ORIGINAL RESEARCH COMMUNICATION

Hypothalamic Apelin/Reactive Oxygen Species Signaling Controls Hepatic Glucose Metabolism in the Onset of Diabetes

Anne Drougard,^{1,2} Thibaut Duparc,^{1,2} Xavier Brenachot,^{1–3} Lionel Carneiro,³ Alexandra Gouazé,³ Audren Fournel,^{1,2} Lucie Geurts,⁴ Thomas Cadoudal,^{1,2} Anne-Catherine Prats,⁵ Luc Pénicaud,³ Didier Vieau,⁶ Jean Lesage,⁶ Corinne Leloup,³ Alexandre Benani,³ Patrice D. Cani,⁴ Philippe Valet,^{1,2} and Claude Knauf^{1,2}

Abstract

Aims: We have previously demonstrated that central apelin is implicated in the control of peripheral glycemia, and its action depends on nutritional (fast versus fed) and physiological (normal versus diabetic) states. An intracerebroventricular (icv) injection of a high dose of apelin, similar to that observed in obese/diabetic mice, increase fasted glycemia, suggesting (i) that apelin contributes to the establishment of a diabetic state, and (ii) the existence of a hypothalamic to liver axis. Using pharmacological, genetic, and nutritional approaches, we aim at unraveling this system of regulation by identifying the hypothalamic molecular actors that trigger the apelin effect on liver glucose metabolism and glycemia. **Results:** We show that icv apelin injection stimulates liver glycogenolysis and gluconeogenesis *via* an over-activation of the sympathetic nervous system (SNS), leading to fasted hyperglycemia. The effect of central apelin on liver function is dependent of an increased production of hypothalamic reactive oxygen species (ROS). These data are strengthened by experiments using lentiviral vector-mediated over-expression of apelin in hypothalamus of mice that present over-activation of SNS associated to an increase in hepatic glucose production. Finally, we report that mice fed a high-fat diet present major alterations of hypothalamic apelin/ROS signaling, leading to activation of glycogenolysis. **Innovation/Conclusion:** These data bring compelling evidence that hypothalamic apelin is one master switch that participates in the onset of diabetes by directly acting on liver function. Our data support the idea that hypothalamic apelin is a new potential therapeutic target to treat diabetes. *Antioxid. Redox Signal.* 20, 557–573.

Introduction

THE HYPOTHALAMUS is the target of numerous factors, including hormones, neurotransmitters, and nutrients (56). After integration of these signals, modification of hypothalamic neuronal activity induces large variations of peripheral tissues functions *via* the autonomous nervous system (ANS). Consequently, abnormal hypothalamic responses may have consequences on liver functions, including over-activation of

glycogenolysis and/or gluconeogenesis in fasting conditions as observed during type 2 diabetes (9). Thus, identifying molecular hypothalamic actors implicated in the alteration of glucose homeostasis is now of crucial importance for therapeutic advance (38).

Apelin and its receptor APJ are now considered potential peripheral targets in the treatment of cardiovascular diseases, obesity, and type 2 diabetes (7). In contrast, recent studies have suggested that central apelin plays a role in the transition

¹Institut National de la Santé et de la Recherche Médicale (INSERM), Toulouse, France.

²Institut des Maladies Métaboliques et Cardiovasculaires (I2MC), Université Paul Sabatier, UPS, Toulouse Cedex, France.

³UMR CNRS 6265-INRA 1324, Centre des Sciences du Goût et de l'Alimentation CSGA, Université de Bourgogne, Dijon, France.

⁴Metabolism and Nutrition Research Group, Louvain Drug Research Institute, LDRI, Université Catholique de Louvain, Brussels, Belgium.

⁵Université de Toulouse, UPS, TRADGENE, Toulouse, France.

⁶Unité Environnement Périnatal et Croissance, EA 4489, IFR 114, Université Lille-Nord de France, Equipe Dénutritons Maternelles Périnatales, Villeneuve d'Ascq Cédex, France.

Innovation

Central apelin is known to control glycemia in a dose-dependent manner. In the brain, a slight physiological increase of apelin stimulates a hypothalamic nitric oxide (NO) pathway, which results in an increase in peripheral glucose utilization. On the contrary, a high increase in central apelin, similar to that observed in obese/diabetic mice, provoked fasted hyperglycemia in normal mice. We demonstrate that central apelin participates in the establishment of a type 2 diabetes state by modulating the release of reactive oxygen species in the hypothalamus. Consequently, the autonomic nervous system provokes an over-activation of glycogenolysis and gluconeogenesis in the liver, resulting in fasted hyperglycemia.

from the normal to diabetic state. First, apelin expression is increased in the hypothalamus of obese/diabetic mice (42). Second, acute and chronic intracerebroventricular (icv) injections of a high quantity of apelin (similar to that observed in obese/diabetic mice) in fasted normal mice induce type 2 diabetes symptoms such as hyperinsulinemia, hyperglycemia, glucose intolerance, and insulin resistance (11). This result suggests that hypothalamic apelin may control liver glucose metabolism, thereby controlling glucose release *via* glycogenolysis and/or gluconeogenesis in fasted state. Reinforcing this hypothesis, we clearly demonstrated that central apelin exerts its glycemic effect by targeting the ventromedial hypothalamic nuclei (11), a region whose electrical stimulation triggers the activation of hepatic glycogenolysis and gluconeogenesis *via* the ANS (44, 49).

One molecular target of apelin actions is the reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). Finely balanced mitochondrial ROS (mROS) production may be at the core of proper metabolic maintenance, and unbalanced mROS production, which is largely documented, might be an important trigger of metabolic disorders (15, 23). Data from literature demonstrate that apelin increases the production of ROS in neuronal cells culture (57). In the brain, hypothalamic hyperglycemia increases mROS hypothalamic production, which, in turn, stimulates peripheral hyperinsulinemia, thereby supporting the implication of brain mROS in the control of glucose homeostasis (24). Under metabolic dysfunction, mitochondria are considered the principal source of H_2O_2 (2), and an elevated mitochondrial H_2O_2 emission is a primary cause of insulin resistance (2, 15).

Thus, unraveling the hypothalamic apelin to liver axis involved in the onset of type 2 diabetes appears to be of major importance. However, the downstream molecular pathways and functional effects of hypothalamic apelin on the liver remain to be established. Given the well-established role of central apelin in the control of glycemia, we tested here the possibility that hypothalamic apelin positively regulates hepatic glucose production. To address this hypothesis, we used pharmacological and genetic approaches to increase hypothalamic level of apelin in normal and high-fat diet (HFD) obese/diabetic mice. These experiments allowed us to examine whether the increased levels of hypothalamic apelin is sufficient to modify hypothalamic ROS/ H_2O_2 production and, consequently, hepatic glucose release *via* ANS.

Results

Antioxidants counterbalanced icv apelin-induced fasted-hyperglycemia in mice

We confirmed here that acute apelin icv injection provoked fasted hyperglycemia in normal mice (Fig. 1A) (11). Since ROS are potential targets of apelin action, we injected icv apelin in mice previously treated with an antioxidant, trolox at 1 mM. At this concentration, trolox is able to prevent hyperinsulinemia generated by glucose-induced hypothalamic ROS production (8). Treatment with icv trolox completely prevented apelin-induced hyperglycemia (Fig. 1A, B). All icv conditions did not modify food intake during re-feeding (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars) and plasma apelin levels (Supplementary Fig. S1B), demonstrating that (i) icv apelin did not modify the behavior of animals after injection and (ii) the specificity of central apelin action on glucose homeostasis.

