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Metformin Supports the Antidiabetic Effect of a Sodium Glucose Cotransporter 2 Inhibitor by Suppressing Endogenous Glucose Production in Diabetic Mice

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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 *db/db* and diabetic 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 counterresponse, and therefore, combination therapy more efficiently maintained glycemic control. Finally, combination treatment blunted postprandial glucose excursions and improved HbA_{1c} levels within 2 weeks. We conclude that coapplication of metformin enhances the glucose-lowering actions of SGLT2I by restraining endogenous glucose production, which may provide long-term improvement of glycemic control in type 2 diabetic patients.

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 et al. (3) recommended a shift in the treatment paradigm away from monotherapies toward the use of combined compounds that lower blood glucose levels via different modes of action early in diabetes history.

Coadministration of metformin and an SGLT2I represents an attractive strategy in type 2 diabetes management, as neither of these compounds targets pancreatic β-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 (HbA_{1c}) levels (9). Inhibition of renal glucose reabsorption is a recent strategy in type 2 diabetes therapy (10). However, clinical studies

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reported that SGLT2Is enhance endogenous glucose production, which undermines their glucose-lowering efficacy (11,12).

As Bailey et al. (13) outlined that the combination of metformin with an SGLT2I more efficiently counteracted hyperglycemia in patients with inadequately controlled type 2 diabetes than metformin alone, 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 antidiabetes drug testing (14,15), and the Tallyho/JngJ, recapitulating broader aspects of human "diabesity" (16). *Dock7*$^{m+}$/*Dock7*$^{m+}$ littermates served as nondiabetic, lean references for *db/db* males. Animals were bred and housed in a temperature- and humidity-controlled environment in compliance with FELASA (the Federation of Laboratory Animal Science Associations) protocols. Animal experiments were approved by the Upper Bavarian government (Gz.55.2-1-54-2531-70-07, 55.2-1-2532-153-11).

At the age of 3 weeks, mice were started on a high-fat diet (Ssniff Spezialdiäten, Soest, Germany) containing palm fat (13.5% of fat), sunflower oil (13.5%), starch (30%), saccharose (10%), casein (20%), lignocellulose (5%), mineral plus vitamin mix (5 and 2%, respectively), safflower oil (0.5%), and linseed oil (0.5). Pharmacological studies were started in 8-week-old *db/db* and wild-type (wt) references, diabetic 9-week-old Tallyho/JngJ males, or, in the case of one study, chronically diabetic 22-week-old Tallyho/JngJ males. Animals received vehicle (5% solutol and 95% hydroxyethylcellulose) without or with metformin (300 mg/kg; Sigma-Aldrich, Taufkirchen, Germany), the SGLT2I AVE2268 (30 mg/kg; Sanofi AG, Frankfurt, Germany [17]), or both compounds (300/30 mg) via gavage.

Subchronic Intervention

Diabetic Tallyho/JngJ and *db/db* mice were treated once per day for 14 days between 5:00 P.M. and 6:00 P.M. before dark-phase onset (6:00 P.M.). Blood samples were collected from 4-h fasting mice prior to the first and 18 ± 2 h after the last dose. In addition, 22-week-old Tallyho/JngJ mice were treated once per day for 7 days. An initial blood sample was collected after 4-h fasting and further from random-fed individuals 12 ± 2 h posttreatment on days 3 and 7.

Acute Intervention

Initial blood samples were collected in random-fed *db/db* mice between 6:00 A.M. and 8:00 A.M. after light-phase onset (6:00 A.M.); then, compounds were administered and diet access was restricted, and the second blood sample was collected 4 h posttreatment. A similar procedure was performed in Tallyho/JngJ mice, but the second blood sample was collected 2 h posttreatment. 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 h after each glucose challenge.

