Brain Glucagon-Like Peptide-1 Regulates Arterial Blood Flow, Heart Rate, and Insulin Sensitivity

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OBJECTIVE—To ascertain the importance and mechanisms underlying the role of brain glucagon-like peptide (GLP)-1 in the control of metabolic and cardiovascular function. GLP-1 is a gut hormone secreted in response to oral glucose absorption that regulates glucose metabolism and cardiovascular function. GLP-1 is also produced in the brain, where its contribution to central regulation of metabolic and cardiovascular homeostasis remains incompletely understood.

RESEARCH DESIGN AND METHODS—Awake free-moving mice were infused with the GLP-1 receptor agonist exendin-4 (Ex4) into the lateral ventricle of the brain in the basal state or during hyperinsulinemic eu-/hyperglycemic clamps. Arterial femoral blood flow, whole-body insulin-stimulated glucose utilization, and heart rates were continuously recorded.

RESULTS—A continuous 3-h brain infusion of Ex4 decreased femoral arterial blood flow and whole-body glucose utilization in the awake free-moving mouse clamped in a hyperinsulinemic-hyperglycemic condition, only demonstrating that this effect was strictly glucose dependent. However, the heart rate remained unchanged. The metabolic and vascular effects of Ex4 were markedly attenuated by central infusion of the GLP-1 receptor (GLP-1R) antagonist exendin-9 (Ex9) and totally abolished in GLP-1 receptor knockout mice. A correlation was observed between the metabolic rate and the vascular flow in control and Ex4-infused mice, which disappeared in Ex9 and GLP-1R knockout mice. Moreover, hypothalamic nitric oxide synthase activity and the concentration of reactive oxygen species (ROS) were also reduced in a GLP-1R–dependent manner, whereas the glutathione antioxidant capacity was increased. Central GLP-1 activated vagus nerve activity, and complementation with ROS donor dose-dependently reversed the effect of brain GLP-1 signaling on peripheral blood flow.

CONCLUSIONS—Our data demonstrate that central GLP-1 signaling is an essential component of circuits integrating cardiovascular and metabolic responses to hyperglycemia. ***Diabetes* 57:2577–2587, 2008**

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There is now compelling evidence supporting the interplay between metabolic and vascular diseases (1,2) in which neuronal circuits in the central nervous system seem to play a critical role in orchestrating the control of glucose homeostasis (3). We recently demonstrated that the central infusion of insulin decreased blood pressure and increased arterial blood flow and heart rate through a molecular mechanism depending on the synthesis of nitric oxide in the hypothalamus (4). Importantly, the central regulation of nitric oxide (NO) metabolism affected whole-body glucose utilization (5). This mechanism was impaired during high-fat diet–induced insulin resistance and diabetes and reverted upon central NO supplementation (4). These findings raise the possibility that signals from peripheral tissues, which act on the brain to control glucose metabolism, could also regulate vascular function.

Enteroendocrine cells have important roles in regulating energy intake and glucose homeostasis through their actions on peripheral target organs, including the endocrine pancreas. Enteroendocrine cells secrete multiple hormones, including glucagon-like peptide (GLP)-1, which controls pancreatic endocrine secretion (6). GLP-1 is also a neuropeptide synthesized by neurons in the caudal regions of the nucleus of the solitary tract (NTS) (7,8). GLP-1 is released into the hypothalamus and controls food intake, blood pressure, and heart rate (9,10). Whereas most of the glucose-lowering actions of GLP-1 have been attributed to the direct effect of the hormone on the endocrine pancreas, i.e., to stimulation of insulin and inhibition of glucagon secretion, we demonstrated the importance of extra-pancreatic GLP-1 receptor–dependent control of insulin secretion (11) and whole-body glucose distribution (12). The infusion into the brain of the GLP-1 receptor antagonist exendin-9 (Ex9) inhibited insulin secretion induced by gut glucose (11). Conversely, central administration of the GLP-1 receptor agonist exendin-4 (Ex4) augmented intravenous glucose-stimulated insulin secretion to a level similar to that obtained during an intragastric glucose infusion (11). Our data suggested that the absorptive state was associated with the stimulation of the gut-to-brain axis leading to the activation of brain GLP-1 signaling and, consequently, to hyperinsulinemia. During the absorptive state, blood flow redistribution toward mesenteric organs is also observed, which has been proposed to favor nutrient redistribution into the liver (13). Importantly, stimulation of the central GLP-1 receptor increases blood pressure and heart rate and activates autonomic regulatory neurons (8,14,15). However, recently it has been shown that GLP-1 reduced islet blood flow after glucose administration (16). There-

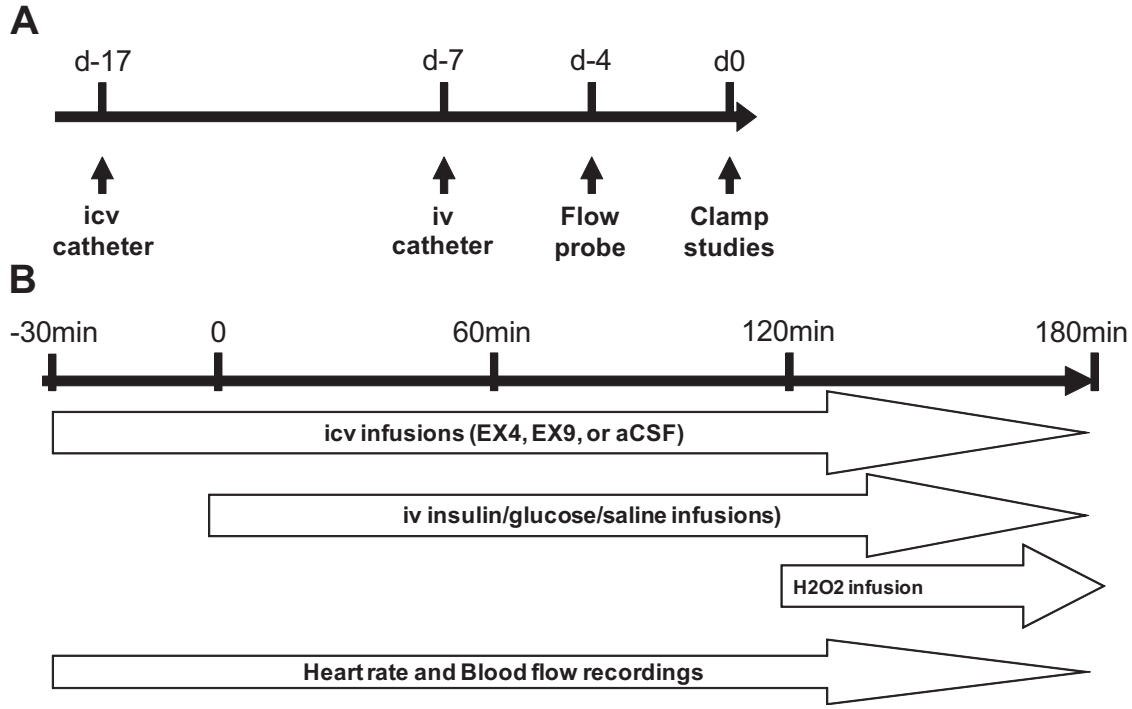


FIG. 1. Surgical and experimental procedures. *A*: Seventeen days (J-17) before infusions and clamp studies, a catheter was indwelled into the brain lateral ventricle. Seven days before the infusion (J-7), a catheter was indwelled into the left femoral vein, and the flow probe was indwelled 4 days (J-4) before the infusions (J0). *B*: Thirty minutes before the infusions, Ex4, Ex9, or aCSF was infused into the lateral ventricle of the mouse until completion of the 3-h clamp procedure. The clamp procedure was initiated by a concomitant infusion of glucose and insulin and was compared with the saline intravenous infusions.

fore, the role of brain GLP-1 signaling also in the control of cardiovascular homeostasis remains incompletely understood.

We have now pursued the importance of GLP-1 action in the central nervous system for control of cardiovascular function using studies in conscious free-moving mice. After central GLP-1 infusion, we simultaneously recorded femoral arterial blood flow, heart rate, and insulin and glucose sensitivity during hyperinsulinemic-euglycemic or hyperglycemic clamps. We now demonstrate that hypothalamic reactive oxygen and nitrogen species are controlled by brain GLP-1 and are essential for the coordinated regulation of metabolic and cardiovascular function.

RESEARCH DESIGN AND METHODS

Animals. Experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee. Eleven-week-old male C57BL/6J (Janvier, Larbresle, France) and GLP-1 receptor knockout mice from our colony (in C57BL/6 background) were used, as previously described (4). Throughout the study period, the mice were housed at 21–22°C with a normal daily cycle and food and water ad libitum.

