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Abstract:

Background: Thiazide diuretics are associated with glucose intolerance and new onset diabetes mellitus. Previous studies demonstrated that thiazides attenuate insulin secretion, but the molecular mechanisms remain elusive. We hypothesized that thiazides attenuate insulin secretion via one of the known molecular thiazide targets in β -cells.

Methods: We performed static insulin secretion experiments with islets of wild-type, NCC (SLC12A3), and NDCBE (SLC4A8) knock-out (KO) mice and with murine Min6 cells with individual knock-down of carbonic anhydrase (CA) isoforms to identify the molecular target of thiazides in β -cells. CA5b KO mice were then used to assess the role of the putative thiazide target CA5b in β -cell function and in mediating thiazide sensitivity *in vitro* and *in vivo*.

Results: Thiazides inhibited glucose- and sulfonylurea-stimulated insulin secretion in islets and Min6 cells at pharmacologically relevant concentrations. Inhibition of insulin secretion by thiazides was CO_2/HCO_3 -dependent, not additive to unselective CA inhibition with acetazolamide, and independent of extracellular potassium. In contrast, insulin secretion was unaltered in islets of mice lacking the known molecular thiazide targets NCC or NDCBE. CA expression profiling with subsequent knockdown of individual CA isoforms suggested mitochondrial CA5b as a molecular target. In support of these findings, thiazides significantly attenuated Krebs cycle anaplerosis through reduction of mitochondrial oxaloacetate synthesis. CA5b KO mice were resistant to thiazide-induced glucose intolerance, and thiazides did not alter insulin secretion in CA5b KO islets.

in β -cells of mice.

Significance Statement:

Thiazide diuretics (thiazides) are among the most widely prescribed drugs worldwide, but their use is associated with glucose intolerance and new onset diabetes mellitus. The molecular mechanisms remain elusive. Our study reveals that thiazides attenuate insulin secretion through inhibition of the mitochondrial carbonic anhydrase isoform 5b (CA5b) in pancreatic β -cells. We furthermore discovered that pancreatic β -cells express only one functional CA isoform, CA5b, which is critical in replenishing oxaloacetate in the mitochondrial tricarboxylic acid cycle (anaplerosis). These findings explain the mechanism for thiazide-induced glucose intolerance and reveal a fundamental role of CA5b in tricarboxylic acid cycle anaplerosis and insulin secretion in β -cells.

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Thiazides Attenuate Insulin Secretion Through Inhibition of Mitochondrial Carbonic Anhydrase 5b in β-islet Cells in Mice

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ABSTRACT

Background: Thiazide diuretics are associated with glucose intolerance and new onset diabetes mellitus. Previous studies demonstrated that thiazides attenuate insulin secretion, but the molecular mechanisms remain elusive. We hypothesized that thiazides attenuate insulin secretion via one of the known molecular thiazide targets in β -cells.

Methods: We performed static insulin secretion experiments with islets of wild-type, NCC (SLC12A3), and NDCBE (SLC4A8) knock-out (KO) mice and with murine Min6 cells with individual knock-down of carbonic anhydrase (CA) isoforms to identify the molecular target of thiazides in β -cells. CA5b KO mice were then used to assess the role of the putative thiazide target CA5b in β -cell function and in mediating thiazide sensitivity *in vitro* and *in vivo*.

Results: Thiazides inhibited glucose- and suffonylurea-stimulated insulin secretion in islets and Min6 cells at pharmacologically relevant concentrations. Inhibition of insulin secretion by thiazides was CO₂/HCO₃⁻-dependent, not additive to unselective CA inhibition with acetazolamide, and independent of extracellular potassium. In contrast, insulin secretion was unaltered in islets of mice lacking the known molecular thiazide targets NCC or NDCBE. CA expression profiling with subsequent knock-down of individual CA isoforms suggested mitochondrial CA5b as a molecular target. In support of these findings, thiazides significantly attenuated Krebs cycle anaplerosis through reduction of mitochondrial oxaloacetate synthesis. CA5b KO mice were resistant to thiazide-induced glucose intolerance, and thiazides did not alter insulin secretion in CA5b KO islets.

Conclusions: Thiazides attenuate insulin secretion via inhibition of the mitochondrial CA5b isoform in β -cells of mice.

INTRODUCTION

Thiazide and thiazide-like diuretics (thiazides) have been the cornerstone for the treatment of essential hypertension and pharmacologic recurrence prevention of kidney stones for more than 50 years. Hence, not surprisingly, thiazides belong to the most widely prescribed drugs worldwide ¹.

Since their introduction into clinical medicine in the 1960ies, thiazides are known to be associated with metabolic side effects, including glucose intolerance and new onset diabetes ²⁻⁸. Several hypotheses have been put forth to explain thiazide-induced glucose intolerance, but the underlying mechanisms remain elusive until today ⁹. Unfortunately, these unpredictable and poorly understood side effects have caused many physicians to avoid the use of these clinically effective, ubiquitously available and cheap drugs. In recognition of this important knowledge gap in a clinically highly relevant area, a working group of the National Heart, Lung and Blood Institute issued a call for research on thiazide-induced dysglycemias a decade ago ⁹.