Euglycemic-Hyperinsulinemic Clamps

Tallyho/JngJ mice received a permanent jugular vein catheter under ketamine/xylazine anesthesia. After a postsurgical recovery period of 6 or 7 days, food access was restricted at 6:00 A.M. Between 10:00 A.M. and 11:00 A.M., 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 min of primed continuous [3-^{3}H]glucose infusion (1.85 kBq/min), we collected a basal blood sample to determine plasma insulin, glucose, and [3-^{3}H]glucose concentrations and calculated basal endogenous glucose appearance rates (R_a) (1.9 ± 0.2 mmol/min · kg vehicle, 2.0 ± 0.6 mmol/min · kg metformin, 2.1 ± 0.2 mmol/min · kg SGLT2I, and 2.5 ± 0.4 mmol/min · kg combination). Between 12:00 A.M. and 1:00 P.M., 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}; Humulin R, Lilly) causing a moderate net increase in plasma insulin concentrations (2.2 ± 0.1-fold increase of vehicle, 2.2 ± 0.3-fold increase of metformin, 2.1 ± 0.2-fold increase of SGLT2I, and 2.2 ± 0.2-fold increase of combination from basal to glucose clamp min 120). Blood glucose concentrations were measured every 10 min, and target glycemia was established by adjusting the rate of a 20% glucose infusion (GIR). At minute 120, we injected 2-deoxy-D-[1-^{14}C]glucose intravenously (370 kBq). Blood samples were collected at minutes 30, 60, 90, 100, 110, 120, 122, 125, 130, and 140. Then, after killing of 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 (R_g) were calculated as previously described (18).

Plasma [^{3}H]- and [^{14}C]radioactivity was determined in deproteinized plasma after [$^{3}H_2O$] evaporation. Glucose fluxes were estimated under basal conditions and between glucose clamp minutes 60–90 and 90–120 as follows: whole-body glucose disappearance rate (R_d) = [3-^{3}H]GIR (dpm/min)/plasma [3-^{3}H]glucose specific activity (dpm/min · μmol), basal EndoR_a = [3-^{3}H]GIR (dpm/min)/plasma [3-^{3}H]glucose specific activity (dpm/min · μmol), and glucose clamp EndoR_a = GIR − R_d.

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

Urinary Glucose Excretion Rates During Euglycemic-Hyperinsulinemic Clamps

Experiments were conducted after single SGLT2I or combination gavage as described above, except that fur was removed in the penis circumference and no radioisotopes were infused. Urine was quantitatively collected between minutes 0–30, 30–60, 60–90, and 90–12 with absorbing tissue pads fitted into a ventral restrainer window. For each 30-min period, total urine volume was calculated as pad mass difference before and after installation. By immersion of single pads in dH_2O, glucose was extracted. Urinary glucose excretion rates (UGEs) were calculated for 30-min periods, and total urinary glucose loss was calculated over the whole 120 min of the glucose clamp.

Assays From Blood, Plasma, and Urine

Blood samples were collected from lateral tail veins. Blood glucose was measured with a glucometer (Contour, Bayer Vital, Germany), urine and plasma glucose were determined with a colorimetric Glucose LabAssay (Wako, Dusseldorf, Germany), and HbA_{1c} was measured with A1cNow+ (Bayer Vital) or Clover Analyzer (Inopia, South Korea).

Statistical Analyses

We estimated appropriate group numbers from pilot studies a priori, considering that a value of 1-β larger than 0.9 was statistically powerful. One- or two-way ANOVA (Bonferroni posttests) or *t* tests were performed.

RESULTS

Subchronic Metformin and SGLT2I Cotreatment Sustained Glycemic Control in *db/db* and Diabetic Tallyho/JngJ Mice

Pharmacological effects were determined ~18 h 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 (Fig. 1*A*) and 1.9-fold in Tallyho/JngJ mice (Fig. 1*B*). Independent from the genetic background, a statistically significant reduction in HbA_{1c} was only observed in combination compared with vehicle-treated animals (Fig. 1*A* and *B*). In 22-week-old, chronically diabetic Tallyho/JngJ mice, combination therapy reduced blood glucose concentrations at least ~1.5-fold after 3 and 7 days' intervention (Fig. 1*C*).

Acute Glucose-Lowering Effects of Metformin and SGLT2I Cotreatment in *db/db* and Diabetic 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 (Fig. 2*A*) and 1.3-fold, 2.1-fold, and 3.3-fold in Tallyho/JngJ mice (Fig. 2*B*) compared with prior treatment. To assess compound effects on postprandial glucose excursions, we subjected Tallyho/JngJ mice to OGTTs. As shown in Fig. 2*B*, metformin and AVE2268 coapplication markedly improved postprandial glycemic control by blunting glucose peaks after each oral glucose challenge. As a result, areas under the curve 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 with vehicle-, metformin-, and SGLT2I-treated mice (Fig. 2*C*).