Surgical procedures. A catheter (Charles River Laboratories, L'Arbresles, France) was inserted into the lateral cerebral ventricle and secured on the top of the skull under anesthesia with isoflurane-oxygen (17). Ten days after the intracerebroventricular surgery, an intravenous catheter was introduced into the left femoral vein, sealed under the skin, and externalized at the back of the neck (Fig. 1). The mice were allowed to recover for 3 days, before an ultrasonic flow probe (Transonic System, Emka Technologies, France) was inserted surrounding the right femoral artery. The probe wire was inserted through the skin at the back of the neck, where it was secured using surgical thread. After surgery, the mice returned to their cages and allowed to recover for at least 4 days before infusions (18). At the end of the recovery period, mice that did not reach their presurgery weights were not used for subsequent experiments (i.e., 15% of the animals).

Infusions. On the day of the study (Fig. 1), the flow probe wire was connected to a Transonic model T403 flowmeter (Transonic System; Emka Technologies, Paris, France) to record the blood flow (ml/min) of the femoral artery and the heart rate (beats/min). The basal femoral arterial blood flow and heart rate were recorded for 30 min in overnight fasted free-moving mice before starting the infusions.

Glucose clamp. A hyperinsulinemic-euglycemic (5.5 mmol/l) or hyperglycemic (20 mmol/l) clamp was performed to activate GLP-1–sensitive cells and to assess whole-body glucose utilization (18) (Table 1). Briefly, insulin was infused through the intrafemoral catheter at a rate of 18 $mU \cdot kg^{-1} \cdot min^{-1}$ for 3 h. Glycemia was clamped at 5.5 or 20 mmol/l by adjusting an intrafemoral glucose infusion. A control group was infused with NaCl 0.9% (saline) for 3 h at a rate that was matched to the mean glucose infusion rate during the hyperinsulinemic clamps. This procedure did not change the hematocrit value, which remained close, ranging between 38.3 and 39.1% when considering all groups.

To ensure the filling of the tubing connected to the brain, intracerebroventricular infusions were started 30 min before the beginning of the clamp procedure and continued throughout the whole study. Briefly, a 5-μl bolus (5 μl) followed by a continuous (12 μl/h) infusion was performed with the cerebral vehicle (artificial cerebrospinal fluid [aCSF], pH 7.35, Na^+ 144 mmol/l, Cl^- 146 mmol/l, K^+ 3 mmol/l, Mg^{2+} 1 mmol/l, Ca^{2+} 1.5 mmol/l, PO^{4-} 1.2 mmol/l, pH 7.35) or with the GLP-1 receptor agonist (Ex4) or antagonist (exendin 9 [Ex9]), at a rate of 0.5 $pmol \cdot kg^{-1} \cdot min^{-1}$ for 3 h, as described (11,12). This Exh infusion is expected to result in cerebral supraphysiological concentrations of GLP-1. However, this exendin infusion rate is 10 times lower than the one required to induce a physiological effect using a systemic infusion (11,12).

Measurement of autonomic nervous system activity. To assess the vagus nerve activity, a subset of mice bearing an intracerebroventricular catheter for 1 week was anesthetized with isoflurane maintained on a heating blanket at 37°C in a Faraday cage. The vagus nerve was isolated from the carotid at the level of the trachea, and one monopolar platinum electrode (Diameter: 125 μm, Phymep, Paris, France) was approximated to the vagal nerve. A second electrode was implanted directly on the skin as a reference. Both electrodes were connected to a BioAMp amplificator system (Ad Instrument; Phymep, Paris, France). The signal was filtered between 0.1 to 1,000 Hz (low and high frequency). The output signal was then directed toward a data acquisition system (PowerLab 8/30, Ad instrument). The neural activity was quantified by counting the frequency of spikes that exceeded a voltage threshold level set just above the electrical noise by using the Spike Histogram software (computer program Chart 5, Ad instrument). Baseline unit activity was recorded for 10 min before and during brain infusions. The neural activity was continuously recorded and quantified for 10 min at different time periods

during the brain infusions. At completion of the recording, 600 μg acetylcholine chloride was injected intraperitoneally to demonstrate appropriate responsivity and recording of the vagus nerve activity.

Hydrogen peroxide infusions. To study the role of reactive oxygen species (ROS) in the brain on arterial blood flow, heart rate, and whole-body glucose utilization, hydrogen peroxide (H_2O_2) was infused into the lateral ventricle of the mice 120 min after the beginning of the hyperglycemic clamp. Hydrogen peroxide was extemporaneously diluted in aCSF and infused at the rates of 2 or 20 nmol/min, as previously described (19). Briefly, 2 μl H_2O_2 was followed by a continuous infusion at a rate of 12 μl/h. At the end of the infusions, the mice were decapitated and the brain was removed from the skull within <15 s. The brain was put into a frozen brain frame and the hypothalamus was dissected out and frozen at −80°C. To validate that the probe was correctly recording the blood flow, at the end of the insulin infusion, some mice were given a flash injection of a rapid NO donor (sodium nitroprusside (10 mg/kg, 25–40 μl i.v.). Upon a correct implantation of the probe, the nitroprusside injection induced at least a 100% increase in blood flow and a rapid increase in heart rate.

TABLE 1
Summary of experimental conditions

Studies protocols	*n*
1: Clamps	
Euglycemic clamps	
aCSF (icv)	7
Ex4 (icv)	6
Hyperglycemic clamps	
aCSF (icv)	7
aCSF (icv) (GLP-1R KO)	6
Ex4 (icv)	7
Ex9 (icv)	5
Ex4 + hydrogen peroxide (20 nmol/min) (icv)	5
2: Saline intravenous infusion	
aCSF (icv)	6
3: Basal blood flow and heart rate studies	
aCSF (icv)	7
Ex4 (icv)	12
Ex9 (icv)	8
PBS (icv)	4
Hydrogen peroxide (2 nmol/min, icv)	4
Hydrogen peroxide (20 nmol/min, icv)	5
4: Vagal activity studies	
aCSF (icv)	5
Ex4 (icv)	4
5: ROS studies	
aCSF (icv, saline iv, WT)	5
aCSF (icv, clamp 5.5 mmol/l, WT)	15
aCSF (icv, clamp 20 mmol/l, WT)	10
Ex4 (icv, clamp 5.5 mmol/l, WT)	8
Ex4 (icv, clamp 20 mmol/l, WT)	14
aCSF (icv, clamp 5.5 mmol/l, GLP-1R KO)	5
aCSF (icv, clamp 20 mmol/l, GLP-1R KO)	5
6: NOS activity studies	
aCSF (icv, clamp 20 mmol/l)	5
Ex4 (icv, clamp 20 mmol/l)	5

The number of mice studied (*n*) is indicated for each experimental condition. aCSF, mice infused with artificial cerebrospinal fluid into the brain (intracerebroventricular [icv]). Some mice were clamped with insulin in euglycemic or hyperglycemic conditions or underwent a saline infusion equivalent to the corresponding insulin and glucose infusion volumes and rates performed during the clamp conditions. Some mice were studied in basal conditions (no insulin, glucose, or saline infusion) only. Hydrogen peroxide was used at the concentration of 2 or 20 nmol/min icv only, as specified. The vagal activity was recorded in response to aCSF or Ex4 icv infusions in a subset of mice. ROS production was assessed in all conditions in WT or GLP-1 receptor knockout mice (GLP-1R KO) mice. The activity of the NOS was studied in a subset of mice during hyperglycemic-hyperinsulinemic clamp conditions in the presence or absence of Ex4 icv.