The classical molecular thiazide target is the Na⁺/Cl⁻ co-transporter NCC (also known as SLC12A3) in distal convoluted tubules (DCT) of the kidney¹⁰. Biallelic pathogenic variants in *SLC12A3* encoding NCC result in Gitelman's syndrome which is characterized by hypotension, hypokalemia, hypomagnesemia and metabolic alkalosis ¹¹. In addition to electrolyte abnormalities, patients affected by Gitelman's syndrome were reported to exhibit an increased prevalence of impaired glucose tolerance ¹²⁻¹⁴. Other molecular thiazide targets than NCC have been described, including the Na⁺-driven Cl⁻/bicarbonate exchanger NDCBE (also known as SLC4A8) and carbonic anhydrase (CA) ^{15, 16}. Thiazides were originally developed by chemical modification of the CA inhibitor acetazolamide (AZM) and retained the ability to inhibit CA ¹⁷. Previous studies demonstrated that AZM or thiazides at high doses attenuate insulin secretion *in vitro*, suggesting that inhibition of CA may play a role in

thiazide-induced glucose intolerance ¹⁸⁻²⁰. If NCC or NDCBE are expressed in pancreatic islets and contribute to insulin secretion has not been explored thus far. The goal of this study was to elucidate the molecular mechanisms underlying thiazide-induced glucose intolerance. We hypothesized that thiazides attenuate insulin secretion in β-cells via a known molecular thiazide target, likely a specific CA isoform.
 METHODS
 Intraperitoneal glucose (IPGTT) and insulin (IPITT) tolerance tests

Tolerance tests were performed in 10-12 weeks old male mice after a 6 AM to 12 PM 6 h fast (IPGTT) or at random fed state at 2 PM (IPITT), as described ^{21, 22}. Blood glucose was measured at time – 30, 0, 15, 30, 60 and 120 min with a Contour glucose monitor (Bayer Healthcare, Germany) by tail vein sampling in duplicates. Vehicle or HCT was applied by intraperitoneal injection at time -30. Glucose (IPGTT; 1 g/kg or 2 g/kg, Sigma-Aldrich) or insulin (IPITT; 0.5 or 1 U/kg Actrapid HM, Novo Nordisk, Denmark) were applied by intraperitoneal injection at time 0. For serum insulin measurements tail vein blood sampling was performed at time -30, 0 and 2 min. Vehicle or HCT was applied at time -30 min and glucose (2 g/kg) was applied at time 0 min. Serum insulin was measured with the ultrasensitive mouse insulin ELISA (CrystalChem, Downers Grove, IL; # 90080).

Isolation of islets and in vitro insulin secretion assays

Pancreata were perfused *in situ* with collagenase solution and islets isolated exactly as described ^{22, 23}. After overnight incubation in RPMI medium with 11 mM glucose, islets were washed twice with KRBH containing (in mM): 115 NaCl, 5 KCl, 25 NaHCO₃, 0.5 NaH₂PO₄, 1.2 MgSO₄, 2.5 CaCl₂, 2 glucose, 10 HEPES pH 7.4 and 0.1 % (w:v) BSA. In case of experiments without CO₂/HCO₃⁻, the incubation buffer contained (in mM): 140 NaCl, 5 KCl,

0 NaHCO3, 0.5 NaH2PO4, 1.2 MgSO₄, 2.5 CaCl2, 2 glucose, 10 HEPES pH 7.4 and 0.1 % (w:v) BSA . After washing, islets were placed in 12-well plates (10 islets/well) containing 1 ml of buffer with 2 mM glucose and pre-incubated for 2.5 hrs at 37 °C. Insulin secretion into the supernatant was then measured for 2 h for all secretagogues except for KCl, which was for 1 h. Supernatants were then harvested, plates put on ice and total cellular insulin extracted by addition of acid ethanol (70 % ETOH, 1.5% HCl conc). Secreted and cellular insulin were determined with the ultra-sensitive mouse insulin ELISA.

Statistical analysis

Data distribution was assessed by D'Agostino-Pearson tests and QQ-plots. In case of deviation from a Gaussian distribution, non-parametric tests were employed and data displayed as median \pm IQR. Data analysis was done with GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). All statistical tests were two-sided and p < 0.05 was considered statistically significant.

RESULTS

Hydrochlorothiazide induces glucose intolerance in mice without affecting insulin sensitivity

We first assessed if thiazides induce glucose intolerance in mice. To this end, intraperitoneal glucose tolerance tests (IPGTT) were performed in 3 months old male C57BL/6J mice treated with intraperitoneally injected 1, 2, 5 or 50 mg/kg body weight hydrochlorothiazide (HCT) or vehicle. As shown in Fig. 1A-H, HCT treated mice exhibited significantly higher glycemic excursions during IPGTTs compared to vehicle treated mice in a dose-dependent manner. The lowest HCT dose associated with glucose intolerance was 5 mg/kg (Fig. 1E,F). In all conditions, body weight was similar in HCT and vehicle treated mice (Fig. S1A-D). After overnight fasting, IPGTT results were similar compared to 6 h fasted mice (Fig. S2A-C).