Apelin stimulates hypothalamic ROS production in mice

The implication of ROS/ H_2O_2 in the control of glucose metabolism in normal and physiopathological states is well established (15). To determine whether hypothalamic apelin requires an H_2O_2 -dependent signaling pathway, our first approach used an indirect method of detection of H_2O_2 release by hypothalamus in response to apelin. We demonstrated that apelin significantly increased H_2O_2 release (Fig. 1C). Based on this result, we developed for the first time in hypothalamic explants a method using specific amperometric probes that was highly and specifically sensitive to H_2O_2 in order to detect its release in real time (54). As observed in Figure 1D, apelin rapidly increased hypothalamic H_2O_2 release from 2 to 20 min. This effect was completely blunted by trolox, thereby demonstrating the existence of an hypothalamic apelin/ H_2O_2 signaling pathway (Fig. 1D). It is well established that the majority of hypothalamic ROS implicated in rapid signaling pathway have a mitochondrial origin (3). Data from literature show that hypothalamic NADPH oxidase is implicated in the control of energy homeostasis (*i.e.*, food intake) *via* ROS production (19). To test whether NADPH oxidase could be a major enzymatic target of hypothalamic apelin action, we tested the effect of icv diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase, on glycemia. icv DPI injection failed to counterbalance hyperglycemia induced by icv apelin (Fig. 1E).

Central apelin controls glycemia via APJ receptor

Nowadays, there is only one known apelin receptor called APJ. To determine the specificity of icv apelin on glycemia, we treated mice with F13A, an APJ antagonist (26). Acute icv F13A injection blocked icv apelin-induced hyperglycemia (Fig. 1F).

Central apelin stimulates glycogenolysis and gluconeogenesis via hypothalamic ROS production and the ANS in mice

During fasting, the liver is the principal organ able to release glucose *via* the activation of glycogenolysis and/or gluconeogenesis. The brain, and more specifically the hypothalamus, contributes to the regulation of liver glucose metabolism during fasting *via* the ANS. To test whether

FIG. 1. Central apelin induces fasting hyperglycemia *via* hypothalamic hydrogen peroxide (H_2O_2) production in normal mice. **(A)** Effect of acute intracerebroventricular (icv) apelin injection on blood-glucose-fasted mice ($n=10$) compared with artificial cerebrospinal fluid (aCSF)-injected fasted mice ($n=9$), to Trolox-Apelin-injected fasted mice ($n=6$) and to Trolox-aCSF (Trolox)-injected fasted mice ($n=8$). **(B)** The average area under the curve (AUC) associated with the previous fasted blood glucose. ^a $p \leq 0.05$ Apelin *versus* Control; ^b $p \leq 0.01$ Apelin *versus* Control; ^c $p \leq 0.05$ Apelin *versus* Trolox-Apelin; ^d $p \leq 0.05$ Apelin *versus* Trolox; ^e $p \leq 0.001$ Apelin *versus* Control; ^f $p \leq 0.01$ Apelin *versus* Trolox; ^g $p \leq 0.01$ Apelin *versus* Trolox-Apelin. **(C)** *Ex vivo* hypothalamic H_2O_2 release measured by indirect enzymatic detection in fasted mice after injection of apelin ($n=10$) or krebs-ringer solution ($n=10$); ^{*} $p < 0.05$. **(D)** *Ex vivo* hypothalamic H_2O_2 release measured by real-time amperometric detection in fasted mice after injection of apelin ($n=5$) or aCSF ($n=5$) or Trolox-Apelin ($n=4$) or Trolox ($n=4$). ^a $p \leq 0.05$ Apelin *versus* Control; ^b $p \leq 0.05$ Apelin *versus* Trolox; ^c $p \leq 0.01$ Apelin *versus* Trolox-Apelin; ^d $p \leq 0.01$ Apelin *versus* Control; ^e $p \leq 0.01$ Apelin *versus* Trolox; ^f $p \leq 0.001$ Apelin *versus* Trolox-Apelin. **(E)** Effect of acute icv apelin injection on blood glucose-fasted mice ($n=10$) compared with aCSF-injected fasted mice ($n=9$), to diphenyliodonium (DPI)-Apelin-injected fasted mice ($n=6$) and to DPI-aCSF (DPI)-injected fasted mice ($n=6$). The adjacent bar graph represents the average AUC. ^a $p \leq 0.05$ Apelin *versus* Control; ^b $p \leq 0.01$ Apelin-DPI *versus* Control; ^c $p \leq 0.01$ Apelin *versus* Control; ^d $p \leq 0.01$ Apelin *versus* DPI; ^e $p \leq 0.05$ Apelin-DPI *versus* DPI; ^f $p \leq 0.001$ Apelin *versus* Control; ^g $p \leq 0.001$ Apelin-DPI *versus* Control; ^h $p \leq 0.05$ Apelin *versus* DPI; ⁱ $p \leq 0.01$ Apelin-DPI *versus* DPI; ^j $p \leq 0.05$ Apelin-DPI *versus* Control. **(F)** Effect of acute icv apelin injection on blood-glucose-fasted mice ($n=10$) compared with aCSF-injected fasted mice ($n=9$), to F13A-Apelin-injected fasted mice ($n=7$) and to F13A-aCSF (F13A)-injected fasted mice ($n=5$). The adjacent bar graph represents the average AUC. ^a $p \leq 0.05$ Apelin *versus* Control; ^b $p \leq 0.01$ Apelin *versus* Control; ^c $p \leq 0.05$ Apelin *versus* F13A; ^d $p \leq 0.05$ Apelin *versus* F13A-Apelin; ^e $p \leq 0.001$ Apelin *versus* Control; ^f $p \leq 0.01$ Apelin *versus* F13A-Apelin; ^g $p \leq 0.01$ Apelin *versus* F13A; ^h $p \leq 0.001$ Apelin *versus* F13A-Apelin.

hypothalamic apelin/ROS triggers fasted hyperglycemia by mechanisms involving the liver, we measured the consequence of icv apelin $\pm$ trolox on glycogenolysis and gluconeogenesis in fasted mice. An acute icv injection of apelin decreased hepatic glycogen content as observed by histological (Fig. 2A, B) and biochemical (Fig. 2C) measurements. Consistent with the effect observed in the hypothalamus, trolox treatment abolished the activation of hepatic glycogenolysis in apelin icv-injected mice (Fig. 2A–C). Then, in response to an intraperitoneal pyruvate injection, apelin significantly increased glycemia, demonstrating an activation of liver gluconeogenesis (Fig. 2D). Similar to glycogenolysis, icv apelin effect on gluconeogenesis is blocked by icv trolox.

Numerous enzymes are implicated in the control of glycogenolysis and/or gluconeogenesis. Among them, the activity of glucose 6-phosphatase (G6Pase), a key enzyme that is known to stimulate glycogenolysis and gluconeogenesis (1), was significantly increased in the liver of icv apelin-treated mice (Fig. 2E). This effect was blocked by icv trolox. No significant variation of phosphoenolpyruvate carboxykinase (PEPCK), glycogen synthase, and glycogen phosphorylase activities was observed in all experimental conditions (Fig. 2F–H). The effects of icv apelin on glucose metabolism were not associated to the modification of plasma triglycerides (TG) (Supplementary Fig. S2A), free fatty acid (FFA) (Supplementary Fig. S2B), ketone bodies (Supplementary Fig. S2C), liver TG (Supplementary Fig. S2D), and lipid (Supplementary Fig. S2E) content.

