Metformin supports the antidiabetic effect of a sodium glucose cotransporter 2 (SGLT2) inhibitor by suppressing endogenous glucose production in diabetic mice

SHORT TITLE

Metformin and SGLT2I combination therapy

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ABSTRACT

Combined use of metformin and a sodium glucose cotransporter 2 inhibitor (SGLT2I) is a promising treatment strategy for type 2 diabetes. The mechanism by which combination treatment provides better glycemic control than metformin or SGLT2I monotherapy remains elusive.

Therefore, we investigated the physiological mechanism, by which both compounds lower blood glucose concentrations in diabetic mice.

We compared the potential of metformin and the SGLT2I AVE2268 alone or in combination to mitigate hyperglycemia and modulate glucose fluxes in diabetic db/db and TALLYHO/JngJ mice.

SGLT2I treatment alone elicited a rapid decline in circulating blood glucose levels, which appeared to induce endogenous glucose production. Supplementation of metformin dampened this counter-response and, therefore, combination therapy more efficiently maintained glycemic control. Finally, combination treatment blunted postprandial glucose excursions and improved HbA1c levels within two weeks.

Taken together, we conclude that co-application of metformin enhances the glucose-lowering actions of SGLT2I by restraining endogenous glucose production what may provide long-term improvement of glycemic control in type 2 diabetic patients.

INTRODUCTION

Unrestrained endogenous glucose production is a hallmark of type 2 diabetes (1). Most monotherapies lack the ability to sustain glycemic control, cause severe side effects, and eventually require other glucose-lowering compounds to maintain blood glucose levels within a normal range (2,3). DeFronzo and colleagues recommended a shift in the treatment paradigm away from monotherapies towards the use of combined compounds that lower blood glucose levels *via* different modes of action early in diabetes history (3).

Co-administration of metformin and a SGLT2I represents an attractive strategy in type 2 diabetes management as neither of these compounds targets pancreatic beta-cells, increases body weight or causes major safety risks (4-6). Metformin dampens unrestrained hepatic glucose output, induces glucose uptake in skeletal muscle, and suppresses lipolysis in adipose tissue (7,8). However, when provided as a monotherapy, metformin fails to durably lower glycated hemoglobin (HbA1c) levels (9). Inhibition of renal glucose reabsorption is a recent strategy in type 2 diabetes therapy (10). However, clinical studies reported that SGLT2Is enhance endogenous glucose production what undermines their glucose-lowering efficacy (11,12).

As Bailey and colleagues outlined that the combination of metformin with an SGLT2I more efficiently counteracted hyperglycemia in inadequately controlled type 2 diabetics than metformin alone (13) we aimed to unravel the *modus operandi* by which combination treatment provides superior glycemic control. Therefore, we evaluated acute and subchronic glucose-lowering properties of metformin and the SGLT2I AVE2268, either as mono- or combination therapy, in two genetically distinct type 2 diabetes mouse models.

RESEARCH DESIGN AND METHODS

Animals and pharmacological treatment.

We conducted studies in two obese, type 2 diabetes models, the BKS.Cg-*Dock7*^{m}+/+ *Lepr*^{db}/J (db/db) presenting a common murine model used for antidiabetic-drug testing (14,15), and the TALLYHO/JngJ, recapitulating broader aspects of human 'diabesity' (16). *Dock7*^{m}+/*Dock7*^{m}+ littermates served as non-diabetic, lean references for db/db-males. Animals were bred and housed in a temperature- and humidity-controlled environment in compliance with FELASA-protocols. Animal experiments were approved by the Upper-Bavarian government (Gz.55.2-1-54-2531-70-07, 55.2-1-2532-153-11).

From an age of three weeks, mice were fed a high-fat diet (Ssniff Spezialdiäten, Germany) containing [gm%] palm fat [13.5], sunflower oil [13.5], starch [30], saccharose [10], casein [20], lignocellulose [5], mineral+vitamin mix [5+2], safflower-oil [0.5], linseed-oil [0.5]. Pharmacological studies were started in diabetic eight-week-old db/db and wt references, diabetic nine-week-old TALLYHO/JngJ males, or in case of one study in chronically diabetic, 22-week-old TALLYHO/JngJ males. Animals received vehicle (5% solutol/95% hydroxyethylcellulose) without or with metformin (300 mg/kg; Sigma Aldrich, Germany), the SGLT2I AVE2268 (30 mg/kg; Sanofi-Aventis AG, Frankfurt, Germany (17)), or both compounds (300/30 mg) *via* gavage.