Plasma potassium levels were unaltered in HCT-treated compared to vehicle-treated mice during the IPGTT (Fig. S2D). Also with a lower glucose challenge (1g/kg instead of 2 g/kg body weight), HCT treated mice displayed significantly higher glycemia compared to vehicle treated mice (Fig. S3). To assess the impact of HCT on insulin sensitivity, we performed intraperitoneal insulin tolerance tests (IPITT). As demonstrated in Fig. 1I-L and Fig. S1E,F, insulin sensitivity, tested by two different insulin doses (0.5 IU/kg and 1 IU/kg body weight, respectively), was not altered by HCT application. Together these findings reveal that HCT induces glucose intolerance with maintained insulin sensitivity in mice. In humans, robust diuretic effects are observed with HCT doses of 1-2 mg/kg body weight 24 . Significantly higher thiazide doses are needed in mice to stimulate natriuresis, in case of HCT typically doses of 20 - 50 mg/kg body weight are applied ²⁵⁻²⁸. The reason for this discrepancy is not clear, but likely due to differences in the pharmacokinetics of thiazides between mice and humans. To this end, we established the dose-response for the natriuretic effect of HCT in mice, which reflects inhibition of the primary thiazide target NCC in the DCT. As shown in Fig. S4, there was a dose-dependent increase in urinary Na⁺ excretion. The 5 mg/kg but not 1 mg/kg HCT dose increased urinary Na⁺ excretion compared to vehicle treated mice, and the natriuretic effect was higher with 50 mg/kg compared to 5 mg/kg. Hence, the 5 mg/kg HCT dose in mice corresponds to a HCT dose typically employed in humans (12.5 - 50 mg daily with corresponding steady-state plasma concentrations of 0.02 - 0.020.2 µM)²⁹⁻³¹.

Thiazides attenuate insulin secretion in vivo and in vitro

To further investigate the basis of HCT-induced glucose intolerance, we measured serum insulin levels in mice treated with HCT or vehicle. As depicted in Fig. 2A,B and Fig. S1G,H, serum insulin levels were significantly lower 2 min after an intraperitoneal glucose challenge

(2 g/kg body weight) in mice pre-treated with either 5 or 50 mg/kg body weight HCT, indicating reduced first-phase insulin secretion. This notion is supported by the observation that HCT-treated mice exhibit hyperglycemia early during the IPGTT, followed by a decrease in blood glucose that parallels the decrease observed in vehicle-treated mice.

We then assessed the impact of HCT on insulin secretion in primary islets isolated from C57BL/6J mice and in the murine β -cell line Min6 (Fig. 2C,D, Fig. S5). HCT significantly attenuated insulin secretion in islets and Min6 cells *in vitro* at pharmacologically relevant concentrations while not affecting cell viability ³¹. We obtained similar findings with other thiazides, including metolazone, chlorthalidone, indapamide and bendroflumethiazide (Fig. S6). HCT also significantly lowered sulfonylurea-induced insulin secretion (250 μ M tolbutamide) while it only had a small effect on basal (2 mM glucose) and direct depolarization-induced insulin secretion by high (30 mM) extracellular K⁺ (Fig. 2E) ³². Together, these results suggest reduced insulin secretion as mechanism of HCT-induced glucose intolerance.

Thiazides target carbonic anhydrase in β-cells

To further define the basis of reduced insulin secretion by thiazides, we assessed mRNA expression of the known thrazide targets including NCC, NDCBE and CA isoforms in Min6 cells and murine islets (Fig. S7A-D). Transcripts of both NDCBE and NCC were detectable in Min6 cells and islets. Of the 15 CA isoforms CA1, CA4 and CA5a were not detectable in both Min6 cells and islets. CA6 and CA7 were expressed in Min6 cells but not islets. We next assed the impact of genetic NDCBE or NCC deletion in islets on insulin secretion. As shown in Fig. S7E,F, islets isolated of NDCBE and NCC knock-out (KO) mice displayed no insulin secretion deficit. To assess the role of NCC in systemic glucose homeostasis, we additionally performed IPGTTs in NCC KO mice. As shown in Fig. S7G,H, genetic loss of