Given that the sympathetic nervous system (SNS) controls glycogenolysis and gluconeogenesis, we measured norepinephrine release in the blood of icv apelin $\pm$ trolox-treated mice as an index of SNS activation. Norepinephrine levels were significantly higher in response to icv apelin than control mice, but not in icv apelin/trolox or trolox-alone treated mice (Fig. 3A). No significant variation of plasma epinephrine was observed (Fig. 3B). Alpha(1) adrenergic receptors are the principle receptors implicated in the control of hepatic glucose metabolism in mice (28). An intraperitoneal injection of prazosin, an alpha(1) adrenergic receptors antagonist, prevented icv apelin-induced hyperglycemia (Fig. 3C), thereby demonstrating the existence of a central apelin-SNS-liver signaling pathway. In addition to an alpha(1) adrenergic receptors-mediated effect on glycemia, activation of SNS by icv apelin is associated to an increase in plasma glucagon release (Fig. 3D), which further stimulates hepatic glucose production (41).

Apelin over-expression in the medio-basal hypothalamus stimulates hepatic glycogenolysis and gluconeogenesis

We have previously demonstrated that the medio-basal hypothalamus (MBH) is a hypothalamic target of apelin action on peripheral glucose homeostasis, as c-Fos expression is increased in the MBH of hyperglycemic apelin icv-injected mice (11). Moreover, electrical stimulation of ventro-median hypothalamus stimulates hepatic glucose release (48). To determine whether apelin controls liver function *via* an action on MBH, we injected apelin-expressing lentivectors specifically in the MBH of normal mice. Four weeks after infection, the level of apelin mRNA was significantly increased in the MBH of mice that received apelin-expressing lentivectors (Fig. 4A). The relative expression of apelin mRNA was increased by about 1.5-fold, similar to that observed at the protein level in

obese/diabetic mice (42), demonstrating that this over-expression of apelin in normal mice corresponds to the level found in pathological conditions. To identify the impact on glucose homeostasis, we performed insulin tolerance test (ITT). Lentivectors apelin-infected mice presented fasted hyperglycemia and an insulin intolerance typical features of type 2 diabetes (Fig. 4B). This metabolic phenotype was associated to an increase in TNFalpha mRNA expression in the hypothalamus of lentivectors apelin-infected mice (Fig. 4C), suggesting an increase in hypothalamic inflammation that is clearly associated to a diabetic state (51); whereas hypothalamic IL1beta mRNA tends to increase in lentivectors apelin-infected mice, but did not reach significance ($p=0.074$, Fig. 4D). This inflammatory state was specific to the hypothalamus, as we did not observe any modification of mRNA TNFalpha and IL1beta expressions in the liver and adipose tissues (Supplementary Fig. S3C–H). In this chronic apelin expressed model, we showed no significant difference in plasma apelin (Supplementary Fig. S4F) and liver apelin/APJ mRNA expressions (Supplementary Fig. S3A, B) between the two experimental groups, suggesting that central control of apelin did not affect its own release in periphery.

Then, we measured the impact of apelin over-expression in the MBH on liver glucose metabolism. Hypothalamic apelin over-expression decreased liver glycogen content (Fig. 5A–C) and increased gluconeogenesis (Fig. 5D). This metabolic phenotype was associated to an increase in G6Pase activity during fasted state (Fig. 5E), but not PEPCK (Fig. 5F), glycogen synthase (Fig. 5G), and glycogen phosphorylase (Fig. 5H). No significant modification of plasma TG (Supplementary Fig. S4A), FFA (Supplementary Fig. S4B), ketone bodies (Supplementary Fig. S4C), liver TG (Supplementary Fig. S4D), and lipid content (Supplementary Fig. S4E) was observed between the two groups. This result clearly demonstrates that the diabetic state generated by apelin over-expression in the MBH is not associated to the modification of lipid metabolism, but specifically links to direct alteration of glucose metabolism.

Similar to that observed in response to an acute apelin injection (Fig. 3A, B), apelin over-expression in the MBH was associated to an increase in plasma norepinephrine (Fig. 6A), but not epinephrine (Fig. 6B), showing the over-activation of SNS. On the contrary, we did not observe any significant difference in plasma glucagon (Fig. 6C), suggesting that SNS over-activation coupled to modification of G6Pase activity was sufficient to generate a diabetic state.

Antioxidants counterbalanced icv apelin-induced fasted hyperglycemia in obese/diabetic mice

We previously found that glycemia of HFD obese/diabetic mice could further increase in response to acute icv apelin injection (11), suggesting that the hypothalamic to liver axis is functionally still able to respond to a central stimulation by releasing hepatic glucose. Here, we showed that icv trolox completely abolished the hyperglycemic effect of apelin in HFD mice (Fig. 7A, B), suggesting that the hypothalamic apelin/ROS pathway was still effective.

Apelin amplifies the H₂O₂ release by the hypothalamus of obese/diabetic mice

The hypothalamic ROS signaling pathway is profoundly altered in obese/diabetic mice participating in an insulin-

FIG. 2. Central apelin stimulates glycogenolysis and gluconeogenesis via hypothalamic reactive oxygen species (ROS) in normal mice. (A) Histological visualization of hepatic glycogen after acute icv apelin injection on fasted mice ($n=5$) compared with aCSF-injected fasted mice ($n=5$), to Trolox-Apelin-injected fasted mice ($n=4$), and to Trolox-aCSF (Trolox)-injected fasted mice ($n=5$). Scale bar: 200 μm . (B) Quantification of coloration associated with the previous histological visualization. (C) Effect of acute icv apelin injection on hepatic glycogen content of fasted mice ($n=12$) compared with aCSF-injected fasted mice ($n=13$), to Trolox-Apelin-injected fasted mice ($n=12$), and to Trolox-aCSF (Trolox)-injected fasted mice ($n=7$). $^{**}p<0.01$ versus Apelin; $^{***}p<0.001$ versus Apelin. (D) Effect of acute icv apelin injection on gluconeogenic activity of fasted mice ($n=8$) compared with aCSF-injected fasted mice ($n=8$), to Trolox-Apelin-injected fasted mice ($n=10$) and to Trolox-aCSF (Trolox)-injected fasted mice ($n=6$). The adjacent bar graph represents the average AUC. $^ap\leq 0.01$ Apelin versus Control; $^bp\leq 0.001$ Apelin versus Control; $^cp\leq 0.01$ Apelin versus Trolox-Control; $^dp\leq 0.01$ Apelin versus Trolox; $^ep\leq 0.05$ Apelin versus Trolox-Apelin; $^fp\leq 0.05$ Apelin versus Control; $^gp\leq 0.05$ Apelin versus Trolox. (E) Effect of acute icv apelin injection on hepatic glucose-6-phosphatase (G6Pase) activity-fast mice ($n=5$) compared with aCSF-injected fasted mice ($n=5$), to Trolox-Apelin-injected fasted mice ($n=5$) and to Trolox-aCSF (Trolox)-injected fasted mice ($n=6$). $^*p<0.05$ versus Apelin; $^{**}p<0.01$ versus Apelin. (F) Effect of acute icv apelin injection on hepatic phosphoenolpyruvate carboxykinase (PEPCK) activity-fast mice ($n=5$) compared with aCSF-injected fasted mice ($n=4$), to Trolox-Apelin-injected fasted mice ($n=4$) and to Trolox-aCSF (Trolox)-injected fasted mice ($n=6$). (G) Effect of acute icv apelin injection on ratio of phosphorylated hepatic glycogen synthase/total hepatic glycogen synthase on fasted mice ($n=3$) compared with aCSF-injected fasted mice ($n=3$), to Trolox-Apelin-injected fasted mice ($n=3$) and to Trolox-aCSF (Trolox)-injected fasted mice ($n=3$). (H) Effect of acute icv apelin injection on hepatic glycogen phosphorylase activity-fast mice ($n=4$) compared with aCSF-injected fasted mice ($n=4$), to Trolox-Apelin-injected fasted mice ($n=4$) and to Trolox-aCSF (Trolox)-injected fasted mice ($n=4$).