Subchronic intervention.

Diabetic TALLYHO/JngJ and db/db mice were treated once/day for 14 days between 5-6 PM before dark-phase onset (6 PM). Blood samples were collected from four-

hour fasting mice prior to the first and 18±2 hours after the last dose. In addition, twentytwo-week-old TALLYHO/JngJ mice were treated once/day for seven days. An inital blood sample was collected following four-hour fasting and further from random-fed individuals 12±2 hours post-treatment on day three and seven.

Acute intervention.

Initial blood samples were collected in random-fed db/db mice between 6-8 AM after light-phase-onset (6 AM), then compounds were administered, diet-access restricted, and the second blood sample was collected four hours post-treatment. A similar procedure was performed in TALLYHO/JngJ mice, but the second blood sample was collected two hours post-treatment. Thereafter TALLYHO/JngJ mice were subjected to two oral glucose tolerance tests (OGTT1/2: 1/2 g glucose/kg body mass) and blood glucose concentrations were determined 0.5, 1, 2, 3, and 4 hours after each glucose-challenge.

Euglycemic-hyperinsulinemic clamps.

TALLYHO/JngJ received a permanent jugular vein catheter under ketamine/xylazine-anesthesia. Six-seven days at 6 AM later food-access was restricted. Between 10-11 AM conscious mice were placed in oversized rat-restrainers and warmed by warming-pads. Catheter-ends were connected to syringes in CMA402-pumps (Axel Semrau, Sprockhoevel, Germany). After 110 minutes primed-continuous [3-^{3}H]glucose infusion (1.85 kBq/min) we collected a blood sample to determine plasma insulin, glucose and [3-^{3}H]glucose concentrations and calculated basal endogenous glucose appearance rates (Ra, mmol/min*kg: 1.9±0.2 vehicle, 2.0±0.6 metformin, 2.1±0.2 SGLT2I; 2.5±0.4 combination). Between 12 AM and 1 PM mice received vehicle,

metformin, SGLT2I, or combination *via* gavage. Subsequently, glucose-clamps were started with a [3-^{3}H]glucose infusion (3.7 kBq/min) containing insulin (36 pmol/kg*min^{-1}; HumulinR, Lilly, USA) causing a moderate net-increase in plasma insulin concentrations (fold-increase basal-glucose-clamp minute 120: 2.2±0.1 vehicle, 2.2±0.3 metformin, 2.1±0.2 SGLT2I, 2.2±0.2 combination). Blood glucose concentrations were measured every 10 minutes and target glycemia established by adjusting the rate of a 20%-glucose infusion (GIR). At minute 120, we injected 2-deoxy-D-[1-^{14}C]glucose (370 kBq) intravenously. Blood samples were collected at minute 30, 60, 90, 100, 110, 120, 122, 125, 130, and 140. Then after killing mice with an intravenous ketamine/xylazine-overdose, gastrocnemius muscle and epididymal adipose tissue were collected, immediately snap-frozen in liquid nitrogen, and stored at -80°C. Tissue 2-[^{14}C]deoxyglucose-6-phosphate was extracted and glucose uptake rates (Rg) were calculated as described previously (18).

Plasma [^{3}H]- and [^{14}C]-radioactivity was determined in deproteinized plasma after [$^{3}H_2O$] evaporation. Glucose fluxes under basal conditions and between glucose-clamp minute 60-90 and 90-120 were estimated as described: whole-body glucose disappearance rate Rd=[3-^{3}H]GIR (dpm/min)/plasma [3-^{3}H]glucose specific activity (dpm/min*µmol); basalEndoRa=[3-^{3}H]GIR (dpm/min)/plasma [3-^{3}H]glucose specific activity (dpm/min*µmol), glucose-clamp EndoRa=GIR-Rd.

Ultima-Gold scintillation-cocktail, radioisotopes, and a Tri-Carb2910TR were from Perkin Elmer (Germany).

Urinary glucose excretion rates (UGE) during euglycemic-hyperinsulinemic clamps.