NCC was not associated with altered glucose tolerance. Furthermore, HCT attenuated insulin secretion to a similar degree in islets of NCC KO mice as in islets of WT mice (Fig. S7I). We then assessed the role of CA in insulin secretion. Inhibition of CA with the non-specific CA inhibitor AZM significantly attenuated insulin secretion in both Min6 cells and islets in vitro (Fig. 3A,B) and induced glucose intolerance in vivo (Fig. 3C-E). Compared to either AZM or HCT alone, the combination of AZM and HCT did not further attenuate insulin secretion (Fig. 3F), and HCT had no effect on insulin secretion in a CO_2/HCO_3^{-1} free condition (Fig. 3G). These results reveal that the effect of HCT on insulin secretion is CO₂/HCO₃⁻ -dependent and not additive to (unselective) CA inhibition, suggesting that HCT targets one or several CA isoform(s) in β -cells that are critical for insulin secretion. Currently, there are no specific CA isoform inhibitors available. To identify the responsible CA isoform(s), we performed siRNA-mediated knock-down of all CA isoforms expressed in Min6 cells (Fig. 3H, Fig. S7C, Fig. S8) and performed static insulin secretion experiments. Only CA2 and CA5b knock-down significantly attenuated insulin secretion in Min6 cells, whereas the other CA isoforms expressed in Min6 cells were dispensable for insulin secretion (Fig. 3H). In addition, omission of CO_2/HCO_3^- in the incubation medium did not further impair insulin secretion in either CA2 or CA5b depleted cells compared to control, indicating critical but non-redundant roles of these two CA isoforms for insulin secretion in Min6 cells (Fig. 3I). We then assessed CA isoform mRNA expression in murine islets and purified β cells. As shown in Fig. 3J,K and Fig. S7D, while many CA isoforms are expressed in islets, primary β -cells only express the two isoforms CA5b and CA10. These findings, together with the fact that CA10 is a catalytically inactive and secreted CA isoform, suggest mitochondrial CA5b as the likely target of thiazides in β -cells³³.

Hydrochlorothiazide inhibits oxaloacetate synthesis in β-cells

The two mitochondrial CA isoforms CA5a and CA5b utilize CO₂, which freely diffuses into mitochondria, to produce HCO₃⁻ (Fig. S11). Several mitochondrial enzymes critically depend on HCO₃⁻ such as pyruvate carboxylase (PC), which generates oxaloacetate (OAA) from pyruvate and HCO₃⁻³⁴. β -cells exhibit high PC activity, and a large fraction of pyruvate entering mitochondria is converted to OAA. PC activity correlates steeply with insulin secretion and OAA is a central metabolite in nutrient-induced insulin secretion (Fig. S11) ³⁵. OAA synthesis by PC (anaplerosis) fuels the tricarboxylic acid cycle (TCA) leading to an increases of TCA intermediates such as citrate, isocitrate, and malate, that are transported from the mitochondria to the cytoplasm (cataplerosis). OAA is also the starting point of phosphoenolpyruvate (PEP) synthesis via the mitochondrial GTP-dependent enzyme PCK2 ^{35,38}. PEP synthesized from OAA exits mitochondria to the cytosol, where pyruvate kinase (PK) converts ADP and PEP into ATP and pyruvate, leading to closure of K_{ATP} channels and initiation of insulin secretion ^{39,40}. The third OAA-dependent pathway contributing to nutrient-stimulated insulin secretion is the pyruvate/malate shuttle, which results in the generation of cytosolic NADPH via malic enzyme ^{35,36}.

In a next step, we measured glucose-induced insulin secretion in Min6 cells in the presence of HCT, the PC inhibitor phenylacetic acid (PAA) or a combination of both, with and without CO₂/HCO₃⁻. As shown in Fig. 4A, inhibition of glucose-stimulated insulin secretion was equal with incubation of HCT or PAA, and the combination of both did not result in a further reduction. Furthermore HCT, PAA or the combination of both had no effect on glucosestimulated insulin secretion in CO₂/HCO₃⁻-free conditions. Similarly, in murine islets, glucose-stimulated insulin secretion was reduced to a similar degree in the presence of HCT or PAA, and the combination of both HCT and PAA did not result in a further reduction (Fig. 4B). We then measured OAA levels in Min6 cells exposed to HCT or PAA during glucose stimulation. As shown in Fig. 4C, both HCT and PAA attenuated OAA levels in Min6 cells by a similar magnitude and the combination of both did not result in a further reduction of OAA levels. Furthermore HCT, PAA or the combination of both had no effect on OAA levels in Min6 cells in CO_2/HCO_3^- -free conditions. Finally, we treated Min6 cells with control or CA5b siRNA, and measured OAA in the presence and absence of CO_2/HCO_3^- . CA5b depletion or HCT were equally effective in reducing OAA levels in Min6 cells, and this effect was again clearly CO_2/HCO_3^- -dependent (Fig. 4D).