FIG. 3. Central apelin stimulates glycogenolysis and gluconeogenesis *via* autonomous nervous system (ANS) activation in normal mice. **(A)** Effect of acute icv apelin injection on blood norepinephrine-fasted mice ($n=7$) compared with aCSF-injected fasted mice ($n=9$), to Trolox-Apelin-injected fasted mice ($n=7$) and to Trolox-aCSF (Trolox)-injected fasted mice ($n=8$). $^{**}p < 0.01$ versus Apelin. **(B)** Effect of acute icv apelin injection on blood epinephrine-fasted mice ($n=7$) compared with aCSF-injected fasted mice ($n=9$), to Trolox-Apelin-injected fasted mice ($n=7$) and to Trolox-aCSF (Trolox)-injected fasted mice ($n=8$). **(C)** Effect of acute icv apelin injection on blood glucose-fasted mice ($n=11$) compared with aCSF-injected fasted mice ($n=8$), to intraperitoneal injection of prazosin before apelin icv injection (Prazosin-Apelin) on fasted mice ($n=6$), and to intraperitoneal injection of prazosin before aCSF icv injection (Prazosin) on fasted mice ($n=6$). The adjacent bar graph represents the average AUC. $^ap \leq 0.05$ Apelin versus Control; $^bp \leq 0.01$ Apelin versus Control; $^cp \leq 0.05$ Apelin versus Prazosin; $^dp \leq 0.05$ Apelin versus Prazosin-Apelin; $^ep \leq 0.001$ Apelin versus Control; $^fp \leq 0.01$ Apelin versus Prazosin; $^gp \leq 0.001$ Apelin versus Prazosin; $^hp \leq 0.01$ Apelin versus Prazosin-Apelin. **(D)** Effect of acute icv apelin injection on blood glucagon-fasted mice ($n=4$) compared with aCSF-injected fasted mice ($n=5$), to Trolox-Apelin-injected fasted mice ($n=5$) and to Trolox-aCSF (Trolox)-injected fasted mice ($n=4$). $^*p < 0.05$ versus Apelin.

resistant state (15). Similar to that performed in normal mice, we measured real-time H_2O_2 release in response to apelin on hypothalamic explants from HFD mice. In this pathological model, apelin stimulated an exacerbated H_2O_2 release in the hypothalamus, and this was counterbalanced by trolox treatment (Fig. 7C).

Central apelin stimulates glycogenolysis but not gluconeogenesis *via* hypothalamic ROS in HFD mice

The liver is one of the most important organ in glucose homeostasis whose activity is deeply disturbed during metabolic diseases (36). Indeed, previous data demonstrate that

gluconeogenic activity of the liver of obese/diabetic mice is exacerbated (55). We demonstrated that HFD mice are able to increase quickly fasted glycemia (Fig. 7A, B) on apelin treatment; this corresponds to the activation of glycogenolysis but not gluconeogenesis (45–48). In accordance to glycemia, hepatic glycogenolysis was activated by icv apelin and blocked when icv trolox was co-injected (Fig. 8A–C). Acute icv apelin injection in HFD mice did not significantly modify gluconeogenic function in the liver (Fig. 8D). The decrease of glycogen content was mirrored by an increase in liver G6Pase activity (Fig. 8E) without modification of PEPCK, glycogen phosphorylase, and glycogen synthase (Supplementary Fig. S5A–C). Plasma norepinephrine, epinephrine, and glucagon

FIG. 4. Apelin over-expression into the medio-basal hypothalamus (MBH) generates insulin intolerance state. (A) Relative expression of apelin mRNA in hypothalamus of lenti-Apelin mice ($n=4$) compared with lenti-GFP mice ($n=5$), $*p<0.05$. **(B)** Insulin tolerance test (ITT) in 6-h-fasted lenti-Apelin mice ($n=5$) compared with lenti-GFP mice ($n=5$), $*p<0.05$. Two-way ANOVA followed by *t*-test *post hoc* reveals time ($***p<0.001$) and treatment ($$$$p<0.001$) effects. The adjacent bar graph represents the average AUC. **(C)** Relative expression of TNF α mRNA in hypothalamus of lenti-Apelin mice ($n=4$) compared with lenti-GFP mice ($n=5$), $*p<0.05$. **(D)** Relative expression of IL1 β mRNA in hypothalamus of lenti-Apelin mice ($n=4$) compared with lenti-GFP mice ($n=5$).

concentrations did not vary in our experimental conditions (Fig. 8G, H).

Blockade of central apelin ameliorates diabetic state of HFD mice

Our data show that a high level of central apelin participates in the establishment of a type 2 diabetes. To test whether blockade of apelin action in the hypothalamus could be a major target to restore a fasted normal glycemia, we have daily injected icv mice with an APJ antagonist, F13A, during 2 weeks after 3 months of HFD. Our data show that icv F13A decreased fasted hyperglycemia and insulin intolerance in HFD mice (Fig. 9A). HFD mice presented a decrease in glycogen content (Fig. 9B–D) at a level similar to that measure in normal mice (Fig. 2C), demonstrating an amelioration of the diabetic state in HFD mice. This phenotype is associated to an increase in liver glycogen phosphorylase activity, which could explain the low level of glycogen store (Fig. 9E). The activities of G6Pase, PEPCK and glycogen synthase did not vary between the two groups (Supplementary Fig. S6A–C). Plasma norepinephrine and epinephrine did not vary between the two groups of mice (Fig. 9F, G). Plasma glucagon concentration was decreased in F13A mice as compared with control HFD mice (Fig. 9H), thereby suggesting that chronic central apelin blockade improves glucose homeostasis regulation during diabetic state.

Discussion

Hypothalamic apelin is implicated in the control of peripheral glucose utilization in normal and pathological con-

ditions (11). In the present study, we have identified for the first time the hypothalamic molecular signaling pathway implicated in deleterious effect of apelin. Moreover, we report that the couple apelin/ROS in the hypothalamus controls hepatic glucose production *via* the SNS to induce peripheral hyperglycemia.