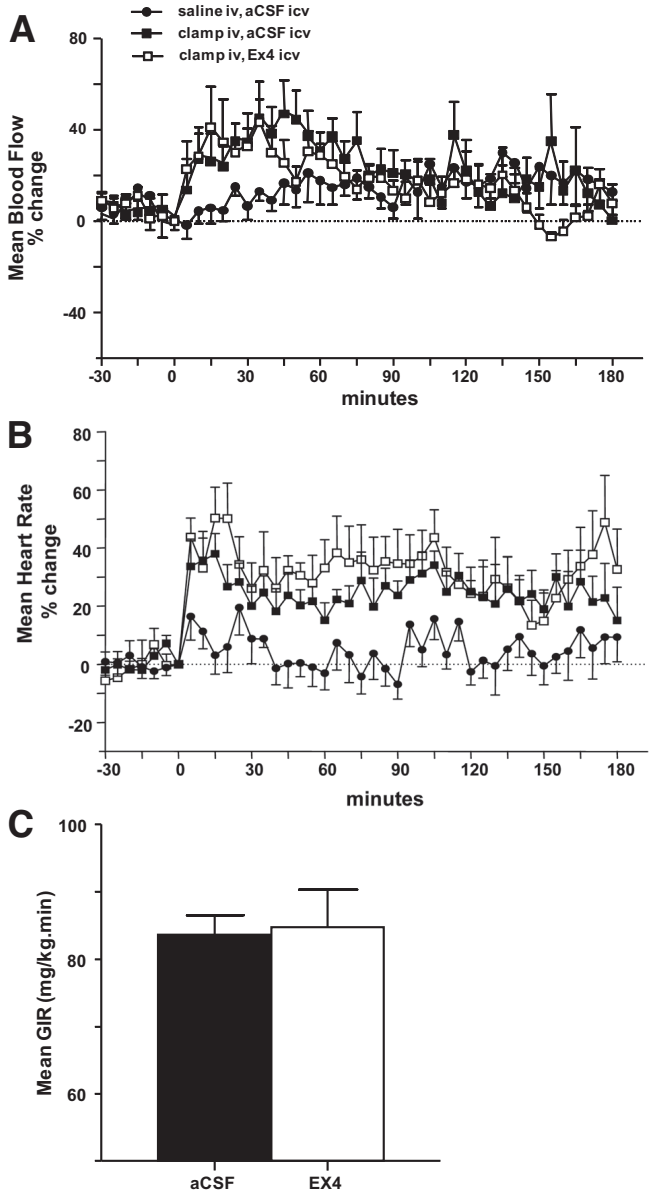
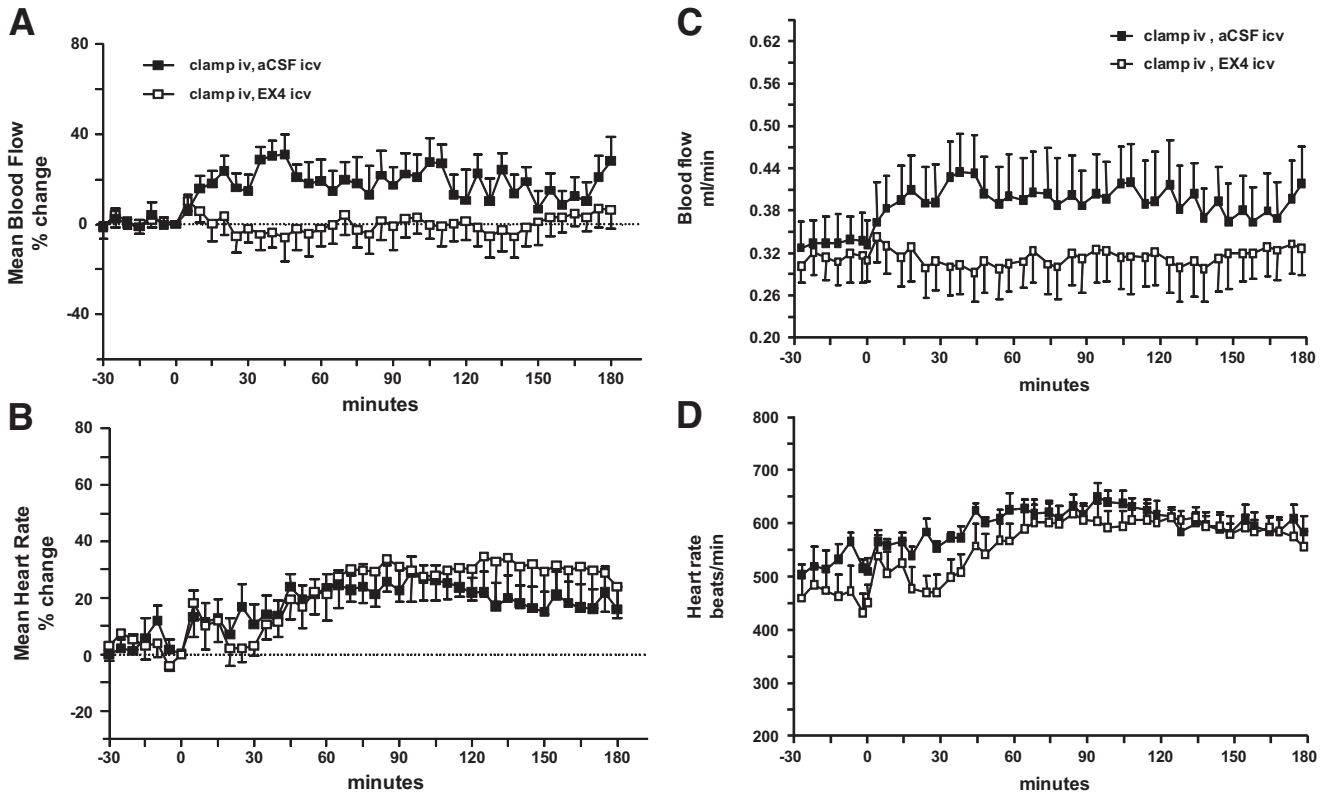


FIG. 2. Hyperinsulinemia controls arterial blood flow, heart, and whole-body glucose utilization rates in wild-type mice. Percent changes are shown from baseline of mean arterial femoral blood flow (*A*) and heart rates (*B*) during 3 h of hyperinsulinemic-euglycemic conditions where aCSF (■) or Ex4 (□) was simultaneously infused into the brain of awake free-moving C57BL/6 J wild-type mice. In a subset of wild-type mice, where aCSF was infused into the brain, intravenous saline was simultaneously infused instead of glucose or insulin (control group, ●). Data are means ± SE for six to seven mice per group and significantly different between the control group and the two other groups ($P < 0.05$). In *C*, whole-body glucose utilization rate, glucose infusion rate (GIR) ($mg \cdot kg^{-1} \cdot min^{-1}$), was calculated in steady-state euglycemic-hyperinsulinemic conditions (5.5 mmol/l) in mice infused with aCSF or Ex4 into the brain. Means ± SE are shown for six to seven mice per group.

Blood sampling. At the end of the intracerebroventricular infusions, blood was collected from the retro-orbital sinus into a tube, mixed with 1 μg/μl aprotinin/0.1 mmol/l EDTA, and centrifuged at 8,000 rpm for 5 min at 4°C. Plasma was stored at −80°C until assay. The insulin level was measured using an ELISA kit (Mercodia, Sweden). Briefly, 10 μl plasma was incubated for 2 h in the anti-insulin antibody–coated 96-well plate. Then, after washing the secondary, antibody conjugated with peroxidase was added for 30 min. The reaction was stopped by adding acid to give a colorimetric end point that is read spectrophotometrically. The interassay variation coefficient was ~8% and the intra-assay coefficient was ~4%.

Hypothalamic ROS and glutathione determinations. Tissue treatment for ROS determination was accomplished according to the method of Szabados et



al. (20). Briefly, we used a rodent brain matrix (World Precision Instrument, Sarasota, FL) to carefully slice out the hypothalamus and the surrounding tissues. This frame allows an accurate and repeated sampling of a 1-mm-thick brain slice. Then, a second frame is used to dissect out the hypothalamus precisely and uniquely from the other part of the brain surrounding the hypothalamus. Hypothalamic pieces were carefully homogenized using a dounce and loaded with the dichlorodihydro-fluorescein diacetate probe that is oxidized to fluorescent dichlorofluorescein by H_2O_2 and classically used to monitor intracellular generation of ROS (CM-H_2DCFDA, Molecular Probes). It is noteworthy that this probe is very specific for H_2O_2 and that the background level is very low. However, the origin of the ROS produced, i.e., mitochondrial or cytoplasmic, cannot be detected. Hypothalamic homogenates were incubated with 4 μmol/l CM-H_2DCFDA in a final 0.5-ml volume for 30 min at 37°C. After centrifugation, ROS measurements (on a 200-μl supernatant) were performed in a Fluorescent Plate Reader (Perkin Elmer). Intensity of fluorescence was expressed as arbitrary units per milligram protein. Oxidized glutathione/total glutathione ratio was assessed in hypothalamic homogenates. They were immediately grounded in 1% EDTA/5% metaphosphoric acid (1:5 vol/vol). After centrifugation, supernatants were used to detect total reduced glutathione and its oxidized form (GSSG) by reverse-phase high-performance liquid chromatography with electrochemical detection as described (21).

Hypothalamic NO synthase activities. To assess hypothalamic NO synthase (NOS) activities, the tissues were collected, weighted, and homogenized in buffer (250 mmol/l Tris-HCl, 10 mmol/l EDTA and EGTA). The NOS assay kit (Calbiochem, Darmstadt, Germany) is based on the biochemical conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline by NOS. The data are hence considered as an index of NOS activity. NOS catalyzes the stoichiometric conversion of L-arginine into L-citrulline in the presence of NADPH, oxygen, calmodulin, and calcium. The tissues were incubated with radiolabeled [^{3}H]-L-arginine (1 μCi/μl) and passed through a column containing a resin that binds L-arginine but not L-citrulline. The quantification of [^{3}H]-L-citrulline radioactivity is performed from the eluate. Results are expressed in counts per minute by milligram of tissues.