Experiments were conducted following single SGLT2I- or combination-gavage as described above, except that fur was removed in the penis circumference and no radioisotopes were infused. Urine was collected over 30 minute periods with absorbing tissue-pads fitted into a ventral restrainer-window. Total urine volume was calculated as pad-mass-difference before and after installation. Glucose was extracted with dH_2O from pads, UGEs calculated for 30 minute periods and total urinary glucose loss over the whole 120 minutes of the glucose-clamp.

Assays from blood, plasma, urine.

Blood samples were collected from lateral tail veins. Blood glucose was measured with a glucometer (Contour, Bayer Vital, Germany), urine and plasma glucose with a colorimetric Glucose LabAssay (Wako, Germany), and HbA1c with A1cNow+ (Bayer Vital) or Clover Analyzer (Inopia, South Korea).

Statistical analyses.

Considering a 1-β larger than 0.9 statistically powerful we estimated appropriate group numbers from pilot studies *a priori*. One- or two-way Analyses of Variance (Bonferroni post-tests) or t-tests were performed.

RESULTS

Subchronic metformin and SGLT2I co-treatment sustained glycemic control in diabetic db/db and TALLYHO/JngJ mice.

Pharmacological effects were determined ~18 hours after the last of 14 daily doses. Metformin lowered blood glucose concentrations at least 1.2-fold in both strains, whereas combination treatment more effectively lowered blood glucose concentrations 2.9-fold in db/db mice (**Figure 1A**) and 1.9-fold in TALLYHO/JngJ mice **(Figure 1B**). Independent from the genetic background a statistically significant reduction in HbA1c was only observed in combination- as compared to vehicle-treated animals (**Figure 1A,B right panels**). In 22 weeks old, chronically diabetic TALLYHO/JngJ mice, combination therapy reduced blood glucose concentrations at least ~1.5-fold after three and seven days intervention (**Figure 1C**).

Acute glucose-lowering effects of metformin and SGLT2I co-treatment in diabetic db/db and TALLYHO/JngJ mice.

Metformin, SGLT2I or combination acutely lowered blood glucose levels 1.4-fold, 2.8-fold, and 3.4-fold in db/db mice (**Figure 2A**) and 1.3-fold, 2.1-fold, and 3.3-fold in TALLYHO/JngJ mice (**Figure 2B**) compared to prior treatment. To assess compound effects on postprandial glucose excursions, we subjected TALLYHO/JngJ mice to OGTTs. As shown in **Figure 2B**, metformin and AVE2268 co-application markedly improved postprandial glycemic control by blunting glucose peaks after each oral glucose challenge. As a result, AUCs of combination-treated animals were 2.6-fold, 2.2-fold and 1.9-fold reduced during OGTT1 and- remained at least 1.4-fold

lower during OGTT2 compared to vehicle-, metformin-, and SGLT2I-treated mice (**Figure 2C**).

Metformin and SGLT2I co-application acutely–restrained endogenous glucose production.

Next we determined acute glucose flux adaptations in diabetic TALLYHO/JngJ mice following pharmacologic treatment. We established comparable steady-state glycemias in all groups within 30-60 minutes–(**Figure 3A**). Combination-treatment increased GIR's between minute 90-120 at least two-fold compared to all other groups (**Figure 3B**), but did not alter skeletal muscle or white adipose tissue glucose uptake rates (**Figure 3C**). However, metformin and SGLT2I co-application suppressed EndoRa's two-fold more efficiently compared to all other groups (**Figure 3D**). We assessed whether co-administration of metformin alters SGLT2I-facilitated glycosuria but did not observe differences between SGLT2I- and combination-treated mice in total urinary glucose content (**Figure 3E**) or mean 30-minute-UGEs (**Figure 3F**).

DISCUSSION

Unrestrained endogenous glucose production is a major factor contributing to fasting hyperglycemia in type 2 diabetes (1), which may arise from peripheral insulin resistance, inadequate insulin release or a combination of both. Despite the multi-causal nature of type 2 diabetes, many therapeutic strategies focus on amplifying insulin output to overcome peripheral insulin resistance. This may be deleterious in the long-term and accelerate the need for insulin treatment. Furthermore, therapeutic strategies promoting the accruement of adipose tissue mass are unattractive for overweight type 2 diabetics considering obesity constitutes a risk factor for the development of insulin resistance.