Due to the tight functional coupling between CA5b and PC, a direct effect of thiazides on PC activity cannot be definitively ruled out with studies employing intact cells. To test for a possible effect of HCT on PC, we performed in vitro PC activity experiments in Min6 cell lysates in the absence of CO_2 with exogenous administration of HCO_3^- . As demonstrated in Fig. 4E, HCT, AZM or the combination of HCT and AZM had no effect on PC activity, further supporting the notion of CA5b as molecular target of HCT in β -cells. To substantiate this claim, we performed IPGTTs in CA5b KO and WT littermate mice (mixed C57BL6/J / SV129 background) treated with 5mg/kg body weight HCT or vehicle ³⁴. HCT-treated WT mice exhibited significantly higher glycemic excursions during IPGTTs compared to vehicle treated mice (Fig. 5A,B, Fig. S9A). In contrast, we observed no difference between HCT and vehicle treated CA5b KO mice (Fig. 5C,D, Fig. S9B). Acid-base parameters and electrolytes were similar in both groups of mice (Table S1). We then performed IPITTs to assess the impact of HCT on insulin sensitivity in 3 months old male WT and CA5b KO mice. As demonstrated in Fig. 5E-H and Fig. S9C,D, insulin sensitivity was similar in all groups of mice. Furthermore, in WT but not CA5b KO mice, HCT treatment was associated with lower serum insulin after the glucose challenge compared to vehicle treatment (Fig. 5I,J, Fig.

S9E,F). Together, these *in vivo* findings demonstrate that genetic deletion of CA5b confers resistance to HCT-induced glucose intolerance.

To assess the role of genetic CA5b deletion on insulin secretion, we performed static insulin secretion experiments with islets isolated from WT and CA5b KO mice (Fig. 6A). No CA5b expression was detectable in islets isolated from CA5b KO mice (Fig. S10A). Furthermore, expression profiling of other CA isoforms did not reveal differences between WT and CA5b KO islets (Fig. S10B). As previously observed with islets isolated from C57BL/6J mice, HCT attenuated insulin secretion in islets isolated from WT littermates of CA5b KO mice (mixed C57BL/6J/SV129 background). While insulin secretion of CA5b KO islets was significantly reduced compared to WT islets in the presence of the vehicle, HCT had no effect on insulin secretion of CA5b KO islets. A similar pattern was observed when we quantified OAA levels in WT and CA5b KQ islets (Fig. 6B). In WT islets, HCT attenuated both high glucose (20 mM)- and sulfonylurea (250 µM tolbutamide)-induced insulin secretion, but did not influence basal (2mM glucose) or direct depolarization-induced insulin secretion by high (30 mM) extracellular K⁺ (Fig. 6C). In contrast, HCT did not attenuate basal or stimulated insulin secretion in CA5b KO islets (Fig. 6D). Together these results demonstrate that mitochondrial OAA synthesis and insulin secretion capacity of CA5b KO islets are resistant to the action of HCT.

DISCUSSION

Our study reveals that thiazides induce acute glucose intolerance in mice via attenuation of insulin secretion through inhibition of CA5b in β -cells. HCT and other frequently used thiazides, such as chlorthalidone, indapamide, metolazone and bendroflumethiazide, inhibited insulin secretion in a pharmacologically relevant, submicromolar range. CA expression profiling in Min6 cells and islets and subsequent siRNA knock-down

experiments of individual CA isoforms in Min6 cells suggested the mitochondrial CA5b isoform as molecular target of thiazides in β -cells. In support of these results, CA5b KO mice were resistant to HCT-induced glucose intolerance, and insulin secretion of CA5b-deficient islets or Min6 cells was unaffected by HCT.

Mitochondrial CA5b provides HCO_3^- for anaplerotic OAA synthesis from pyruvate by PC (Fig. S11)³⁵. Deletion of CA5b or treatment with HCT greatly attenuated OAA levels in islets or Min6 cells. In line with these findings, purified full length (human) CA5b was previously shown to be directly inhibited by HCT and other thiazides *in vitro* with a K_i in the nanomolar range ¹⁷. While direct PC inhibition by PAA in islets mimicked the findings obtained with HCT treatment or CA5b deletion, our *in vitro* studies in CO₂-free conditions with exogenous administration of HCO_3^- demonstrate that PC is not inhibited by HCT or other thiazides up to the concentration of 100° M. This experiment enabled to functionally separate the two closely interacting enzymes CA5b and PC and led to the conclusion that PC *per se* is not thiazide-sensitive. In support of these results, structural studies demonstrated direct interaction of thiazides with CA isoforms ^{17, 41}.

OAA is a well-established central metabolite in nutrient-induced insulin secretion 35 . In support of this, we found that secretagogue-induced insulin secretion was severely impaired in CA5b KO islets. Attenuation of insulin secretion was more pronounced in CA5b KO islets compared to acute knock-down with siRNA or treatment with thiazides, primarily due to significantly increased basal insulin secretion in CA5b KO islets. CA5b KO islets were completely unresponsive to stimulation by high glucose, tolbutamide and high extracellular K⁺.

In Min6 cells but not WT islets, HCT also slightly reduced basal and high K⁺-stimulated insulin secretion. The reason for these differences is not clear at the moment. We speculate that residual CA5b activity (inhibition, siRNA knock-down) versus complete loss of CA5b

activity (KO model), and acute (inhibition, siRNA knock-down) versus chronic CA5b deficiency (KO model) may play a role.