Data analysis and statistics. Data are expressed as means ± SE. Data were analyzed using the GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). Statistical significance was determined by applying, respectively, a Student's *t* test, a Mann-Whitney test, a Kruskal-Wallis followed by a Dunn's multiple comparison test, a Pearson test, or a two-way ANOVA test for repeated measurements with fixed factors of treatment/genotype, time, and treatment/genotype × time followed by post hoc test (Bonferroni's multiple comparison test) when appropriate. The acceptable level of significance was defined as $P < 0.05$.

The Student's *t* test was used for the glucose infusion rate and NOS activities, since it is designed to compare two means together within a binomial distribution. The Kruskal Wallis followed by the Dunn post hoc test was used to compare more than two means together with single values obtained during different experiments and was used for the comparison of ROS activities. Eventually, we used the two-way ANOVA for repeated measurements with fixed factors followed by the Bonferroni post hoc test for all time courses: heart rate, blood flow, and vagal activities.

RESULTS

The activation of the central GLP-1 receptor regulates femoral arterial blood flow, heart rate, and whole-body glucose utilization under hyperglycemic conditions. The systemic infusion of insulin during a euglycemic-hyperinsulinemic clamp increased femoral blood flow and heart rate (Fig. 2*A* and *B*). The simultaneous brain infusion of the GLP-1 receptor agonist Ex4 did not affect hemodynamic parameters and whole-body glucose utilization (Fig. 2*A*–*C*) when the mice were clamped under euglycemic conditions. Because we previously demonstrated that brain GLP-1 signaling was glucose-dependent (11), the blood glucose was raised to 20 mmol/l during the hyperinsulinemic clamp. Hyperglycemia modestly diminished the relative increase in femoral arterial blood flow (compare Fig. 3*A* and 2*A*). Remarkably, coinfusion of Ex4 into the brain under hyperglycemic conditions abolished the increase in femoral artery blood flow (Fig. 3*A* and C). Simultaneously, hyperglycemia diminished the increase in heart rate (Fig. 3*B* and *D*) observed under euglycemic clamp conditions (compare Fig. 3*B* and 2*B*), but unlike blood flow, heart rate was not affected by Ex4. Concomitantly, the whole-body glucose utilization rate was also reduced by Ex4 (Fig. 3*E*). In contrast to the effects seen under conditions of systemic hyperglycemia and hyperinsulinemia, infusion of Ex4 into the lateral ventricle

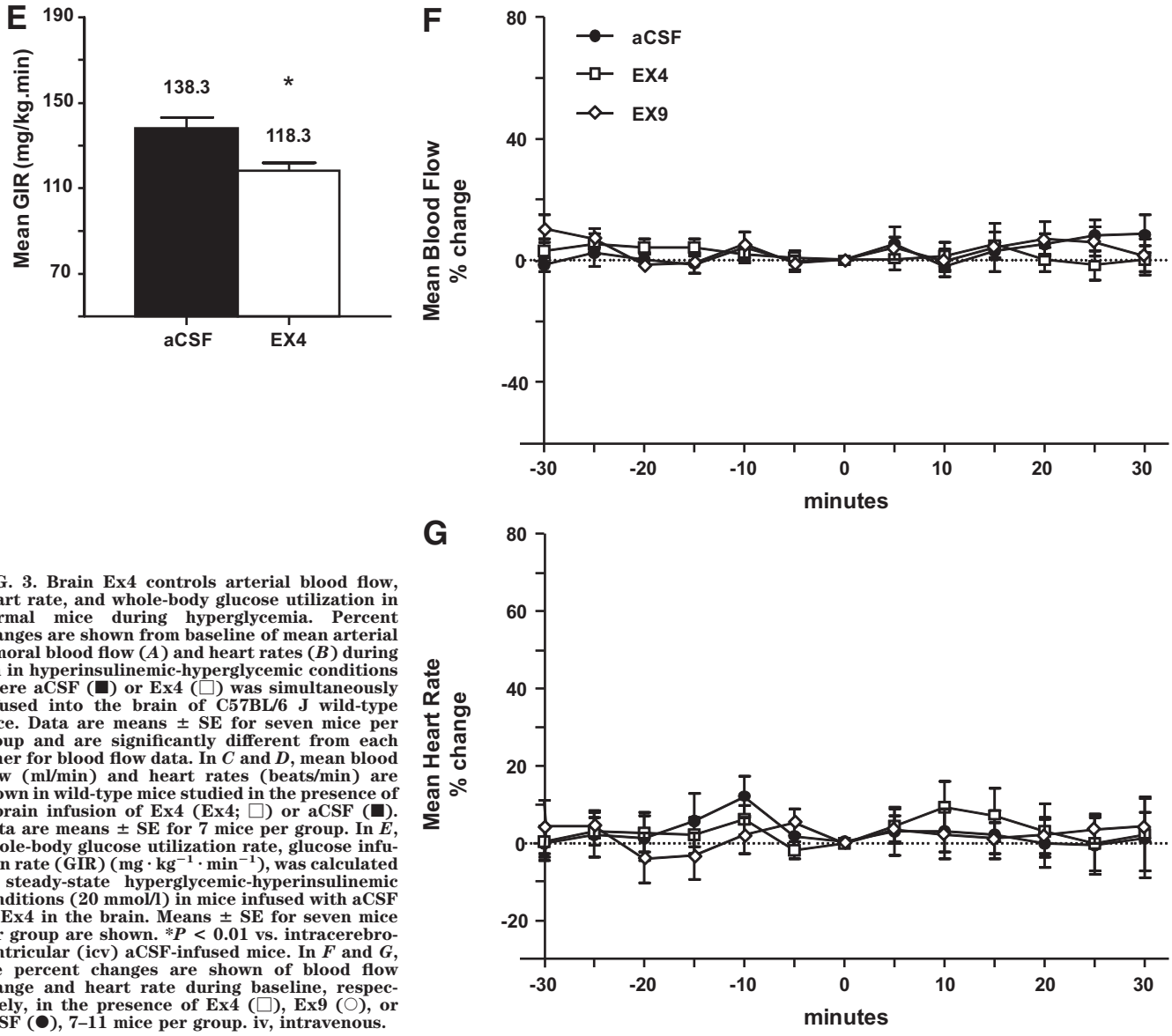


FIG. 3. Brain Ex4 controls arterial blood flow, heart rate, and whole-body glucose utilization in normal mice during hyperglycemia. Percent changes are shown from baseline of mean arterial femoral blood flow (*A*) and heart rates (*B*) during 3 h in hyperinsulinemic-hyperglycemic conditions where aCSF (■) or Ex4 (□) was simultaneously infused into the brain of C57BL/6 J wild-type mice. Data are means ± SE for seven mice per group and are significantly different from each other for blood flow data. In *C* and *D*, mean blood flow (ml/min) and heart rates (beats/min) are shown in wild-type mice studied in the presence of a brain infusion of Ex4 (Ex4; □) or aCSF (■). Data are means ± SE for 7 mice per group. In *E*, whole-body glucose utilization rate, glucose infusion rate (GIR) (mg · kg^{-1} · min^{-1}), was calculated in steady-state hyperglycemic-hyperinsulinemic conditions (20 mmol/l) in mice infused with aCSF or Ex4 in the brain. Means ± SE for seven mice per group are shown. *P < 0.01 vs. intracerebroventricular (icv) aCSF-infused mice. In *F* and *G*, the percent changes are shown of blood flow change and heart rate during baseline, respectively, in the presence of Ex4 (□), Ex9 (○), or aCSF (●), 7–11 mice per group. iv, intravenous.

had no effect on blood flow and heart rate in the fasting basal state (Fig. 3*F* and *G*).

To further examine the importance of endogenous GLP-1 receptor signaling for the control of femoral arterial blood flow and whole-body glucose utilization, we used *1*) GLP-1 receptor knockout and *2*) wild-type mice infused into the brain with the GLP-1 receptor antagonist Ex9 under hyperinsulinemic-hyperglycemic conditions. Remarkably, transient attenuation or genetic elimination of central GLP-1 receptor signaling markedly augmented femoral arterial blood flow (Fig. 4*A*). Similarly, heart rate was increased in GLP-1 receptor knockout mice, and whole-body glucose utilization rates were also increased by the disruption of central GLP-1 receptor signaling (Fig. 4*B* and *C*).

