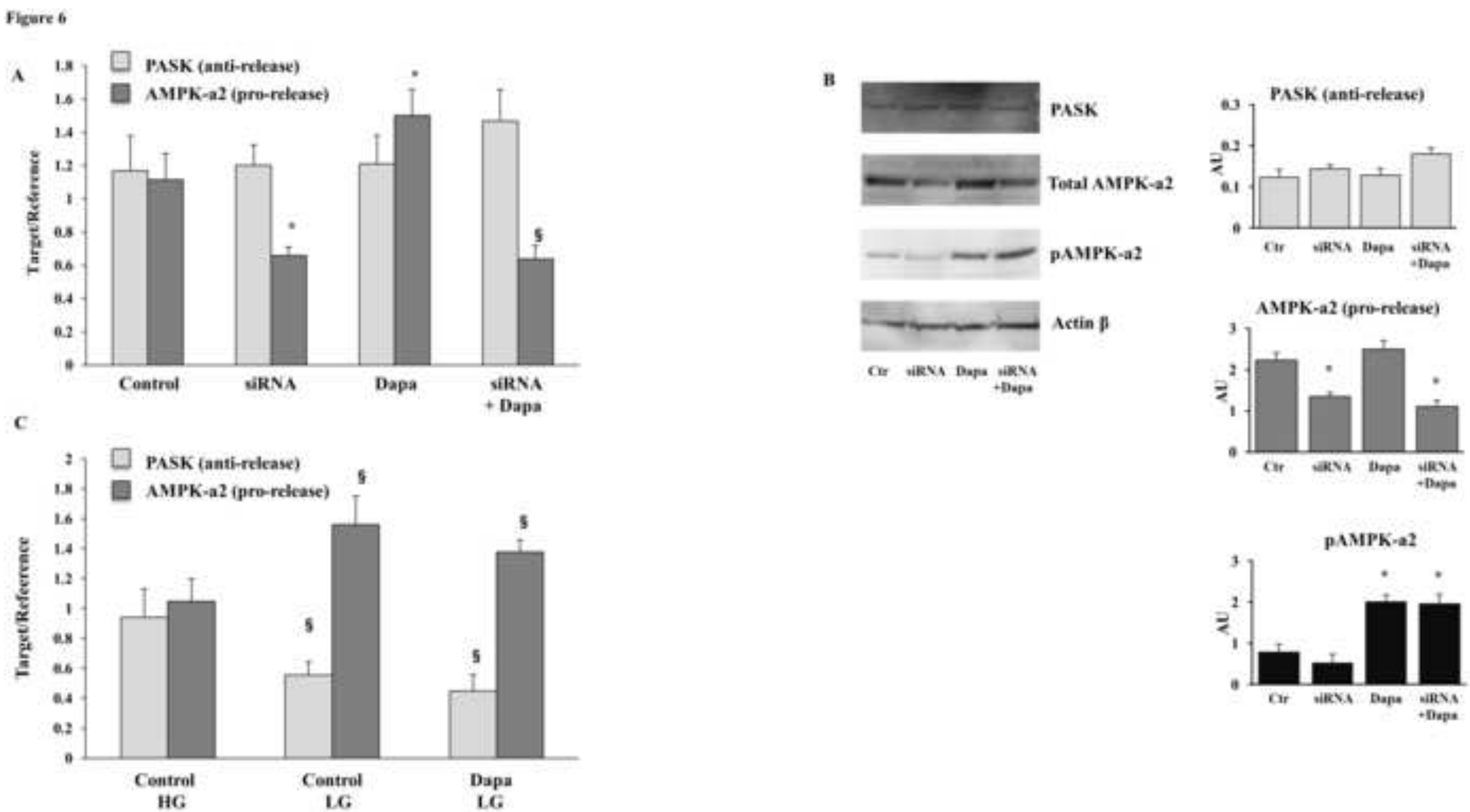

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