Interestingly, Min6 cells express several CA isoforms, and seem to depend on cytosolic CA2 activity in addition to mitochondrial CA5b for insulin secretion. In contrast, primary murine β-cells only express CA5b and CA10. While CA2-positive pancreatic cells are progenitors of both exocrine and endocrine pancreatic cells, CA2 remains highly expressed in the exocrine but not endocrine pancreas upon differentiation ⁴². Likely, immortalization procedures, clonal selection artifacts and/or adaptations to cell culture conditions are responsible for the altered CA expression profile and the acquired dependence on CA2 for insulin secretion in Min6 cells. As is the case for CA5b, also CA10 is expressed in both Min6 cells and primary β -cells. CA10 is a catalytically inactive, secreted glycoprotein that was recently shown to physically interact with neurexins, a family of presynaptic adhesion molecules, and to facilitate their surface transport $^{33, 43}$. Hence, our data intriguingly suggest that β -cells do not express a cytoplasmic CA isoform. Furthermore, only one of the two mitochondrial CA isoforms is expressed in β -cells. CA5b KO mice do not exhibit an overt phenotype ³⁴. In contrast, CA5a knock-out mice exhibit reduced growth, poor fertility and hyperammonemia as a result of defective ureagenesis in the liver. This suggests non-redundant physiological roles of the two mitochondrial CA isoforms. Purified CA5a can also be inhibited by thiazides in vitro, but the K_i values for thiazides are much higher compared to CA5b ¹⁷. Nevertheless, an effect of thiazides on CA5a activity with therapeutic doses of thiazides would theoretically still be possible and should be investigated further.

In the past, several hypotheses have been proposed for thiazide-induced glucose intolerance, including decreased peripheral ⁴⁴⁻⁴⁶ or hepatic insulin sensitivity ^{47, 48}, activation of the sympathetic nervous and the renin angiotensin system by thiazides ⁹ or attenuation of insulin secretion due to thiazide-induced hypokalemia ⁴⁹. In our acute model, insulin sensitivity was

not affected by HCT and not different between WT and CA5b KO mice. Also, we observed thiazide-induced attenuation of insulin secretion in the absence of changes in extracellular K^+ .

Our study has also limitations, such as the lack of a conditional CA5b KO mouse model to exclude the possibility that pathways outside the β -cells contribute to thiazide-induced glucose intolerance. Also, while insulin tolerance tests did not display differences between vehicle and thiazide treated groups, we did not perform hyperinsulinemic euglycemic clamping experiments to definitively rule out an impact of HCT on hepatie gluconeogenesis. Further, we did not assess the impact of chronic thiazide administration on glucose metabolism. It is possible that additional mechanisms contribute to thiazide-induced glucose intolerance during long-term administration of thiazides. Clearly, however, acute thiazide administration results in CA5b-dependent attenuation of insulfn secretion with subsequent glucose intolerance. Furthermore, chronic mitochondrial CA5b deficiency in β -cells cannot be fully compensated. While the basal insulin secretion is increased, islets of mice with a constitutive CA5b deletion secrete significantly less insulin when stimulated with glucose or sulfonylureas compared to islets of WT mice.

In summary, our results demonstrate that thiazides induce glucose intolerance by an attenuation of insulin secretion in β -cells through inhibition of mitochondrial CA5b.

AUTHOR CONTRIBUTIONS

Patrycja Kucharczyk: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing

Giuseppe Albano: Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – review & editing

Christine Deisl: Data curation, Investigation, Methodology, Writing - review & editing

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Matteo Bargagli: Formal analysis, Investigation, Writing – review & editing

Manuel Anderegg: Investigation, Writing – review & editing

Stephan Wueest: Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing

Daniel Konrad: Formal analysis, Funding acquisition, Methodology, Supervision, Writing – review & editing

Daniel Fuster: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing

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DISCLOSURES

M. Anderegg reports Ownership Interest: small numbers: BioNTech, Moderna, Alnylam Pharmaceuticals, Bayer AG, Novartis, Roche. D. Fuster reports Consultancy: Otsuka Pharmaceuticals Switzerland, Alnylam Pharmaceuticals, Kyowa Kirin; Research Funding: Otsuka Pharmaceuticals Switzerland, Boehringer Ingelheim; Honoraria: Otsuka Pharmaceuticals Switzerland, Alnylam Pharmaceuticals; and Advisory or Leadership Role: Otsuka Pharmaceuticals Switzerland, Alnylam Pharmaceuticals, Kyowa Kirin. D. Konrad reports Research Funding: NovoNordisk. The remaining authors have declared that no conflict of interest exists.

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DATA SHARING STATEMENT

All raw data, processed data and corresponding metadata will be made available on Dryad (https://datadryad.org/stash) upon publication of the manuscript.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at XX.

- Supplemental Methods
- Supplemental Figure 1. Body weights of mice of *in vivo* experiments displayed in Fig. 1.
- Supplemental Figure 2. Glucose tolerance test with hydrochlorothiazide treatment after overnight fasting.
- Supplemental Figure 3. Glucose tolerance test with hydrochlorothiazide treatment with reduced dose (1 g/kg) glucose challenge.
- Supplemental Figure 4. Dose-dependence of the natriuretic effect of hydrochlorothiazide in mice.
- Supplemental Figure 5. Assessment of cell viability in murine islets or Min6 cells treated with

hydrochlorothiazide.