Femoral arterial blood flow correlates with whole-body glucose utilization in the presence of an activated GLP-1 receptor. To determine whether the control of whole-body glucose utilization by brain GLP-1 signaling is intimately related to the rate of femoral arterial blood flow, we correlated both parameters for each mouse independently, under different experimental conditions. The data show that femoral arterial blood flow and whole-body glucose utilization rates were positively correlated under hyperglycemic-hyperinsulinemic conditions (Fig. 5*A*) and the mean values of each group for both parameters exhibited a highly significant correlation (Fig. 5*B*). In contrast, no correlation between femoral blood flow and glucose utilization was observed in mice with transient central or complete genetic disruption of GLP-1 receptor signaling (Fig. 5*D*) when compared with WT control mice (Fig. 5*C*).

GLP-1 receptor activation decreases hypothalamic nitric oxide synthase activity and reactive oxygen species concentration. We previously showed that hyperinsulinemia increases femoral arterial blood flow by a mechanism requiring eNOS activation (4). Therefore, we assessed the activity of nitric oxide synthases in the hypothalamus of mice clamped in hyperglycemia and infused with Ex4 into the brain. Central Ex4 significantly reduced total nitric oxide synthase activity (Fig. 6*A*). Because ROS are also regulated by insulin and glucose in the brain (22), we assessed the impact of glucose and Ex4 in vivo on ROS production. Hyperglycemia significantly reduced ROS production, which was even more markedly

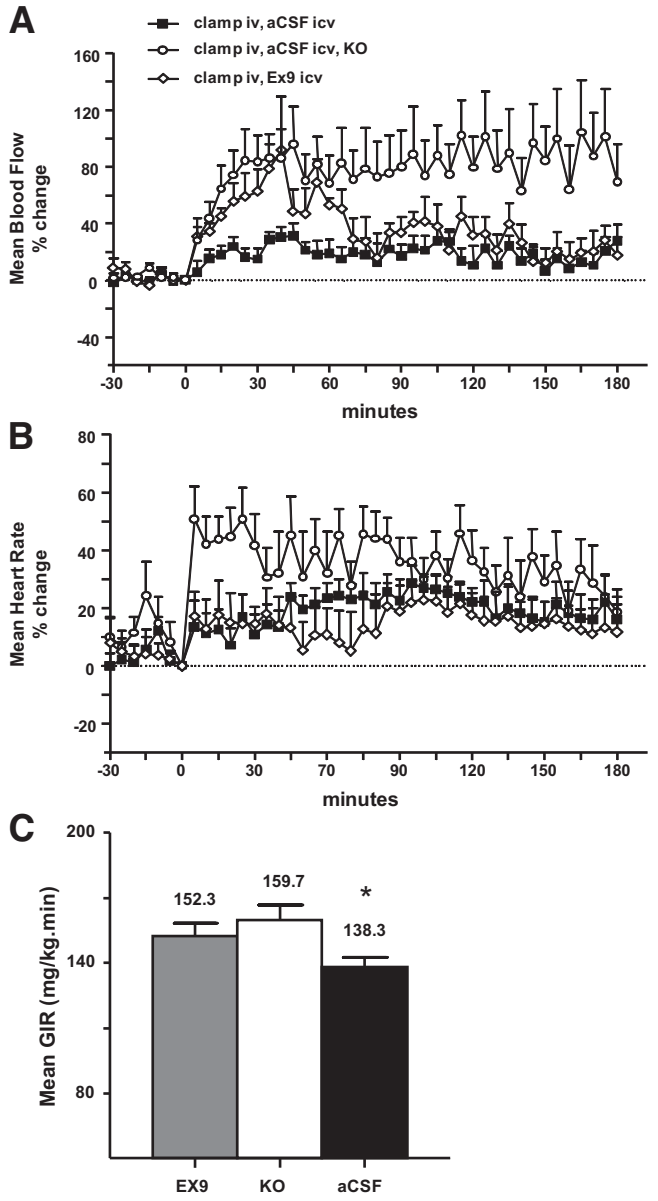


FIG. 4. Brain GLP-1 receptor inactivation modulates arterial blood flow, heart rate, and whole-body glucose utilization in mice. Percent changes from baseline of mean arterial femoral blood flow (*A*) and heart rates (*B*) during 3 h in hyperinsulinemic-hyperglycemic conditions, where aCSF was infused in the brain of wild-type (WT, ■) or GLP-1 receptor knockout mice (KO, ○), or in WT infused with Ex9 into the brain (◇). Data are means ± SE for 5–7 mice per group. Statistical significance was determined between mice infused with Ex9 or aCSF and between GLP-1R KO mice and wild-type mice infused with aCSF ($P < 0.05$ for the mean blood flow data only). In *C*, whole-body glucose utilization rate, glucose infusion rate (GIR) ($mg \cdot kg^{-1} \cdot min^{-1}$), was calculated in steady-state hyperglycemic-hyperinsulinemic conditions (20 mmol/l) in wild-type mice infused with aCSF or Ex9 in the brain and in GLP-1 receptor knockout (KO) mice. *$P < 0.05$ vs. intracerebroventricular (icv) aCSF-infused KO mice. iv, intravenous.

suppressed by Ex4 in a glucose-dependent manner (Fig. 6*B*). Remarkably, the ability of glucose to diminish hypothalamic ROS was completely abrogated in GLP-1 receptor knockout mice (Fig. 6*B*). This reduced ROS concentration was associated with an increased antioxidant capacity in response to the Ex4 treatment and in hyperglycemia only, since the oxidized glutathione–to–total glutathione ratio was slightly reduced (Fig. 6*C*). However, we cannot rule out that another antioxidant mechanism could also have controlled the ROS production in response to brain GLP-1 signaling.

ROS in the central nervous system controls femoral arterial blood flow and whole-body glucose utilization in a glucose-dependent manner. Because we previously demonstrated that the pharmacological modulation of brain NO controlled femoral arterial blood flow and whole-body glucose utilization rates (4), we focused here on the role of ROS. To determine whether changes in ROS could link GLP-1 receptor activation to the control of femoral arterial blood flow and whole-body glucose utilization rates, we performed a continuous infusion of oxygen peroxide into the lateral ventricular cavity of control mice under basal conditions. H_2O_2 induced a dose-dependent increase in femoral arterial blood flow (Fig. 7*A*). We next determined whether H_2O_2 could reverse the effects of Ex4 on blood flow. The ROS donor was injected 120 min after the beginning of the simultaneous hyperglycemic-hyperinsulinemic clamp and Ex4 infusion (Fig. 7*B*). H_2O_2 dramatically and rapidly increased the arterial blood flow in the presence of Ex4 (Fig. 7*B*). Conversely, the heart rate was reduced in the same experiments (Fig. 7*C*).

Vagus nerve activity is increased in response to brain Ex4 administration. To determine whether the autonomic nervous system is a candidate for transmission of the brain GLP-1 signal to peripheral tissues, we recorded vagus nerve activity during brain Ex4 infusion. Ex4 administration rapidly increased the firing rate of the vagus nerve within 5 min (Fig. 8*B* and *C*), which lasted for 55 min, i.e., until the end of the infusion (Fig. 8*B* and *C*).

DISCUSSION

The present data demonstrate that in the awake free-moving mouse, brain GLP-1 receptor signaling simultaneously controls femoral arterial blood flow, heart rate, and glucose utilization. We further show that reactive nitric oxide and oxygen species are regulated by brain GLP-1 signaling and likely important for the coordinated regulation of metabolic and cardiovascular function. The brain to periphery signal was associated with an increased vagus nerve activity. Notably, the action of GLP-1R signaling for control of nitric oxide and ROS was strictly glucose dependent.

We previously reported that brain GLP-1 induced insulin resistance and increased insulin secretion to favor hepatic glucose storage (11). We now extend these findings by demonstrating that brain GLP-1 signaling reduces hindlimb arterial blood flow under conditions of hyperglycemia. Hence, these new data strongly suggest that brain GLP-1 signaling may modify the metabolic activity of hindlimb muscles by a mechanism involving changes in blood flow. Consistent with this hypothesis, a strong correlation was observed between glucose utilization and blood flow rates. Importantly, this correlation was no longer present in experimental conditions where brain GLP-1 signaling was abolished in GLP-1 receptor knockout mice and, even more selectively, in mice infused with the GLP-1 receptor antagonist Ex9 into the brain. It is noteworthy that there is an important difference between the blood flow profiles obtained in the KO mice and those obtained with the GLP-1 receptor antagonist Ex9. It is suggested that either the GLP-1 receptor KO mice have adapted to the genetic mutation or that the GLP-1 receptor is also important to regulate blood flow somewhere else in the body. Recent evidence suggests that GLP-1 regulates