The role of central apelin in the control of glucose homeostasis is poorly defined. We were the first to demonstrate that central apelin may have beneficial or deleterious effects on glucose homeostasis depending on the injected dose and/or the nutritional state (11). Indeed, an injection of a low dose of icv apelin induces an increase in glucose tolerance in normal mice. This result suggests that the slight increase of plasma apelin concentration observed in fed state in normal mice may reach the hypothalamus, a neurohemal structure which facilitates communications with peripheral hormones (25), to decrease glycemia in fed state (11). On the contrary, obese/diabetic mice fed a HFD show a disruption of circadian apelin regulation (11). Then, over-increased hypothalamic apelin expression observed in obese/diabetic (11, 42) is associated to abnormal regulation of glycemia (11). To reinforce this hypothesis, an icv injection of a high dose of apelin (used in this study and similar to that observed in obese/diabetic mice) in normal mice induces fasted hyperglycemia and insulin resistance (11), characterizing a type 2 diabetes. The liver is the main organ that is able to release glucose in fasted state *via* glycogenolysis and/or gluconeogenesis (30). Thus, we focused our attention on the hypothalamic apelin to liver axis in normal and physiopathological conditions. In all the models investigated, central apelin stimulated hepatic glucose

FIG. 5. Apelin overexpression into the MBH stimulates hepatic glycogenolysis and gluconeogenesis. (A) Histological visualization of hepatic glycogen of fasted Lenti-Apelin mice ($n=4$) compared with fasted Lenti-GFP mice ($n=5$). Scale bar: 200 μm . (B) Quantification of coloration associated with the previous histological visualization. $**p < 0.01$. (C) Hepatic glycogen content of fasted Lenti-Apelin mice ($n=5$) compared with fasted Lenti-GFP mice ($n=5$). $*p < 0.05$. (D) Gluconeogenic activity of fasted Lenti-Apelin mice ($n=9$) compared with fasted Lenti-GFP mice ($n=9$). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Two-way ANOVA followed by t -test *post hoc* reveals time ($***p < 0.001$) and treatment ($$$$p < 0.01$) effects. (E) Hepatic G6Pase activity in fasted Lenti-Apelin mice ($n=7$) compared with fasted Lenti-GFP mice ($n=9$). $**p < 0.01$. (F) Hepatic PEPCK activity in fasted Lenti-Apelin mice ($n=8$) compared with fasted Lenti-GFP mice ($n=8$). (G) Ratio of phosphorylated hepatic glycogen synthase/total hepatic glycogen synthase in fasted Lenti-Apelin mice ($n=3$) compared with fasted Lenti-GFP mice ($n=3$). (H) Hepatic glycogen phosphorylase activity in fasted Lenti-Apelin mice ($n=5$) compared with fasted Lenti-GFP mice ($n=5$).

production and release *via* glycogenolysis and/or gluconeogenesis. Importantly, the previously described kinetics of glucose release by the liver (glycogenolysis within minutes; gluconeogenesis within hours) (45, 46, 48) clearly fits with our two waves of increased glucose release induced by icv apelin injections. The only exception of this concept is observed in HFD mice, where an icv apelin injection did not modify gluconeogenesis (Fig. 8D). This result is in accordance with data from literature showing that liver gluconeogenesis is overactivated during metabolic disorders (55), leading to hy-

pothesize that icv apelin treatment is not able to stimulate more than possible the hepatic glucose production. Moreover, these results could be correlated to the absence of stimulatory action of icv leptin on liver gluconeogenesis in over-feeding rats as opposed to normal chow-fed rats (34). In our experimental procedure, it is difficult to evaluate the potential variation of glycogenic and/or gluconeogenic enzymes activities in response to icv apelin. It is worth noting that the electrical stimulation of the hypothalamus stimulates hepatic glucose release in a few minutes, but the kinetic of activation of

FIG. 6. Apelin over-expression into the MBH stimulates hepatic glycogenolysis and gluconeogenesis *via* ANS activation. (A) Plasma norepinephrine in fasted lenti-Apelin mice ($n=5$) compared with fasted lenti-GFP mice ($n=5$); * $p<0.05$. (B) Plasma epinephrine in fasted lenti-Apelin mice ($n=5$) compared with fasted lenti-GFP mice ($n=5$). (C) Plasma glucagon in fasted lenti-Apelin mice ($n=4$) compared with fasted lenti-GFP mice ($n=4$).

enzymes precedes the effect. In fact, in response to hypothalamic stimulation, the activities of G6Pase in the liver are increased in ~ 30 s (46). However, in a longer period of time, a sustained electrical stimulation of the ventro-median hypothalamus (20 s periods once every 3 min for 4 h) increases liver PEPCK activity after 120 min (49). Whether an acute stimulation of hypothalamic nuclei in response to icv apelin

modifies enzymes activities in the liver within seconds or minutes remains to be determined. Our data suggest a kinetic of activation closed to this, as variations of glycemia are observed from 15 to 210 min in our protocol. For this reason, we evaluate the activities of four enzymes implicated in the control of glycogenolysis and/or gluconeogenesis in the liver in all experimental conditions after 30 min of icv treatment.

FIG. 7. Central apelin stimulates fasting hyperglycemia *via* H₂O₂ hypothalamic production in obese and diabetic mice. (A) Effect of acute icv apelin injection on blood glucose fasted high-fat diet (HFD) mice ($n=10$) compared with aCSF-injected fasted HFD mice ($n=9$), to Trolox-Apelin-injected fasted HFD mice ($n=10$) and to Trolox-aCSF (Trolox)-injected fasted HFD mice ($n=6$). (B) The average AUC associated with the previous fasted blood glucose. ^a $p\leq 0.001$ Apelin *versus* Control; ^b $p\leq 0.05$ Apelin *versus* Trolox-Apelin; ^c $p\leq 0.05$ Apelin *versus* Trolox; ^d $p\leq 0.01$ Apelin *versus* Control; ^e $p\leq 0.01$ Apelin *versus* Trolox; ^f $p\leq 0.01$ Apelin *versus* Trolox-Apelin. (C) *Ex vivo* hypothalamic H₂O₂ release measured by real-time amperometric detection in fasted HFD mice after injection of apelin ($n=5$) or aCSF ($n=5$) or Trolox-Apelin ($n=4$) or Trolox-aCSF ($n=4$). ^a $p\leq 0.05$ Apelin *versus* Control; ^b $p\leq 0.05$ Apelin *versus* Trolox-Apelin; ^c $p\leq 0.01$ Apelin *versus* Trolox; ^d $p\leq 0.01$ Apelin *versus* Control; ^e $p\leq 0.01$ Apelin *versus* Trolox-Apelin; ^f $p\leq 0.001$ Apelin *versus* Trolox; ^g $p\leq 0.001$ Apelin *versus* Trolox-Apelin.

FIG. 8. Central apelin stimulates glycogenolysis via hypothalamic ROS release and ANS activation in obese and diabetic mice. (A) Histological visualization of hepatic glycogen after acute icv apelin injection on fasted HFD mice ($n=6$) compared with aCSF-injected fasted HFD mice ($n=6$), to Trolox-Apelin-injected fasted HFD mice ($n=5$) and to Trolox-aCSF (Trolox)-injected fasted HFD mice ($n=4$); scale bar: 200 μm . (B) Quantification of coloration associated with the previous histological visualization. $*p < 0.05$ versus Apelin; $**p < 0.01$ versus Apelin. (C) Effect of acute icv apelin injection on hepatic glycogen content of fasted HFD mice ($n=6$) compared with aCSF-injected fasted HFD mice ($n=7$), to Trolox-Apelin-injected fasted HFD mice ($n=13$), and to Trolox-aCSF (Trolox)-injected fasted HFD mice ($n=4$). $*p < 0.05$ versus Apelin; $**p < 0.01$ versus Apelin. (D) Effect of acute icv apelin injection on gluconeogenic activity of fasted HFD mice ($n=7$) compared with aCSF-injected fasted HFD mice ($n=6$), to Trolox-Apelin-injected fasted HFD mice ($n=5$) and to Trolox-aCSF (Trolox)-injected fasted HFD mice ($n=4$). The adjacent bar graph represents the average AUC. (E) Effect of acute icv apelin injection on hepatic G6Pase activity-fastest HFD mice ($n=4$) compared with aCSF-injected fasted HFD mice ($n=4$), to Trolox-Apelin injected in fasted HFD mice ($n=4$) and to Trolox-aCSF (Trolox)-injected fasted HFD mice ($n=4$). $*p < 0.05$ versus Apelin. (F) Effect of acute icv apelin injection on blood norepinephrine in fasted HFD mice ($n=7$) compared with aCSF-injected fasted HFD mice ($n=9$), to Trolox-Apelin-injected fasted HFD mice ($n=7$) and to Trolox-aCSF (Trolox)-injected fasted HFD mice ($n=4$). (G) Effect of acute icv apelin injection on blood epinephrine in fasted HFD mice ($n=7$) compared with aCSF-injected fasted HFD mice ($n=9$), to Trolox-Apelin-injected fasted HFD mice ($n=7$) and to Trolox-aCSF (Trolox)-injected fasted HFD mice ($n=4$). (H) Effect of acute icv apelin injection on blood glucagon in fasted HFD mice ($n=4$) compared with aCSF-injected fasted HFD mice ($n=5$), to Trolox-Apelin-injected fasted HFD mice ($n=4$), and to Trolox-aCSF (Trolox)-injected fasted HFD mice ($n=5$).