- Supplemental Figure 6. Effect of thiazide diuretics and thiazide-like diuretics on insulin secretion.
- Supplemental Figure 7. Expression of thiazide targets in Min6 cells and islets and insulin secretion studies of islets isolated from NCC and NDCBE KO mice.
- Supplemental Figure 8. Knock-down of CA isoforms in Min6 cells by siRNA.
- Supplemental Figure 9. Body weights of mice of in vivo experiments displayed in Fig. 4.
- Supplemental Figure 10. CA isoform expression in islets of WT and CA5b KO mice.
- Supplemental Figure 11. Schematic of a islet β -cell.
- Supplemental Table 1. Acid-base parameters and electrolytes of WT and CA5b KO mice.

SUPPLEMENTAL MATERIAL -- http://links.lww.com/JSN/E387

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Figure 1. Hydrochlorothiazide induces glucose intolerance in mice without affecting insulin sensitivity. Intraperitoneal glucose tolerance tests of C57BL/6J mice with corresponding AUCs treated with 1 mg/kg (**A**,**B**), 2 mg/kg (**C**,**D**), 5 mg/kg (**E**,**F**) and 50 mg/kg (**G**,**H**) hydrochlorothiazide (HCT) or vehicle in 6 h fasted mice. HCT or vehicle i.p. (arrow H) was applied at time point -30 min, glucose (2g/kg; arrow G) was applied at time point 0 min. Whole blood glucose was measured at indicated time points. Intraperitoneal insulin tolerance tests of C57BL/6J mice with corresponding AUCs treated with 5 mg/kg HCT or vehicle i.p. (arrow H) at time point -30 min and insulin (arrow **D** at time point 0 min. (**I**,**J**) insulin 0.5 IU/kg i.p., (**K**,**L**) insulin 1 IU/kg i.p. (**A**-**H**) N=10 mice per group in all experiments. Data are shown as mean ± SD (panels **A**,**C**,**E**,**G**,**I**,**K**) or median ± IQR (panels **B**,**D**,**F**,**H**,**J**,**L**). Asterisks denote significance for the indicated comparisons (two-tailed unpaired Student's t-test panels **A**,**C**,**E**,**G**,**I**,**K**, Mann, Whitney test panels **B**,**D**,**F**,**H**,**J**,**L**; *p <0.05,**p <0.01, ***p <0.001/****p <0.0001).



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Figure 2. Hydrochlorothiazide attenuates insulin secretion *in vivo* **and in** *vitro*. Serum insulin of C57BL/6J mice treated with 5 mg/kg (**A**) or 50 mg/kg (**B**) HCT or vehicle i.p. (arrow H) at time point -30 min and glucose (2g/kg; arrow G) at time point 0 min. Data are shown as median \pm IQR. (**A**,**B**) Asterisks denote significance for comparisons between groups of mice at indicated time points (Mann-Whitney test; ***p <0.001). (**C**) Glucose-stimulated insulin secretion (20 mM glucose; GSIS) of islets isolated from C57BL/6J mice incubated with vehicle (DMSO 1:1000) or HCT at indicated concentrations. Each dot represents islets isolated of an individual mouse. (**D**) GSIS of Min6 cells incubated with vehicle or HCT at indicated concentrations. Data represent three-individual experiments combined. (**E**) Insulin secretion of Min6 cells in the presence of indicated concentrations of glucose (2 or 20 mM) and KCl (5 or 30 mM), treated with 10⁻⁸ M HCT or 250 μ M tolbutamide. Data represent three individual experiments combined. (**C**-**E**) Data are shown as individual observations with mean \pm SD. Asterisks denote significance for the indicated comparisons (ANOVA with Tukey post-hoc test; **p <0.01, ***p < 0.001).



Figure 3. Hydrochlorothiazide targets carbonic anhydrase in β -ells. (A) Glucose-

stimulated insulin secretion (20 mM glucose; GSIS) of Min6 cells incubated with vehicle (DMSO 1:1000) or the carbonic anhydrase inhibitor acetazolamide (AZM) at indicated concentrations. (B) GSIS of islets isolated from C57BL/6J mice incubated with vehicle or AZM at indicated concentrations. Each dot represents islets isolated of an individual mouse. (C-E) Intraperitoneal glucose tolerance tests with corresponding AUCs and body weights of C57BL/6J mice treated with 5 mg/kg AZM. AZM or vehicle i.p. (arrow A) was applied at time point – 30 min, glucose (2g/kg; arrow G) was applied at time point 0 min. Whole blood glucose was measured at indicated time points. N=8 mice per group. (F) GSIS of Min6 cells treated with vehicle or indicated concentrations (M) of either AZM or HCT, or AZM and HCT combined. (G) GSIS of Min6 cells in the presence (+) or absence (-) of CO₂/HCO₃, treated with vehicle or HCT at indicated concentrations. (H) GSIS of Min6 cells treated with control siRNA or siRNAs targeting indicated carbonic anhydrase (CA) isoforms. (I) GSIS of Min6 cells treated with control siRNA or siRNAs targeting CA2 or CA5b, respectively, in the presence (+) or absence (-) of CO_2/HCO_3 . (J) Expression profiling of CA isoforms in purified murine β -cells by real-time PCR and normalized to GAPDH. Each dot represents β cells isolated from an individual mouse. (K) Assessment of purity of isolated islets and purified β -cells by transcript expression analysis of amylase (marker of exocrine pancreas), glucagon (marker of α -cells) and insulin (marker of β -cells) in pancreas, islets and purified β cells by real-time PCR and normalized to GAPDH. Each dot represents tissue or cells isolated from an individual mouse.