SGLT2I's present a promising treatment strategy for type 2 diabetes by facilitating the excretion of excess circulating glucose *via* the urine (4). However, SGLT2Is impede their glucose-lowering effects by enhancing endogenous glucose production in diabetic rats and humans (11,12,19). We reasoned that supplementation of metformin counteracts the SGLT2I-mediated increase in endogenous glucose production, thereby, providing more efficient glycemic control than SGLT2I-monotherapy. We addressed this hypothesis by comparing the glucose lowering actions of AVE2268 and metformin, either as mono- or combination therapy in two different diabetes mouse models.

SGLT2I and combination acutely decreased blood glucose concentrations much more effectively than metformin in both mouse models. Therefore, we concluded that SGLT2-inhibition accounted for most of the acute glucose lowering effects observed following combination treatment.

Subchronic metformin treatment caused a modest blood glucose reduction whereas the effects of the SGLT2I were diminished ~18 hours after the last dose. This was anticipated considering the plasma half-lives of metformin and AVE2268 with approximately six and three hours. Unexpectedly, in both diabetes mouse models, subchronic metformin and AVE2268 co-treatment normalized blood glucose concentrations up to 18 hours post-treatment and almost comparable to acute effects. In subchronic experiments we provided the compounds prior to the onset of the activity and main food intake period of mice. We speculated that when combined, both compounds extend their acute blood glucose-lowering actions by abrogating postprandial glucose excursions what was confirmed by results from two consecutive oral glucose challenges. Next we investigated, whether blunted postprandial glycemic excursions in combination compared to monotherapy-treated mice were mediated by a more efficient suppression of endogenous glucose production.

To compare acute pharmacological effects on *in vivo* glucose fluxes, euglycemia was maintained by means of an external glucose infusion. However, the interpretation of the GIR – crucial for estimating endogenous glucose production – was challenged by SGLT2I-induced glycosuria. For this reason we developed a method to quantitatively assess urinary glucose loss. As depicted in Figure 3F, in the period between minute 90 and 120 post-SGLTI-application, the UGE approximated 0.5 mmol/kg*min. In parallel and as shown in Figure 3B, the GIR was increased by ~0.7 mmol/kg*min in SGLT2I- compared to vehicle-treated mice and thus, primarily compensated a glycosuria-induced drop in blood glucose concentrations rather than increased glucose turnover. The same GIR-calculation in combination- and vehicle-treated mice yielded a discrepancy of ~1.7 mmol glucose/kg*min, however UGEs in both SGLT2I-treated groups were comparable (see Figure 3B and F). Supported by a similar skeletal

muscle and adipose tissue glucose uptake, we conclude, that the two-fold higher GIR in combination- compared to SGLT2I-treated diabetic mice primarily resulted from a markedly suppressed endogenous glucose production.

Finally, improved glycemic control following subchronic metformin and SGLT2I co-therapy was associated with a HbA1c-reduction in both diabetes mouse models. However, vehicle-treated mice exhibited strain-specific differences probably resulting from the more progressive, earlier-onset diabetes phenotype of db/db compared to TALLYHO/JngJ males. Also, db/db mice express polyphagia caused by a mutation in the leptin-receptor long cellular domain what might make them less prone than TALLYHO/JngJ mice to adapt food intake behavior or meal frequency in response to single housing at the experiment start.

In conclusion, supplementation of metformin maximizes the glucose-lowering potential of an SGLT2I by restraining the SGLT2I-mediated increase in endogenous glucose production. Therefore, combination therapy with metformin and SGLT2Is may improve sustained glycemic control in human type 2 diabetics.

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S.N. wrote manuscript, collected and analyzed data, designed experiment; A.S., J.S., M.W. collected and analyzed data, edited manuscript; P.H. wrote manuscript; M.S., B.R. collected data, edited manuscript; K.S. edited manuscript, designed experiment; E.W., J.B., W.W. edited manuscript; M.H.A. designed experiment, provided funding. S.N. is the guarantor of this work and, as such, had full access to all data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflict of Interests:

MFS was employed at Helmholtz Zentrum München GmbH during the execution of this study. This paper was written while he was an employee of the diabetes medical department of Bristol-Myers Squibb GmbH & Co. KGaA. (Arnulfstr. 29, 80636 Munich, Germany) and he is currently an employee of the diabetes medical department of AstraZeneca GmbH (Tinsdaler Weg 183, 22880 Wedel, Germany). Both pharmaceutical companies had no involvement in the design of the experiments or drafting the manuscript. No other author declared a conflict of interest.