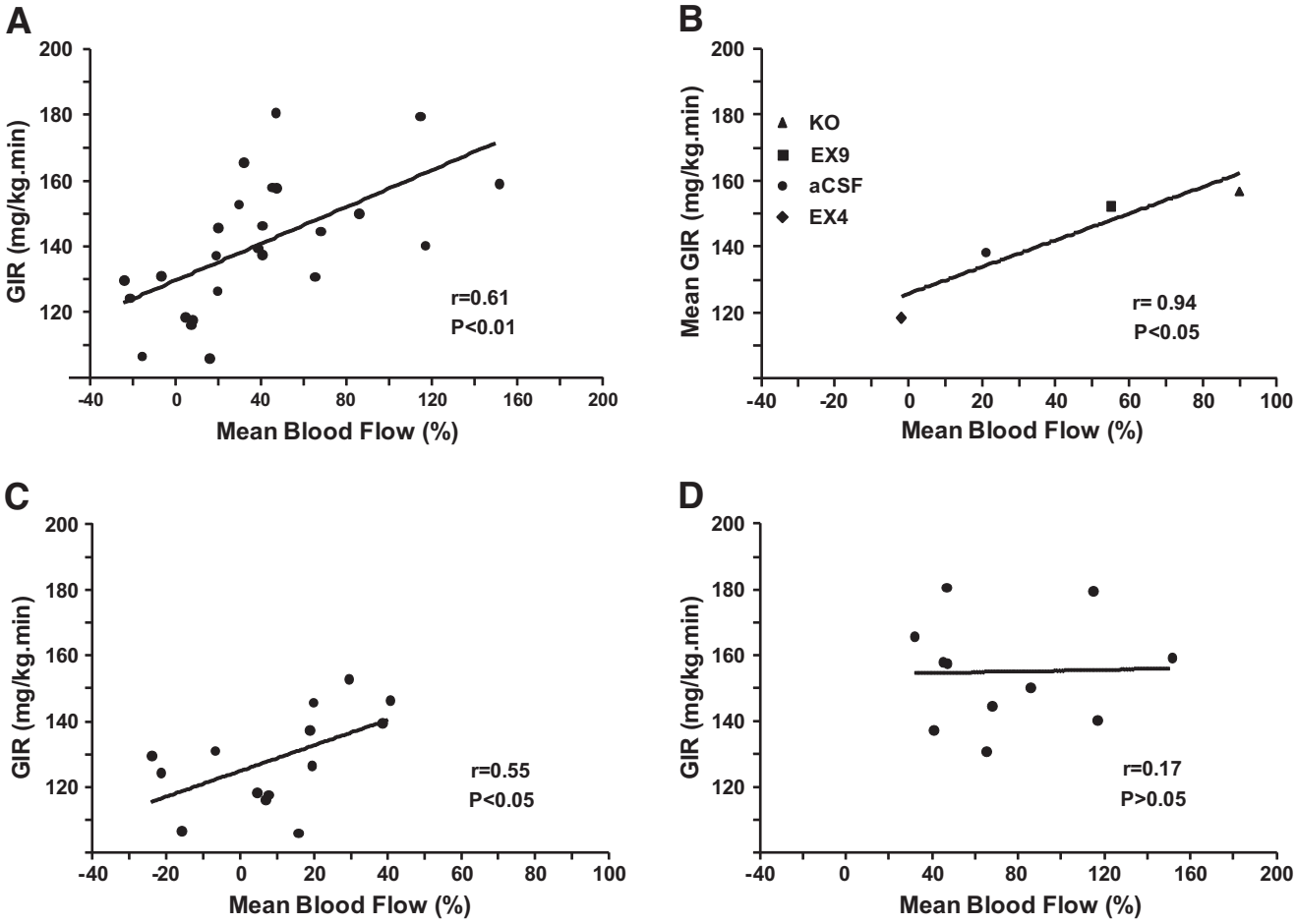


FIG. 5. Correlations between femoral arterial blood flow and whole-body glucose utilization rates under hyperglycemic conditions. The maximal blood flow rates (%) calculated during the hyperinsulinemic-hyperglycemic clamp were plotted in *A* against the glucose infusion rate (GIR, mg · kg^{-1} · min^{-1}) for individual mice (n = 25). In *B*, the mean values are represented for mice infused into the brain with Ex4 (♦), aCSF (●), or Ex9 (■) or in GLP-1 receptor knockout mice (KO, ▲). In *C*, individual data are presented for mice with active brain GLP-1R signaling (wild-type aCSF or Ex4-infused mice, n = 14). In *D*, individual data are presented for mice where brain GLP-1 receptor signaling was blocked by Ex9 or in GLP-1R knockout mice (KO) (n = 11). The Pearson r value was calculated and a positive correlation was statistically significant when $P < 0.05$.

endothelial function. GLP-1 relaxed femoral artery in a dose-response manner (23), and GLP-1 is associated with vasodilation induced by acetylcholine (24). In humans, the systemic infusion of GLP-1 or its analogs does not induce hypertension but, rather, has protective effects (25,26). Therefore, the increased vascular resistance demonstrated by the present data seems to be specific for brain GLP-1 signaling and not observed in response to a systemic infusion. It is noteworthy that Ex4 is infused into the brain and that the rate of infusion is so low that no systemic effect could be detected (11). It is noteworthy that our animal model was designed to study the pharmacological effect of brain GLP-1 signaling on vascular and metabolic functions. We performed hyperinsulinemic-hyperglycemic clamps that do not correspond to any physiological situation.

Therefore, we conclude that brain GLP-1 signaling contributes to the regulation of both metabolic and cardiovascular functions. Importantly, we cannot directly demonstrate a causal relationship between metabolic and vascular function in response to brain GLP-1 signaling.

It has been previously shown that both intravenous and intracerebroventricular administration of GLP-1 receptor agonists increased blood pressure and heart rate (8). We do not have direct evidence of changes in blood pressure under our experimental conditions. However, because the arterial femoral blood flow was reduced in response to brain Ex4 infusion, we could suggest that our data fit previous data from the literature. We here further demonstrate that this increased blood pressure could be due to an increased peripheral vascular resistance.

Moreover, GLP-1 receptors are also expressed in the area postrema of the rat brain, which could interact with circulating GLP-1 (27,28). In our experimental procedure, hyperglycemia was induced by a systemic glucose infusion; therefore, under these conditions, GLP-1 secretion from the gut is not increased. Our data strongly support the idea that central GLP-1 is a direct regulator of peripheral cardiovascular function. Furthermore, previous studies showed that the intracerebroventricular administration of GLP-1R agonists increased arterial blood pressure and heart rate, which was blocked by previous intracerebroventricular administration of Ex9 (14). This is in agreement with our data suggesting that this increased blood pressure could be due to the reduced blood flow that we observed in the present study. Bilateral vagotomy blocked the stimulating effect of intracerebroventricular GLP-1 on arterial blood pressure and heart rate in rats (14). These findings, taken together with our current data, suggest that neural information emerging in the brain after GLP-1 receptor activation is transmitted to the periphery through the vagus nerve. We previously reported that brain insulin