Min6 cell experiments represent three individual experiments combined. Data are shown as mean \pm SD except in panels **D**,**F** where median \pm IQR is displayed. Asterisks denote significance for the indicated comparisons (ANOVA with Tukey post-hoc test panels **A**,**B**,**I**;



two-tailed unpaired Student's t-test panel **C**; Mann-Whitney test panel **D**; Kruskal-Wallis with Dunn's post-hoc test panel **F**; *p <0.05, **p <0.01, ***p <0.001; ****p <0.0001)

Figure 4. Hydrochlorothiazide attenuates oxaloacetate synthesis in β -cells. (A) Glucosestimulated insulin secretion (20 mM glucose; GSIS) of Min6 cells in the presence (+) or absence (-) of CO₂/HCO₃, treated with vehicle (DMSO 1:1000), hydrochlorothiazide (HCT), the pyruvate carboxylase inhibitor phenylacetic acid (PAA) or a combination of PAA and HCT at indicated concentrations. (B) GSIS of islets isolated from C57BL/6J mice incubated vehicle, HCT, PAA or a combination of PAA and HCT at indicated concentrations. (C) Oxaloacetate (OAA) content normalized to protein of Min6 cells stimulated with 20 mM glucose, in the presence (+) or absence (-) of CO_2/HCO_3^- and treated with either vehicle, HCT, PAA or a combination of HCT and PAA at indicated concentrations. (D) OAA content normalized to protein of Min6 cells stimulated with 20 mM glucose, in the presence (+) or absence (-) of CO₂/HCO₃, treated with control siRNA or siRNA targeting CA5b, and incubated with either vehicle or HCT at indicated concentrations. (E) Pyruvate carboxylase activity in lysates of Min6 cells cultured for 4 h in the absence of CO₂ prior to lysis, measured in CO₂-free condition in the presence of indicated concentrations of HCT or AZM. Data are shown as individual observations with mean \pm SD and represent three individual experiments combined. Asterisks denote significance for the indicated comparisons (ANOVA with Tukey post-hoc test; ****p < 0.0001)



Figure 5. CA5b KO mice are resistant to hydrochlorothiazide-induced glucose

intolerance. Intraperitoneal glucose tolerance tests in WT (**A**,**B**) and CA5b KO (**C**,**D**) mice with corresponding AUCs treated with 5 mg/kg hydrochlorothiazide (HCT). HCT or vehicle i.p. (arrow H) was applied at time point – 30 min, glucose (2g/kg; arrow G) was applied at time point 0 min. Whole blood glucose was measured at indicated time points. Intraperitoneal insulin tolerance tests in WT (**E**,**F**) and CA5b KO (**G**,**H**) mice with corresponding AUCs treated with 5 mg/kg HCT or vehicle i.p. (arrow H) at time point – 30 min and insulin (1 IU/kg i.p.; arrow I) at time point 0 min. Serum insulin in WT (**I**) and CA5b KO (**J**) mice treated with 5 mg/kg HCT or vehicle i.p. (arrow H) at time point – 30 min and insulin (2g/kg; arrow G) at time point 0 min. N=10 mice per group in all experiments. Data are show as mean \pm SD. Asterisks denote significance for the indicated comparisons (two-tailed unpaired Student's t-test; ****p < 0.0001).



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Figure 6. Hydrochlorothiazide does not attenuate insulin secretion and oxaloacetate levels in CA5b KO islets. (A) Glucose-stimulated insulin secretion (20 mM glucose) of WT (filled circles) and CA5b KO (open circles) islets treated with vehicle (DMSO 1:1000) or hydrochlorothiazide (HCT) at indicated concentrations. (B) Oxaloacetate content normalized to protein of WT (filled circles) and CA5b KO (open circles) islets stimulated with 20 mM glucose and incubated with vehicle or HCT at indicated concentrations. Insulin secretion of WT (C) or CA5b KO (D) islets in the presence of indicated concentrations of glucose (2 or 20 mM) and KCl (5 or 30 mM), incubated with vehicle, HCT (10^{-8} M) or tolbutamide (250 µM). Data are shown as individual observations with mean \pm SD. Each dot represents islets isolated from an individual mouse. Asterisks denote significance for the indicated comparisons (ANOVA with Tukey post-hoc test; ****p < 0.0001).