Metformin and SGLT2I Coapplication Acutely Restrained Endogenous Glucose Production

Next, we determined acute glucose flux adaptations in diabetic Tallyho/JngJ mice after pharmacologic treatment. We established comparable steady-state glycemias in all groups within 30–60 min (Fig. 3*A*). Combination treatment increased GIRs between minutes 90 and 120 at least twofold compared with all other groups (Fig. 3*B*) but did not alter skeletal muscle or white adipose tissue glucose uptake rates (Fig. 3*C*). However, metformin and SGLT2I coapplication suppressed EndoR_a twofold more efficiently compared with all other groups (Fig. 3*D*). We assessed whether coadministration of metformin alters SGLT2I-facilitated glycosuria but did not observe differences between SGLT2I- and combination-treated mice in total urinary glucose content (Fig. 3*E*) or mean 30-min UGEs (Fig. 3*F*).

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 multicausal 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 patients with type 2 diabetes, considering that obesity constitutes a risk factor for the development of insulin resistance.

SGLT2Is 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 as 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 after combination treatment.

Subchronic metformin treatment caused a modest blood glucose reduction. In contrast, the effects of the SGLT2I were diminished ~18 h after the last dose, likely due to the

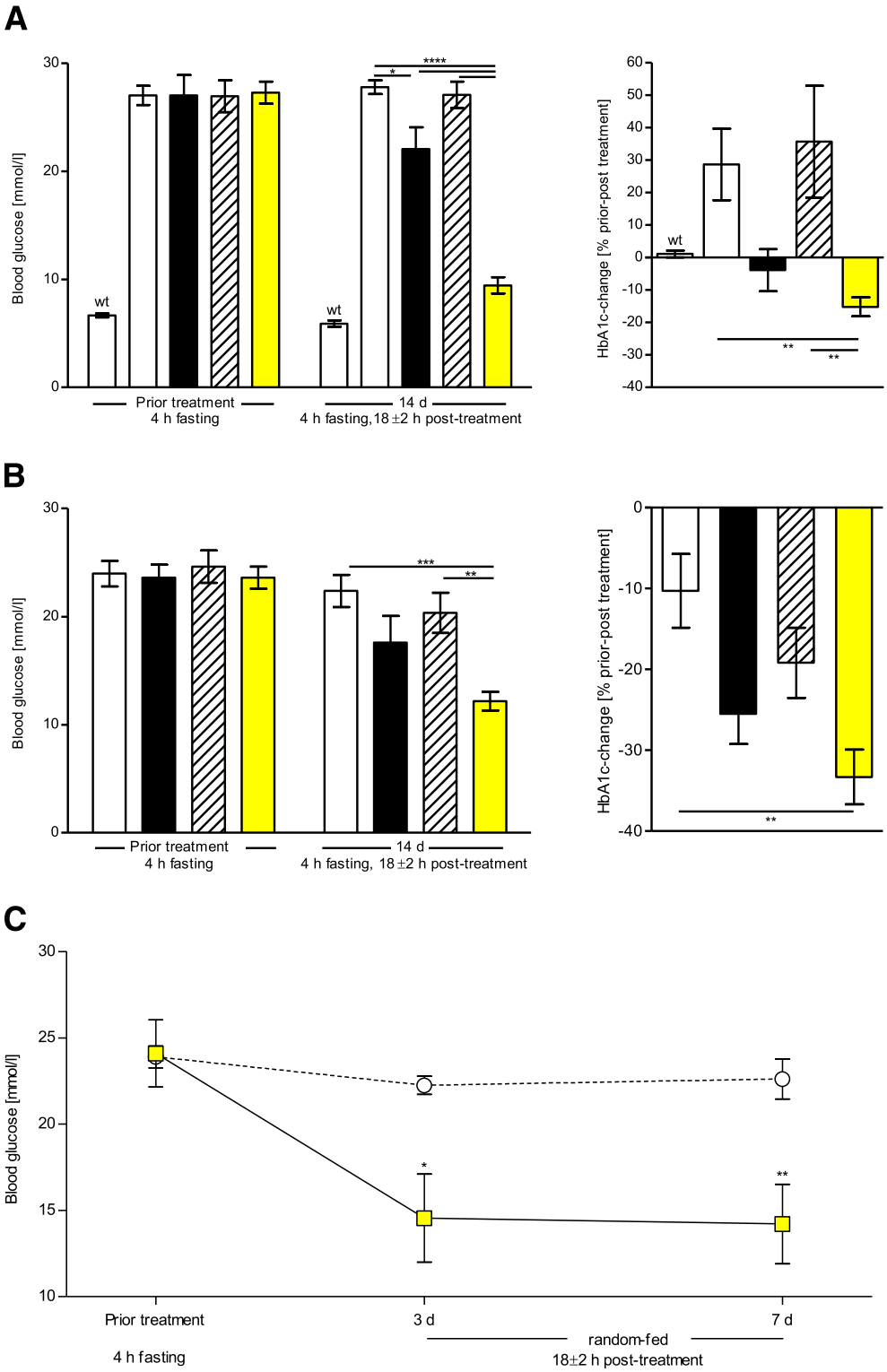

Figure 1—Subchronic glycemic effects after vehicle, metformin, SGLT2I, or combination treatment. After an initial blood glucose and HbA_{1c} measurement, 8-week-old (*A*) *db/db* and normoglycemic wt males (n = 10/group) or 9-week-old (*B*) diabetic Tallyho/JngJ males (n = 10/group) were treated orally once per day prior to the dark-phase onset. After 14 intervention days and 18 ± 2 h posttreatment, blood glucose concentrations and HbA_{1c} were determined again. For *db/db* mice (n = 4–7/group) (*A* [right panel]) and Tallyho/JngJ mice (n = 7/group) (*B* [right panel]), the relative HbA_{1c} change between prior and posttreatment is shown. *C*: In chronically diabetic, 22-week-old Tallyho/JngJ males, blood glucose concentrations were measured after three and seven treatments 12 ± 2 h posttreatment (n = 6/group). Data represent means ± SEM (ANOVA, Bonferroni). d, day. **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.