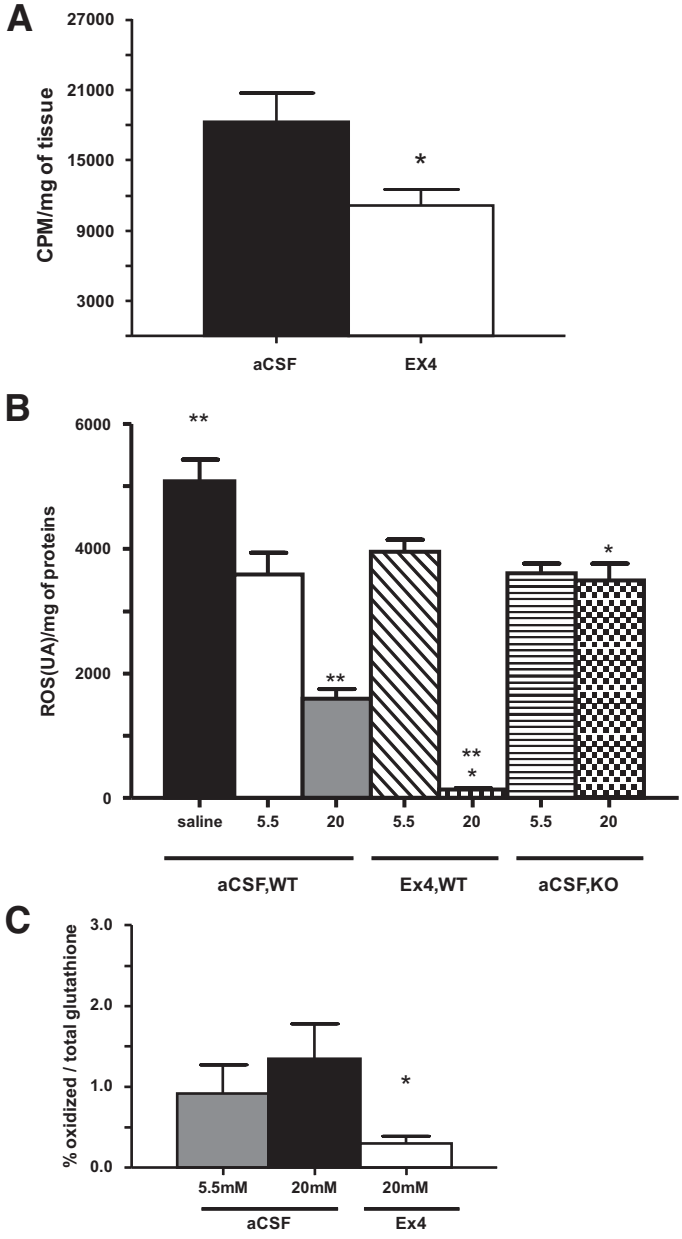


FIG. 6. NOS activity and the concentration of ROS are reduced in the hypothalamus of mice infused with Ex4 into the brain under hyperglycemic conditions. In *A*, hypothalamic nitric oxide activity (cpm/mg tissue) in hyperglycemic-hyperinsulinemic conditions in wild-type mice is infused with Ex4 or aCSF into the brain. The mean of five mice per group is shown. *$P < 0.05$ vs. intracerebroventricular (icv) aCSF-infused mice. *B*: ROS (arbitrary units [AU]/mg protein) in euglycemic (5.5 mmol/l) or hyperglycemic (20 mmol/l) conditions during a hyperinsulinemic clamp or during an intravenous saline infusion (saline) in wild-type mice simultaneously infused with aCSF or Ex4 into the brain. GLP-1 receptor knockout mice (KO) were studied in hyperinsulinemic-euglycemic or hyperglycemic conditions. Data are means ± SE for 5–14 mice per group. *$P < 0.05$ vs. icv aCSF-infused wild-type or mice in hyperglycemic conditions; $P < 0.05$ vs. 5.5 nmol/l condition. *C*: Oxidized glutathione–to–total glutathione ratio in hyperinsulinemic-euglycemic or hyperinsulinemic mice clamped in the presence or absence of Ex4 in the lateral ventricle of the brain. Data are means of 5–8 mice per group. *$P < 0.05$ vs. hyperglycemic icv aCSF-infused mice.

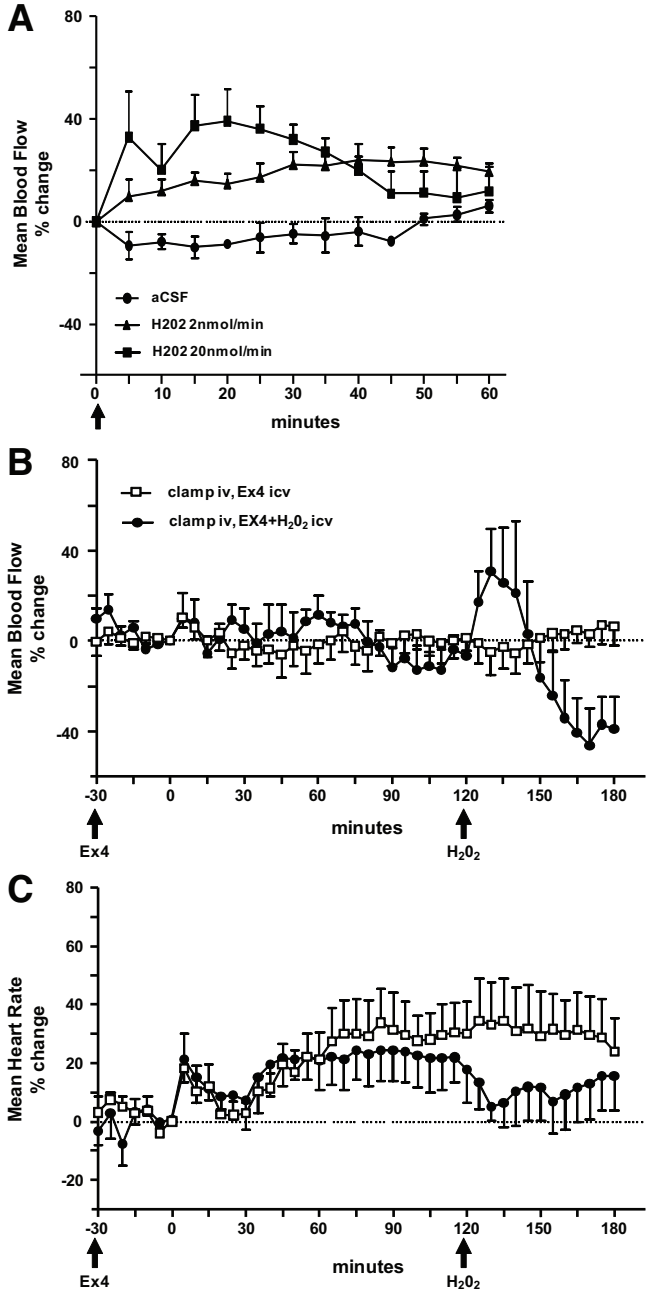
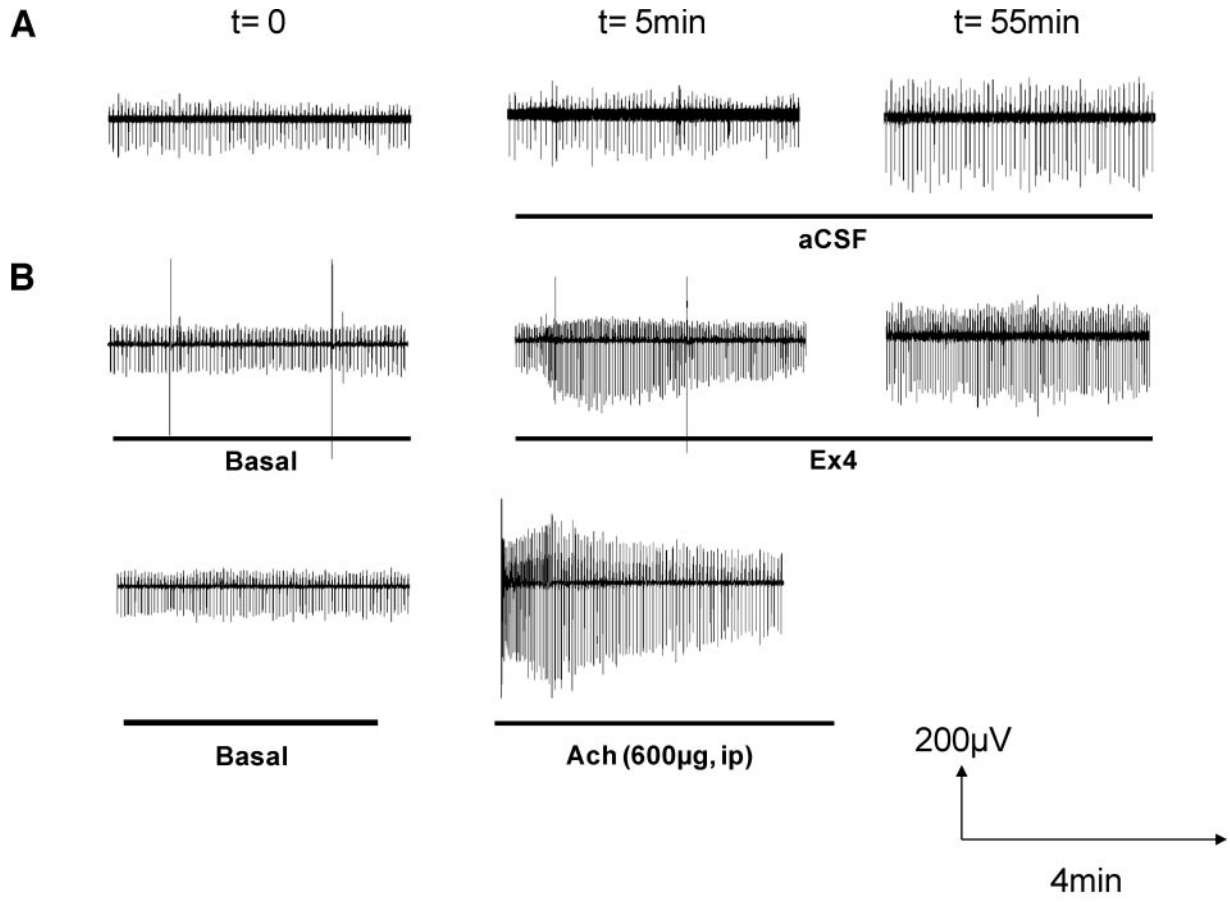