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ABBREVIATIONS

SGLT2I, sodium glucose cotransporter 2 inhibitor; HOMA, homeostasis model assessment; GLP-1, glucagon-like peptide-1; HbA1c, glycated hemoglobin; AUC, area under the curve; GIR, glucose infusion rate; Rd, whole-body glucose disappearance rate; Ra, whole-body glucose appearance rate; Rg, glucose uptake rate; EndoRa, endogenous glucose production rate; OGTT, oral glucose tolerance test; UGE, urinary glucose excretion rate.

FIGURE LEGENDS

Figure 1. S**ubchronic glycemic effects following vehicle, metformin, SGLT2I, or combination treatment.** After an initial blood glucose and HbA1c measurement **A)** eight weeks old, diabetic db/db and normoglycemic wt males (*n* = 10/group) or **B)** nine weeks old, diabetic TALLYHO/JngJ males (*n* = 10/group) were treated orally, once/day prior to the dark-phase onset. After 14 intervention days and 18±2 hours post-treatment, blood glucose concentrations were determined again. For db/db (*n* = 4-7/group, right panel in **A**) and TALLYHO/JngJ mice (*n* = 7/group, right panel in **B**) the relative HbA1c-change between prior- and post-treatment is shown (right panels. **C)** In chronically diabetic, 22 weeks old TALLYHO/JngJ males blood glucose concentrations were measured after three and seven treatments 12±2 hours post-treatment (*n* = 6/group). Data represent means±SEM (ANOVA, Bonferroni). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Open bars/circles = vehicle; black bars = metformin; striped bars = SGLT2I; yellow bars/squares = combination metformin and SGLT2I.

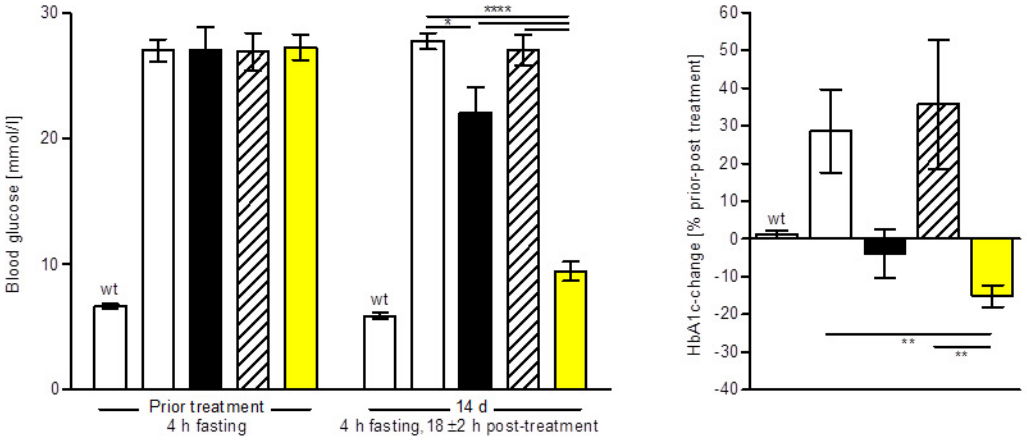
Figure 2. **Acute glycemic effects following single vehicle, metformin, SGLT2I, or combination treatment.**

After an initial blood glucose measurement eight weeks old, diabetic db/db and normoglycemic wt males (*n* = 10/group) or nine weeks old, diabetic TALLYHO/JngJ males (*n* = 7-8/group) received one single oral treatment. **A)** In db/db and wt mice blood glucose concentrations were determined again four hours post-treatment whereas in **B)** TALLYHO/JngJ mice two hours post-treatment. To simulate repeated carbohydrate ingestion TALLYHO/JngJ mice were then subjected to two oral glucose