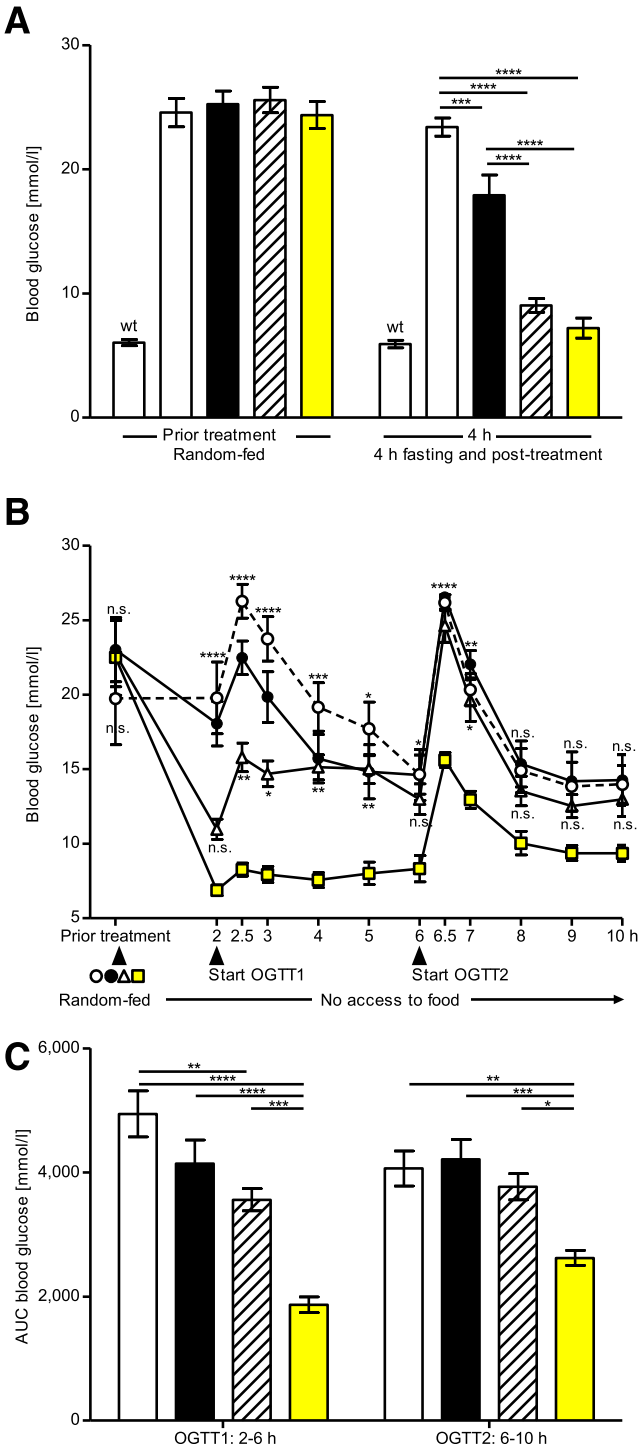

Figure 2—Acute glycemic effects after single vehicle, metformin, SGLT2I, or combination treatment. After an initial blood glucose measurement, 8-week-old *db/db* and normoglycemic wt males (n = 10/group) or 9-week-old diabetic Tallyho/JngJ males (n = 7–8/group) received one single oral treatment. In *db/db* (*A*) and wt mice, blood glucose concentrations were determined again 4 h posttreatment, but in Tallyho/JngJ mice (*B*), concentrations were determined again 2 h posttreatment. For simulation of repeated carbohydrate ingestion, Tallyho/JngJ mice were then subjected to (*B*) OGTT1 and next OGTT2 (1 g and 2 g glucose/kg body mass), and blood glucose excursions and (*C*) areas under the glucose curves (AUC) were calculated. Data represent means ± SEM (ANOVA, Bonferroni). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. (In *B*, upper asterisk series represent comparisons of combination with both vehicle- or metformin-treated groups, and lower asterisk series represent comparisons of combination with SGLT2I-treated group.) Open bars/circles, vehicle; black bars/circles, metformin; striped bars/white triangles, SGLT2I; yellow bars/squares, combination metformin and SGLT2I.

plasma half-lives of metformin (~6 h) and AVE2268 (~3 h). In both diabetes mouse models, subchronic metformin and AVE2268 cotreatment normalized blood glucose concentrations up to 18 h posttreatment in what closely resembled the observed acute effect of combination treatment. 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, which was confirmed by results from two consecutive oral glucose challenges. Next, we investigated whether blunted postprandial glycemic excursions in combination compared with monotherapy-treated mice were mediated by a more efficient suppression of endogenous glucose production.

For comparison of 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 Fig. 3*F*, 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 Fig. 3*B*, the GIR was increased by ~0.7 mmol/kg · min in SGLT2I- compared with 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 Fig. 3*B* and *F*). Comparison of minute 0–30 and 30–60 UGEs widely rules out that differences in glycosurea prior to minute 60 accounted for the differences in endogenous glucose fluxes in combination compared with SGLT2I-treated mice observed later in the glucose clamp.

In support of a similar skeletal muscle and adipose tissue glucose uptake, we conclude that the twofold-higher GIR in combination-treated compared with SGLT2I-treated diabetic mice primarily resulted from a markedly suppressed endogenous glucose production.

Finally, improved glycemic control after subchronic metformin and SGLT2I cotherapy was associated with an HbA_{1c} reduction in both diabetes mouse models. However, vehicle-treated mice exhibited strain-specific differences probably resulting from the more progressive, earlier-onset