FIG. 7. A brain ROS donor increases femoral arterial blood flow and reduces heart rate in a dose-dependent manner. In *A*, the femoral arterial blood flow rate was recorded under basal conditions. aCSF or hydrogen peroxide (H_2O_2, low 2 nmol/min and high 20 nmol/min) were infused in the lateral ventricle at the time indicated by the arrow. Statistical significance ($P < 0.05$) was determined between mice infused with aCSF or H_2O_2. In *B*, the femoral arterial blood flow rate was recorded in hyperglycemic conditions and in the presence of Ex4 infused into the brain (arrow at −30 min). After 120 min, an infusion of hydrogen peroxide was performed in a separate group of mice (see arrow at 120 min, 20 nmol/min). aCSF infusion under the same experimental conditions did not modify the blood flow (not shown). In *C*, the mean heart rate was recorded simultaneously. Data are means ± SE for 5–7 mice per group. icv, intracerebroventricular; iv, intravenous.

signaling increases the arterial femoral blood flow by a mechanism that does not require the cholinergic tone, since the central insulin signal was not affected by atropine (4). We suspected that noncholinergic fibers would be present in the vagus nerve. We here show that Ex4 activates the firing activity of the vagus nerve and is associated with vasoconstriction. Therefore, in the mouse, the reduction of the arterial blood flow might be associated with an increased activity of the vagus nerve. It is noteworthy that we studied anesthetized mice in basal conditions, where Ex4 alone was infused into the brain. Therefore, more experiments should be done to validate this conclusion in awake free-moving mice in response to hyperglycemic-hyperinsulinemic clamps.



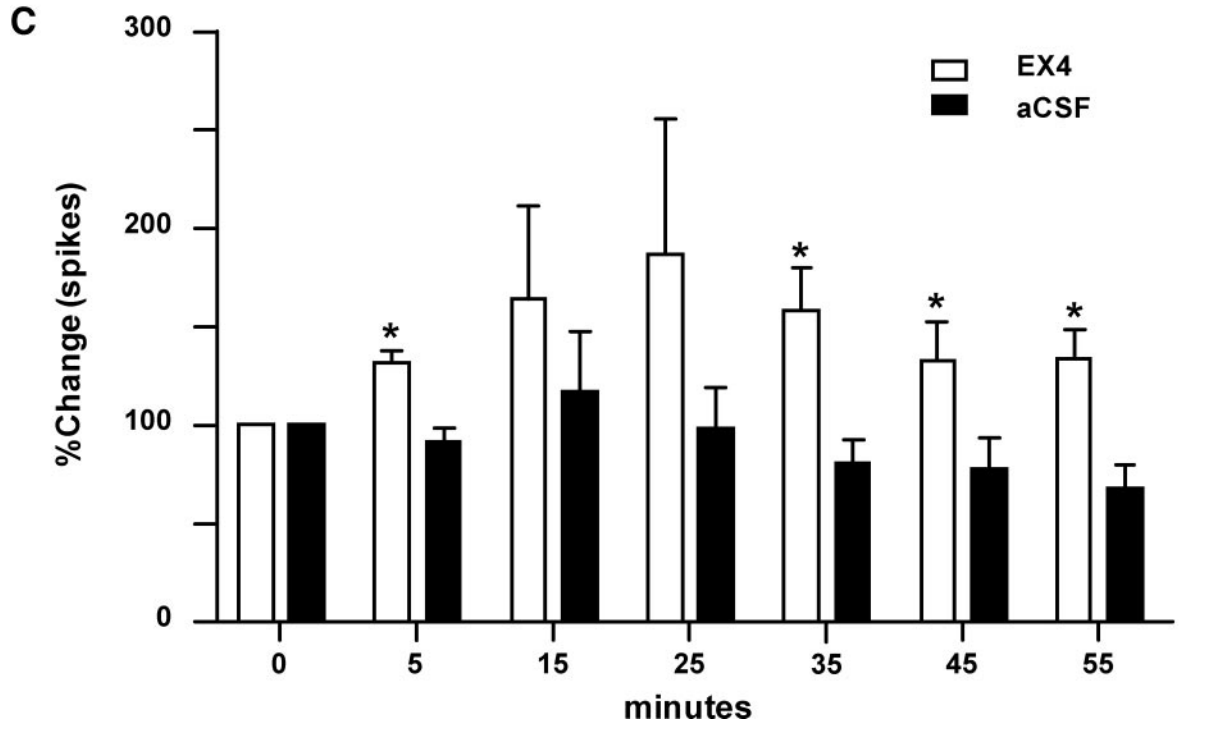


FIG. 8. Brain Ex4 infusion rapidly increases vagus nerve activity. *A*: Firing rate activity of the vagus nerve in microvolt/time shown before (t = 0) and 5 (t = 5) and 55 (t = 55) min after the beginning of the intracerebroventricular (icv) aCSF (*A*) or Ex4 (*B*) infusions. At completion of the experiment, an intraperitoneal flash injection of acetylcholine chloride was performed as a positive control and compared with the basal value. In *C*, the firing rate activity was quantified by assessing the number of spikes per minute and represented in percent of change versus T0 in aCSF and Ex4-infused mice. *Significantly different from aCSF-infused mice when $P < 0.05$.

Importantly, in our experimental procedure, brain GLP-1 signaling was strictly glucose dependent. The glucose dependency of GLP-1 signaling is a main general feature. This characteristic minimizes the risk of hypoglycemia by blunting insulin secretion when glycemia returns to the basal value. Here, we suggest that the vascular effect of GLP-1 would also be blunted when the blood glucose concentration reaches euglycemia. The mechanisms responsible for the glucose dependency of GLP-1 signaling into the brain are unknown but could be related to those that we described into the portal vein (12,29). For example, the glucose transporter GLUT2 is probably a key of the brain glucose sensitivity (3,30).

Brain GLP-1 receptor signaling is activated in response to oral glucose absorption (11), such as that in the fed condition. Glucose and insulin are two other important regulators of brain signaling (31,32). We previously showed that brain insulin increases femoral arterial blood flow by a mechanism depending on the production of NO by eNOS (4) and muscle glucose utilization (17). We have now demonstrated that brain GLP-1 signaling conversely decreased NOS activity and prevented the vasodilatory effect of insulin in a glucose-dependent manner. Hyperglycemia is absolutely required for the control of GLP-1 action on glucose homeostasis (6), and recent data showed that brain glucose sensing requires ROS signaling. The transient increase from 5 to 20 mmol/l glucose increased ROS concentration in hypothalamic slices ex vivo, which is reversed by adding antioxidants (33). In our in vivo experimental condition, i.e., 3 h of hyperglycemia and hyperinsulinemia, the ROS concentration was reduced in the hypothalamus. This was associated with an increased antioxidative activity, since the oxidized glutathione/total glutathione ratio was reduced. We cannot rule out that other ROS scavenging mechanisms could have been activated in response to Ex4. Our current data extend these findings by demonstrating that *1*) exogenous GLP-1R activation markedly reduces glucose-dependent ROS generation and *2*) basal endogenous GLP-1R signaling is required for the transmission of the signal linking hyperglycemia to diminished ROS generation. However, we cannot directly demonstrate that the ROS concentration and GST activity changes were located exclusively in GLP-1 receptor–expressing cells. Recent data from our group and from the literature showed that GLP-1 receptor–expressing cells are located at a high density in the arcuate nucleus of the hypothalamus in the rats and mice. Such cells are mainly associated with pro-opiomelanocortin (POMC)– and neuropeptide Y (NPY)–expressing neurons (34,35). The interpretation would rather be that the hypothalamic GLP-1–sensitive cells would transmit the GLP-1 signal to multiple different cells in connection with the former one. In addition, we used Ex4 instead of GLP-1 in our infusion procedures to demonstrate the role of the GLP-1 receptor. We previously validated that brain Ex4 effects on glucose metabolism and insulin sensitivity specifically depended on brain GLP-1 receptors by studying GLP-1 receptor knockout mice. Although we cannot exclude any unknown Ex4-specific effect, our data strongly suggest that most if not all the effects of Ex4 mimic native brain GLP-1 signaling.

In conclusion, the central nervous system is tightly involved in the integrated control of blood glucose and cardiovascular homeostasis. We have shown that, although at a supraphysiological concentration, central GLP-1 behaves as a molecular signal linking both metabolic and cardiovascular functions. Recently, two new therapeutic strategies based on GLP-1 have been approved for the treatment of diabetes. One involves GLP-1 receptor activation using GLP-1R agonists such as Ex4. The second mechanism involves the prevention of endogenous GLP-1 degradation by the use of inhibitors of the GLP-1 degrading enzyme dipeptidyl peptidase-4. Although both strategies lead to similar reduction of A1C in patients with type 2 diabetes, it seems reasonable to postulate that GLP-1R agonists and dipeptidyl peptidase-4 inhibitors may produce different effects on brain mechanisms regulating glucose homeostasis and cardiovascular function, an issue that will be important to address in future studies.

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