FIG. 9. Blockade of central apelin ameliorates diabetic state in HFD mice. (A) ITT in 6-h-fasted HFD mice with F13A chronic treatment ($n=6$) compared with HFD mice with aCSF chronic treatment ($n=5$), $*p<0.05$, $**p<0.01$. Two-way ANOVA followed by t -test *post hoc* reveals time ($***p<0.001$) and treatment ($*p<0.05$) effects. The adjacent bar graph represents the average AUC. **(B)** Histological visualization of hepatic glycogen of fasted HFD mice with F13A chronic treatment ($n=5$) compared with HFD mice with aCSF chronic treatment ($n=4$). Scale bar: 200 μ m. **(C)** Quantification of coloration associated with the previous histological visualization. $*p<0.05$. **(D)** Hepatic glycogen content of fasted HFD mice with F13A chronic treatment ($n=5$) compared with HFD mice with aCSF chronic treatment ($n=4$). $*p<0.05$. **(E)** Hepatic glycogen phosphorylase activity in fasted HFD mice with F13A chronic treatment ($n=5$) compared with HFD mice with aCSF chronic treatment ($n=4$). $*p<0.05$. **(F)** Plasma norepinephrine in fasted HFD mice with F13A chronic treatment ($n=6$) compared with HFD mice with aCSF chronic treatment ($n=5$). **(G)** Plasma epinephrine in fasted HFD mice with F13A chronic treatment ($n=6$) compared with HFD mice with aCSF chronic treatment ($n=5$). **(H)** Plasma glucagon in fasted HFD mice with F13A chronic treatment ($n=5$) compared with HFD mice with aCSF chronic treatment ($n=3$). $*p<0.05$.

Our data show that the central apelin effect on glycemia is associated to an increase in G6Pase activity in the liver, an enzyme which is known to be implicated in the control of glycogenolysis and gluconeogenesis, which is in close correlation with hyperglycemia. In spite of the absence of variations in PEPCK, glycogen synthase, and glycogen phosphorylase in normal conditions, lentivectors expressed apelin or HFD mice models, we cannot totally exclude an implication of those enzymes on hepatic glucose production in response to icv apelin. Indeed, due to a large range of time

of kinetics of glycemia (from 0 to 210 min) which could make us miss interesting results, it will be easy to speculate that specific variations of one or more types of enzymes may vary during this time-course period.

The importance of ROS in the pathological state is well documented. Obese and insulin-resistant rodents present a profoundly altered hypothalamic mROS signaling (8). Indeed, metabolic abnormal responses are in close correlation with increased hypothalamic mROS level, leading to a constitutive oxidized environment at both the cellular and

mitochondrial levels associated to a dysfunction in mitochondrial respiration (8). Here, we found that hypothalamic action of apelin needs an ROS/H₂O₂-dependent production to stimulate hepatic glucose release to induce fasted hyperglycemia. Using specific probes that detect H₂O₂ in real time, we demonstrate that exogenous apelin applied on hypothalamic explants from normal mice stimulates H₂O₂ release. Interestingly, this H₂O₂ release is exacerbated in hypothalamic explants from HFD mice. Those data are in accordance with literature linking high levels of hypothalamic mROS, and, in particular H₂O₂, are deleterious for the control of glucose homeostasis, and, more specifically, in diabetic models (23). Indeed, mitochondrial respiration that is considered the primary source of H₂O₂ (3) is profoundly increased in the hypothalamus of diabetic rodents in association with high levels of ROS (8). Our original approach to measure H₂O₂ is used here for the first time in *ex vivo* hypothalami and gives access to a new technology to determine the exact quantity of H₂O₂ release directly in tissue, which is of crucial importance to unravel the role of ROS in the biological process (32). Then, levels of H₂O₂ measured in our experimental conditions reinforced the hypothesis that an increase in H₂O₂ release by tissue is clearly associated to a pathological state (53).

In spite of the fact that apelin is known to have an antioxidant effect (16), some publications reveal that apelin may be considered a new oxidative marker. First, Yu *et al.* (58) suggest that high levels of plasma apelin observed in obese/diabetic patients may be not only the results of compensatory response to insulin resistance, but also a causative factor of insulin resistance. Then, apelin can participate in the establishment of an inflammatory and oxidative stress state observed in metabolic disorders. In neuronal cell culture, exogenous apelin treatment increases ROS production (57). Our data show that apelin effects on hepatic glucose metabolism are mediated by ROS in *in vivo* and *ex vivo* conditions. Such a deleterious environment caused by apelin in the hypothalamus may be one of the first signals triggering a diabetic state, that is, fasted hyperglycemia. The hypothesis of the existence of a link between apelin and inflammation is reinforced by our results demonstrating that apelin over-expression in the ventromedian hypothalamus increase the mRNA expression of the pro-inflammatory marker TNF α in the hypothalamus. This specific variation in hypothalamic inflammation generated by apelin, without modification of peripheral inflammation, is responsible for the emergence of a type 2 diabetic state, including fasted hyperglycemia and insulin intolerance (6). This diabetic phenotype is clearly linked with an increase in (i) G6Pase activity in the liver and (ii) plasma norepinephrine concentration. Then, alteration of hypothalamic signaling (*i.e.*, ROS and inflammation) by apelin leads to type 2 diabetes characteristics.

The role of ANS in the control of glucose homeostasis is well established. Metabolic disorders are characterized by increased basal SNS activity but an impaired sympathetic response to various stimuli, such as insulin (18). Here, we show that basal norepinephrine level is increased in hypothalamic apelin over-expressing mice, suggesting an increase in basal SNS tone activity. These data are in accordance with those of Masaki *et al.* (27), who demonstrate that a microinjection of apelin into the paraventricular nucleus of the hypothalamus increases sympathetic nerve activity.