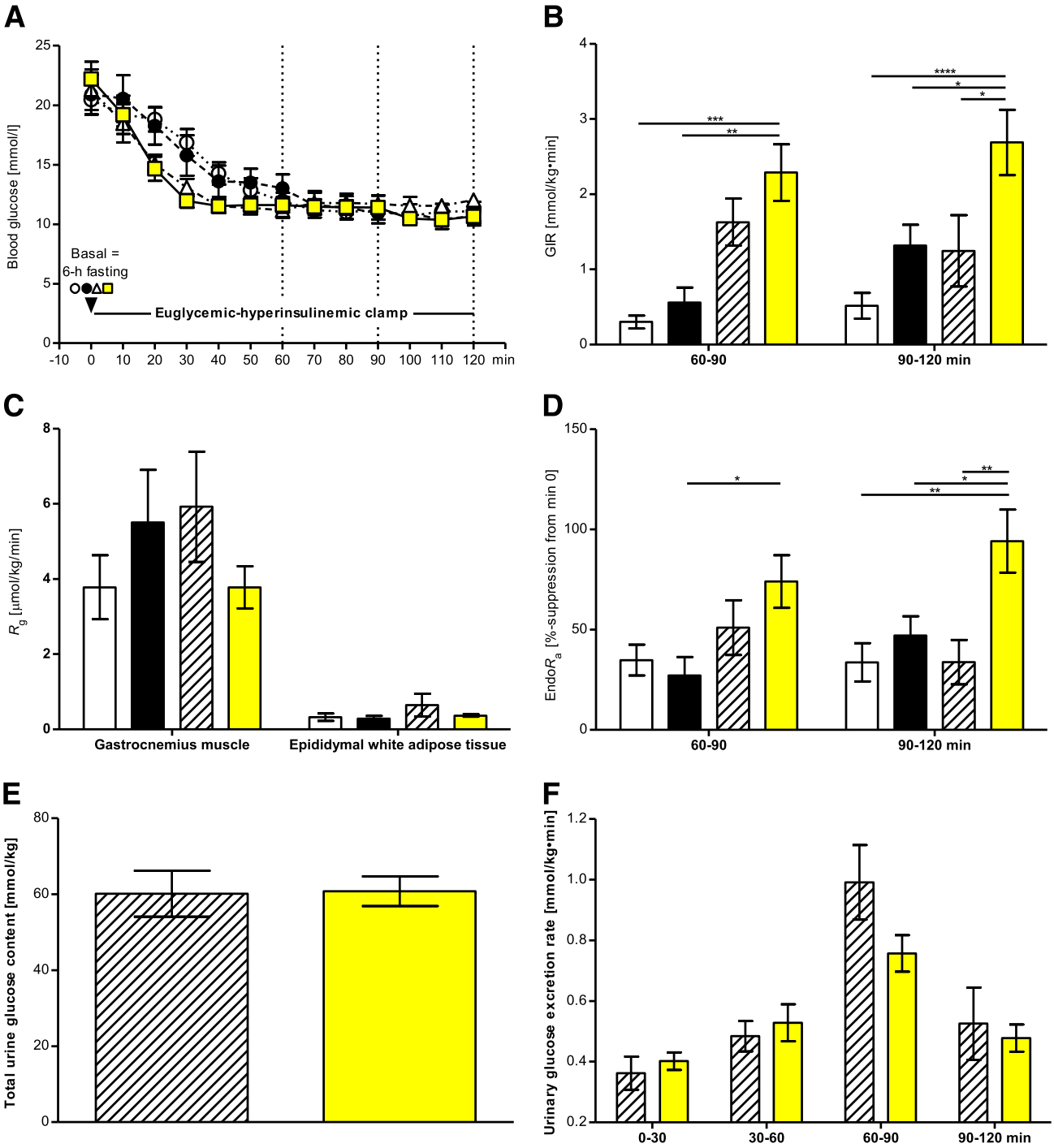

Figure 3—Acute glucose flux adaptations under euglycemic-hyperinsulinemic conditions after single oral vehicle, metformin, or SGLT2I or combination treatment. In 9-week-old, awake Tallyho/JngJ males, blood glucose concentrations (*A*) regulated by the GIRs (*B*) during physiological hyperinsulinemia, R_g (*C*) in gastrocnemius muscle and epididymal white adipose tissue, and the suppression of EndoR_a (*D*) in percent of basal values were calculated. Once target glycemia was reached in all groups by minute 60 of the glucose clamp, GIRs and EndoR_a were estimated for two 30-min intervals (indicated by dotted vertical lines in *A*). In a second euglycemic-hyperinsulinemic clamp experiment, total urinary glucose loss (*E*) and UGEs (*F*) calculated for four 30-min intervals throughout the 120 min 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.

diabetes phenotype of *db/db* compared with Tallyho/JngJ males. Furthermore, *db/db* mice express polyphagia caused by a mutation in the leptin-receptor long cellular domain, which 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 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 diabetic patients.

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Duality of Interest. M.S. was employed at Helmholtz Zentrum München GmbH during the execution of this study, the manuscript was written while he was an employee of the Diabetes Medical Department of Bristol-Myers Squibb (Munich, Germany), and he is currently an employee of the Diabetes Medical Department of AstraZeneca (Wedel, Germany). No other potential conflicts of interest relevant to this article were reported.

Both pharmaceutical companies had no involvement in the design of the experiments or drafting the manuscript.

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

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