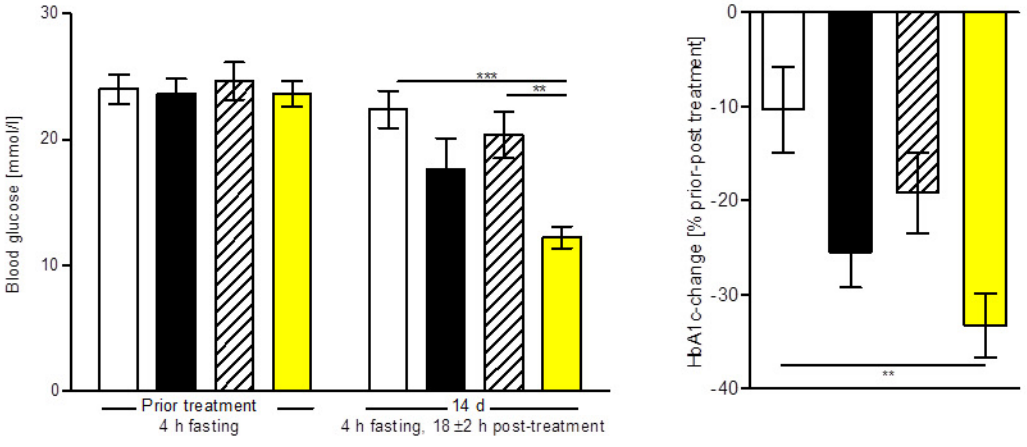
tolerance tests (OGTT1/2: 1 g/2 g glucose/kg body mass). Blood glucose excursions were determined. **C)** depicts total areas under the glucose curves. Data represent means±SEM (ANOVA, Bonferroni). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (in B) a single asterisk/time-point reflects vehicle and metformin *versus* combination- treated mice or SGLT2I *versus* combination-treated mice). Open bars/circles = vehicle; black bars/circles = metformin; striped bars/white triangles = SGLT2I; yellow bars/squares = combination metformin and SGLT2I.

Figure 3. **Acute glucose flux adaptations under euglycemic-hyperinsulinemic conditions following single, oral vehicle, metformin, SGLT2I, or combination treatment.** In nine weeks old, awake TALLYHO/JngJ males **A)** blood glucose concentrations regulated by the **B)** glucose infusion rates (GIR) during physiological hyperinsulinemia, **C)** glucose uptake rates (Rg) in gastrocnemius muscle and epididymal white adipose tissue, and the **D)** suppression of endogenous glucose production rates (EndoRa) in % of basal values were calculated. Once target glycemia was reached in all groups by minute 60 of the glucose clamp GIRs and EndoRa's were estimated for two 30 minute intervals (indicated by dotted vertical lines in the panel A). In a second euglycemic-hyperinsulinemic clamp experiment **E)** total urinary glucose loss and **F)** urinary excretion rates (UGE) calculated for four 30 minute intervals throughout the 120 minutes of the experiment were compared between SGLT2I and combination-treated mice. Data represent means±SEM (n = 8-9/group); *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (ANOVA, Bonferroni). Open bars/circles = vehicle; black bars/circles = metformin; striped bars/white triangles = SGLT2I; yellow bars/squares = combination metformin and SGLT2I.

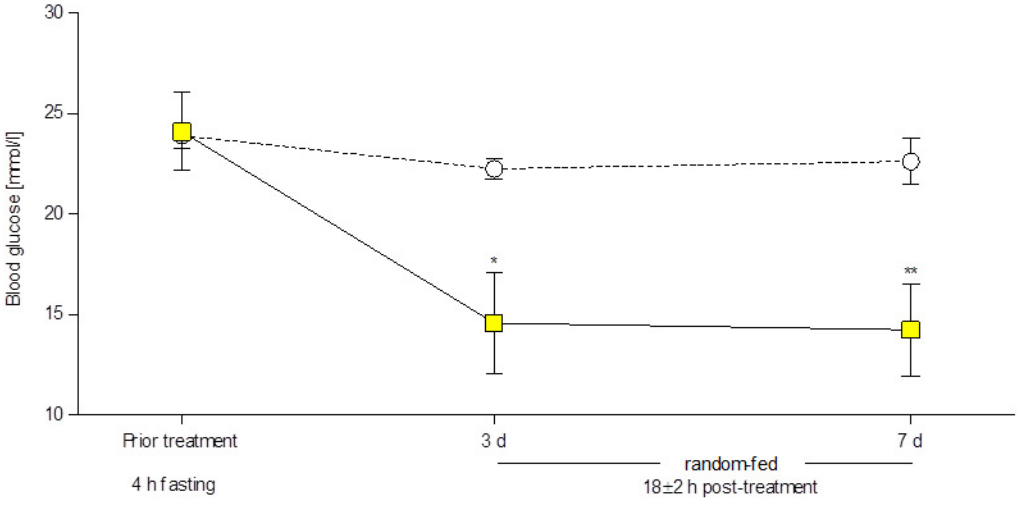
A Subchronic effects: eight-week-old db/db