Norepinephrine can exert its action in the whole body *via* α (1–2) and/or β (1–2) receptors. In the liver of adult rodents, hepatic glucose production is regulated *via* α (1) receptors (28), as (i) blockade of α (2) receptors did not modify glycogenolysis (31), and (ii) β receptors become irrelevant in the livers of mice at adulthood (13). Our data confirm the importance of α (1) regulatory pathway of glycemia, as blockade of α (1) adrenergic receptors failed to increase icv apelin-induced hyperglycemia. Whether SNS activation occurs early in the process or secondary to long-standing metabolic syndrome is unclear, but our data obtained after an icv acute injection of apelin strongly supports the first hypothesis. Indeed, Prior *et al.* (37) show that a short-term HFD (4 weeks) drives toward sympathetic activation associated to an increase in visceral fat depot. On the contrary, we were not able to observe a significant increase in plasma norepinephrine levels in our 3 months HFD mice. The logical explanation of this result is the fact that over time, with obesity, adrenoreceptors down-regulation and/or reduced sensitivity are likely to develop in peripheral tissue, resulting in reduced sympathetic responsiveness (18). Then, this possibility could also explain the absence of activation of gluconeogenesis but not glycogenolysis in HFD mice.

Additional to SNS regulation, hepatic glucose production is under the influence of glucagon (41). Here, we show that acute icv apelin stimulates plasma glucagon secretion in normal mice, suggesting that glucagon is able to participate to fasted hyperglycemia. In accordance to our results, the group of Cherrington clearly demonstrates that in response to a physiological rise in glucagon, the increase observed in hepatic glucose production is entirely attributable to glycogenolysis but not (or little to quote the authors) to gluconeogenesis (41). Then, glucagon could be one important molecular actor participating in deleterious central action of apelin during the onset of a type 2 diabetes. In longer time, when type 2 diabetes is well established, icv apelin cannot increase glucagon secretion as observed in HFD mice, suggesting that hyperglucagonemia of those diabetic mice is too important to be even more increased. For all these reasons, therapeutic strategies cannot rule out the importance of pancreatic α cells as potential good targets to treat hyperglycemia and insulin resistance (5). In our central apelin to liver axis, the participation of glucagon to hyperglycemia is real, but lentivectors approach tends to focus on a direct norepinephrine action on liver, as plasma norepinephrine is increased in lentivectors apelin mice, but not glucagon.

Our data show that central apelin participates in the establishment of type 2 diabetes. We have previously shown that obese/diabetic mice present an increase in plasma apelin (11) and in hypothalamic apelin expression (42). The hypothalamus is a neurohemal structure that facilitates communication with peripheral signal, including hormones and metabolites. Then, in physiological and pathological conditions, the hypothalamus may be influenced by hypothalamic and/or peripheral apelin. To test whether targeting central apelin in an advanced type 2 diabetes could ameliorate the metabolic phenotype, we have injected obese/diabetic mice (3 months HFD) during 14 consecutive days with icv APJ antagonist. This experimental approach blocks the potential effect of hypothalamic and peripheral apelin on hypothalamic neurons. Then, we found that icv APJ antagonist-treated mice present a clear amelioration of their diabetic state, including a decrease in insulin

intolerance, fasted hyperglycemia, plasma glucagon, and liver glycogen content associated to an increase in glycogen phosphorylase activity. No significant variation in plasma norepinephrine and epinephrine was observed between the two groups, reinforcing the hypothesis of a possible adrenoceptor down-regulation in specific tissue and/or reduced sympathetic responsiveness as explained next. These data confirm that the decrease in glycogen content and fasted hyperglycemia is the result of an increase in insulin sensitivity provoked by blockade of central apelin. We can speculate that a longer time treatment may modify the activities of others targets such as G6Pase, PEPCK, and glycogen synthase. In fact, we show that in a well-established diabetic models (HFD), central apelin may be a potential target to treat diabetes, with a similar peripheral target to prediabetic mice (mimicked by icv apelin injected mice and apelin expressed lentivirals mice), that is, the liver.

Although the present set of experiments link hypothalamic apelin/ROS with hepatic glucose metabolism, we acknowledge that the exact molecular and cellular mechanisms implicated in the cross-talks observed between these key organs remained to be elucidated.

In conclusion, we identify a hypothalamic apelin/ROS signaling pathway that is implicated in the control of liver glucose metabolism *via* the SNS. Abnormal hypothalamic level of apelin observed in obese/diabetic mice participates in over-activation of SNS, which improves fasted hyperglycemia a characteristic of type 2 diabetes. Thus, we propose that central apelin could be one new target at the core of the regulation of glucose homeostasis and metabolic disorders.

Materials and Methods

Mice

Animals were handled in accordance with the principles and guidelines established by the National Institute of Medical Research (INSERM) and by the local ethical committee. C57Bl6/J mice were obtained from Charles River Laboratory. Mice were housed conventionally at a constant temperature (20°C–22°C) and humidity (50%–60%) animal room and with a 12/12 h light/dark cycle (lights off at 7:00 AM) and free access to food and water through the 24 h period. All injections and experiments were performed in 13–15-week-old males. A group of male mice was subjected to HFD containing 20% protein, 35% carbohydrate, and 45% fat (SAFE). HFD-fed mice were followed at regular intervals with measure of weight and blood parameters (glucose, insulin) until they were obese and insulin resistant corresponding to 3 months of HFD. Experiments were performed on 6 h-fasted mice. During the time of the experiment, mice fed overnight were placed in a new clean cage without food. In all experiments, icv apelin did not modify body weight of mice (11) and general behavior (unpublished data).

Plasmid construction and lentivector production

The lentiviral plasmid pTRIP-DU3-CMV-MCS was derived from the pTRIP-DU3-EF1a-EGFP plasmid by replacing the EF1a promoter and EGFP by the cytomegalovirus (CMV) promoter and a multiple cloning site (MCS), respectively (50). The cDNA encoding the murine apelin gene (EMBL accession number AJ290423), corresponding to the coding region flanked by 100 and 23 nucleotides of 5'- and 3'-untranslated regions, respectively, was subcloned into the lentivector pTRIP-DU3-

CMV-MCS (43). The lentivectors were produced by tri-transfection of pTRIP-DU3-CMV-apelin and pLvPack and pLvVSVg packaging plasmids (Sigma-Aldrich) in HEK 293FT cells (4).

Surgical procedures

For *in vivo* acute perfusions, an indwelling icv catheter (Alzet Brain Perfusion Kit 3; 1–3 mm (Charles River Laboratory) – 1 mm lateral to the sagittal suture, – 0.2 mm posterior to the bregma and – 1.7 mm below the skull surface) was implanted in anesthetized mice with isoflurane (Abbott).

Acute injections

Apelin. Bolus injection of 2 μ l of [Pyr]apelin-13 (concentration 20 nM corresponding to 40 fmol; Bachem), the most active apelin isoform (10), was injected directly into the icv catheter completed to 2 μ l of artificial cerebrospinal fluid (aCSF) for treated mice, or 4 μ l of aCSF for control mice. Blood glycemia was measured every 30 min from – 30 to 210 min (time 0 corresponding to icv apelin injection). The dose of apelin (2 μ l of 20 nM, *i.e.*, 40 fmol) corresponds approximately to the half quantity of total tissular apelin found in a normal mouse hypothalamus (near to 74 fmol per hypothalamus), and this injection leads approximately to the quantity found in an HFD mouse (near to 111 fmol per hypothalamus).

Antioxidant. Similar experiments were performed with an antioxidant, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; Sigma), which was dissolved extemporaneously in aCSF, and then infused at a concentration of 1 mM (2 μ l). At this concentration, trolox is known to prevent peripheral hyperinsulinemia by blocking hypothalamic ROS production *in vivo* (24). Trolox infusion was started 30 min before the beginning of the apelin injection.