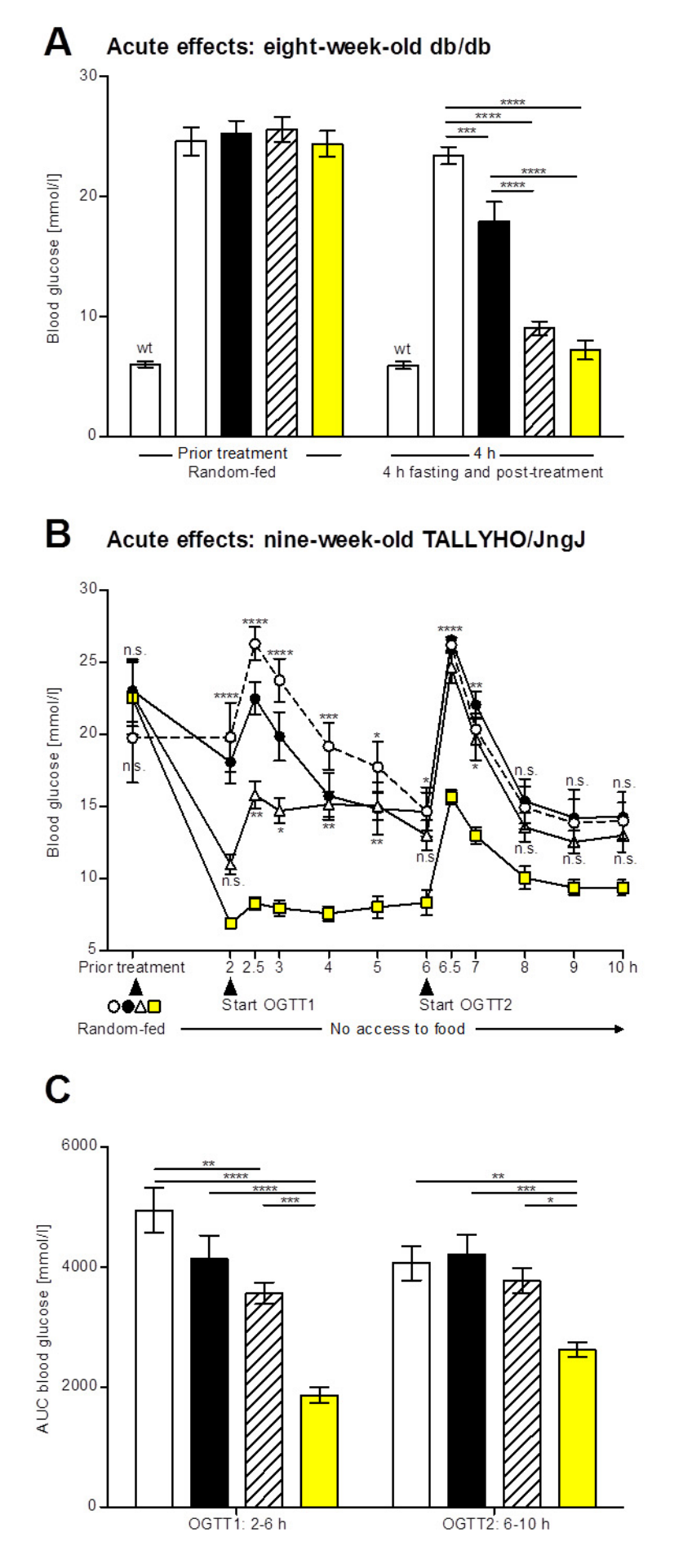


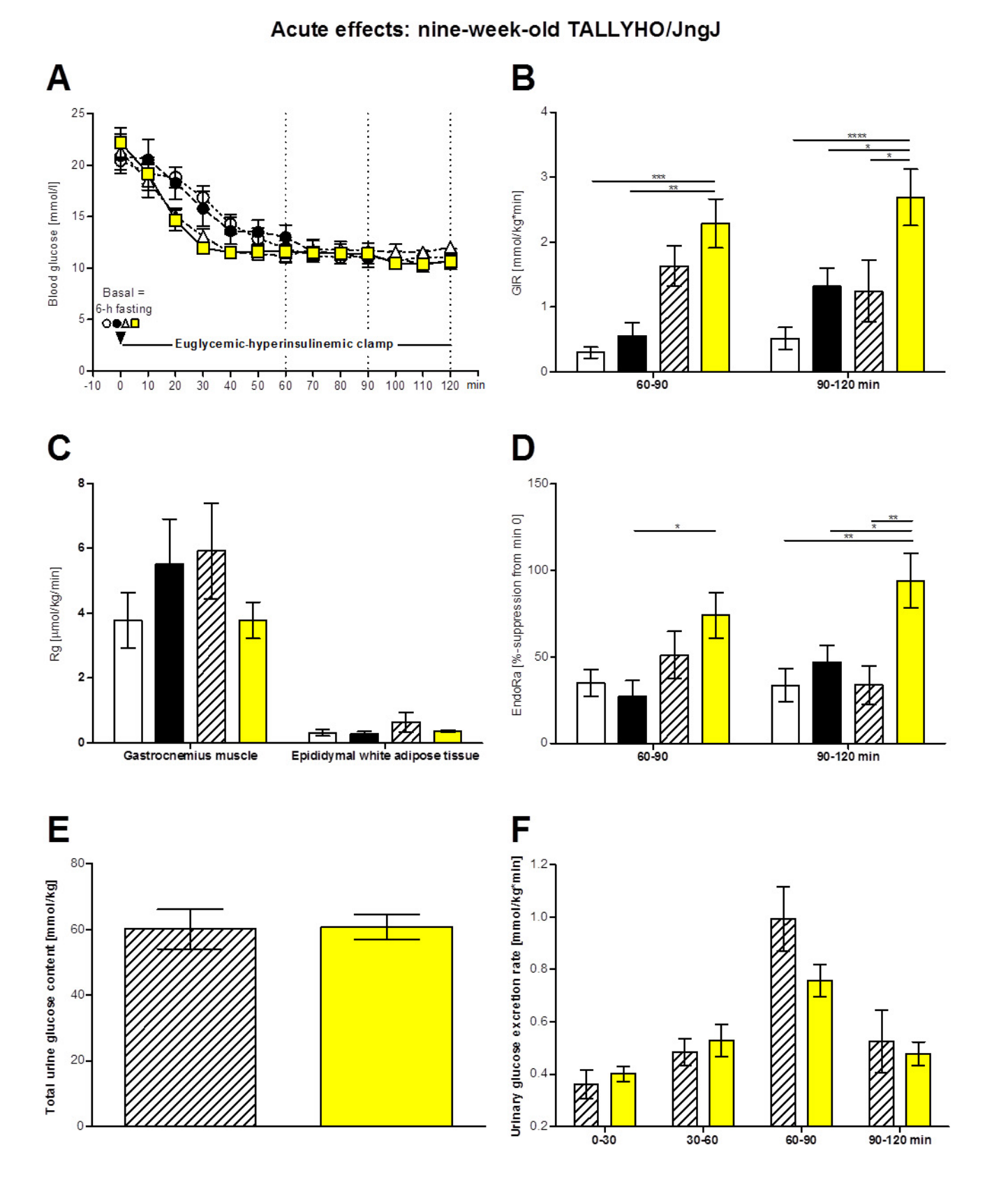
B Subchronic effects: nine-week-old TALLYHO/JngJ



C Subchronic effects: 22-week-old TALLYHO/JngJ




A
Acute effects: eight-week-old db/db
Blood glucose [mmol/l]
wt
wt
Prior treatment
Random-fed
4 h
4 h fasting and post-treatment
B
Acute effects: nine-week-old TALLYHO/JngJ
Blood glucose [mmol/l]
Prior treatment
Start OGTT1
Start OGTT2
Random-fed
No access to food
C
AUC blood glucose [mmol/l]
OGTT1: 2-6 h
OGTT2: 6-10 h


Acute effects: nine-week-old TALLYHO/JngJ
A
Blood glucose [mmol/l]
Basal =
6-h fasting
Euglycemic-hyperinsulinemic clamp
min
B
GIR [mmol/kg*min]
60-90
90-120 min
C
Rg [µmol/kg/min]
Gastrocnemius muscle
Epididymal white adipose tissue
D
EndoRa [%-suppression from min 0]
60-90
90-120 min
E
Total urine glucose content [mmol/kg]
F
Urinary glucose excretion rate [mmol/kg*min]
0-30
30-60
60-90
90-120 min