Inhibitor of NADPH oxidase. Similar experiments were performed with an inhibitor of NADPH oxidase, DPI chloride (Sigma), which was dissolved extemporaneously in aCSF, and then infused at a concentration of 1 mM (2 μ l). At this concentration, DPI inhibits NADPH oxidase in the brain (19). DPI infusion was started 30 min before the beginning of the apelin injection.

APJ antagonist. Similar experiments were performed with an APJ antagonist, F13A (polypeptide) (22), which was dissolved extemporaneously in aCSF, and then infused at a concentration of 2 μ g/mouse (2 μ l). At this concentration, F13A inhibits apelin effects in the brain (26). F13A infusion was started 30 min before the beginning of the apelin injection.

Intraperitoneal injection of antagonist of α 1-adrenergic receptors. 120 min before apelin or aCSF icv injection, prazosin (Prazosin hypochloride; Sigma) was injected intraperitoneally as previously described (39). Prazosin was dissolved extemporaneously in saline solution, and then infused at a concentration of 0.3 mg/kg. At this concentration, prazosin blocks α 1-adrenergic receptors without perturbation of fasted glycemia (20).

Chronic injections

F13A. Male mice fed a HFD during 3 months received every day during 2 weeks a bolus injection of 2 μ l of F13A

(2 μ g/mouse) that was injected directly into the icv catheter completed to 2 μ l of aCSF for treated HFD mice, or 4 μ l of aCSF for control HFD mice.

Bilateral lentivector injection in the MBH

Protocol is explained in details in Supplementary Materials and Methods section.

H₂O₂ measurements by fluorometric methods

Amplex Red (10-acetyl-3,7-dihydrophenoxazine) was used as a fluorescent probe for the detection of H₂O₂. Protocol is explained in detail in Supplementary Materials and Methods section.

Real-time amperometric H₂O₂ measurements

Mice were sacrificed after 6 h of fasting conditions. Protocol is explained in details in Supplementary Materials and Methods section. Data are expressed as delta variation of H₂O₂ release from basal as previously used for amperometric nitric oxide (NO) measurement (11, 12).

Tissues

Mice were fasted for 6 h, and blood samples were collected between 15 and 30 min after the acute injection of apelin or aCSF (these times correspond to the period of release of glucose by the liver glycogenolysis), or after the 6 h fasted for mice with lentivirus injection. Plasma was removed and frozen, powdered in liquid nitrogen, and kept at -80°C until catecholamine assays were performed. Mice were also decapitated without anesthesia. After dissection, liver fragments were also rapidly removed, frozen, powdered in liquid nitrogen, and kept at -80°C until glycogen assays were performed.

Liver glycogen determination

Glycogen content was determined as previously described (21) and explained in the Supplementary Materials and Methods section.

Enzymatic activities

Hepatic G6Pase activity was determined as previously described in detail (40). Results are expressed as μ moles/min per gram of wet tissue. Hepatic PEPCK activity was determined with the methods of Pogson and Smith (35). Results are expressed as nmol/min per gram of wet tissue. Hepatic glycogen phosphorylase activity assay was determined as previously described (33). Results are expressed as a percentage of activity. Glycogen Synthase activity was evaluated by the ratio of phosphorylated protein/total protein. Immunoblots were performed as previously described (11). Membranes were incubated overnight at 4°C with a primary polyclonal antibody against total Glycogen Synthase (dilution 1/1000; Cell Signaling Technology) or the phosphorylated active form (Ser641) of Glycogen Synthase (dilution 1/1000; Cell Signaling Technology).

Histological revelation of glycogen or lipid content

Liver lobe was taken along with the tissues for different assays. The samples were included in Carnoy solution for

24 h, then in 20% sucrose solution for 48 h, and then frozen at -80°C in tissue Teck (Polyfreeze; Sigma). Ten-micrometer-thin cryostat sections were made from livers. Glycogen was visualized with Periodic Acid-Schiff staining. The quantifying of the staining was performed with the software Adobe® Photoshop®. Lipids were visualized by Oil-Red staining. Hepatic TGs content was determined as described (17).

Plasma assays

Plasma catecholamine concentration was measured after 6 h of fasting in all protocols and after 6 h of fasting and 30 min for acute icv treatment. Catecholamine content of the plasma was determined as previously described in detail using high-performance liquid chromatography coupled with electrochemical detection after alumina extraction (29). Plasma glucagon content was determined with a commercial available enzyme-linked immunosorbent assay (ELISA) kit (Glucagon Quantikine; R&D Systems). Plasma ketone bodies content was determined with a commercial kit (Ketone bodies assay kit, Antibodies). Plasma apelin content was determined with a commercial available ELISA kit (Phoenix Pharmaceuticals). Plasma TGs were determined as described (17).

Pyruvate tolerance test

Six-hour-fasted mice were injected with an intraperitoneal pyruvate injection (2 mg/g) after an icv acute injection or unless icv injection (lentivirus). Blood was collected from the tail vein at -30 , 0 , 15 , 30 , 45 , 60 , 90 , and 120 min later for determination of glucose levels.

Insulin tolerance test

Six-hour-fasted mice were injected with an intraperitoneal insulin injection (0.5 mU/g for mice with lentivirus, or 1 mU/g for HFD mice with chronic icv F13A treatment), as previously described (52). Blood was collected from the tail vein at -30 , 0 , 15 , 30 , 45 , 60 , 90 , and 120 min later for determination of glucose levels.

Real-time PCR

Total RNA from tissues was prepared using the TriPure reagent (Roche) as described (14, 17). Quantization and integrity analysis of total RNA was performed as previously described (17). Protocol is explained in detail in Supplementary Materials and Methods section.

Statistical analysis

Results are presented as mean $\pm$ SEM. The statistical significance of differences was analyzed by student t-test, by two-way ANOVA followed by a *post hoc* t-test, or one way followed by *post hoc* Bonferroni's or Dunnett's multiple comparison test, when appropriate. Statistical analyses were assessed by using GraphPad Prism version 5.00 for windows (GraphPad Software). Results were considered statistically significant when $p < 0.05$.

Acknowledgments

The authors are grateful to Valérie Montel and Edith Renaud for technical assistance and Yannick Delpu and Hlne Le Bras for helpful discussion. P.D.C. is a research associate

from the FRS-FNRS (Fonds de la Recherche Scientifique), Belgium. This work was supported by the Société Française de Nutrition (SFN).

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Dr. Claude Knauf

I2MC, CHU Rangueil

1 Avenue Jean Poulhès, BP 84225

31432 Toulouse Cedex 4

France

E-mail: claud.knauf@inserm.fr

Date of first submission to ARS Central, January 4, 2013; date of final revised submission, July 3, 2013; date of acceptance, July 22, 2013.

Abbreviations Used

aCSF = artificial cerebrospinal fluid
ANS = autonomous nervous system
AR = Amplex Red
AUC = area under the curve
CMV = cytomegalovirus
DPI = diphenyleneiodonium
ELISA = enzyme-linked immunosorbent assay
FFA = free fatty acid

G6Pase = glucose-6 phosphatase
H₂O₂ = hydrogen peroxide
HFD = high-fat diet
HRP = horseradish peroxidase
icv = intracerebroventricular
ITT = insulin tolerance test
MBH = medio-basal hypothalamus
MCS = multiple cloning site
mROS = mitochondrial reactive oxygen species
NO = nitric oxide
PBS = phosphate-buffered saline
PEPCK = phosphoenol pyruvate carboxykinase
ROS = reactive oxygen species
SNS = sympathetic nervous system
TG = triglycerides