Original Article

Mitochondrial Reactive Oxygen Species Are Required for Hypothalamic Glucose Sensing

Corinne Leloup,[1] Christophe Magnan,[2] Alexandre Benani,[1] Emilie Bonnet,[1] Thierry Alquier,[1] Géraldine Offer,[1] Audrey Carriere,[1] Alain Périquet,[1] Yvette Fernandez,[1] Alain Ktorza,[2] Louis Casteilla,[1] and Luc Pénicaud[1]

The physiological signaling mechanisms that link glucose sensing to the electrical activity in metabolism-regulating hypothalamus are still controversial. Although ATP production was considered the main metabolic signal, recent studies show that the glucose-stimulated signaling in neurons is not totally dependent on this production. Here, we examined whether mitochondrial reactive oxygen species (mROS), which are physiologically generated depending on glucose metabolism, may act as physiological sensors to monitor the glucose-sensing response. Transient increase from 5 to 20 mmol/l glucose stimulates reactive oxygen species (ROS) generation on hypothalamic slices ex vivo, which is reversed by adding antioxidants, suggesting that hypothalamic cells generate ROS to rapidly increase glucose level. Furthermore, in vivo, data demonstrate that both the glucose-induced increased neuronal activity in arcuate nucleus and the subsequent nervous-mediated insulin release might be mimicked by the mitochondrial complex blockers antimycin and rotenone, which generate mROS. Adding antioxidants such as trolox and catalase or the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone in order to lower mROS during glucose stimulation completely reverses both parameters. In conclusion, the results presented here clearly show that the brain glucose-sensing mechanism involved mROS signaling. We propose that this mROS production plays a key role in brain metabolic signaling. *Diabetes* 55:2084–2090, 2006

Elucidating the signaling mechanisms by which cells sense nutrient or metabolic status, a vital process in energy homeostasis, is of prime importance. Glucose-sensing mechanisms have been mainly characterized in two tissues, both in the pancreas (at the β-cell level) and in the brain (the so-called "glucose-stimulated" or "glucose-inhibited" neurons) (1,2). The cellular and molecular mechanisms underlying such glucose responsiveness appear to share similarities in the two glucose responsive cells (i.e., transport and phosphorylation by GLUT2 and glucokinase, respectively) and the consequent closure of ATP-sensitive K^+ channels (K_{ATP} channels) and calcium influx (3–5). Although ATP production used to be considered the main metabolic signal, recent studies show that the glucose-excited signaling in pancreatic β-cells and neurons is not totally dependent on this production. Within the hypothalamus, a previous work showed that glucose challenge monitors K_{ATP} closure independently of ATP level (6), and more recent data demonstrated that glucose-induced depolarization might occur through a new K_{ATP} channel-independent mechanism, at least in some hypothalamic arcuate neurons (7). These studies suggest that ATP-independent intracellular signaling mechanisms leading to the stimulation of hypothalamic neurons by glucose might be present.

Transient increase in glucose metabolism generates the key substrates NADH and $FADH_2$ for the mitochondria, and their use increases electron formation without modifying other complex constraints along the respiratory chain, ultimately leading to increased superoxide anion production, also called mitochondrial reactive oxygen species (mROS) (8). Although the link between substrates, mROS production, and their deleterious effects has been fully exemplified, for example, during prolonged hyperglycemia (9), emerging data now also demonstrate a physiological role for mROS, including a role as fuel-sensor components (gene transcription, direct enzyme activation, or signal transduction activation) (10,11). Therefore, we investigated whether hypothalamic glucose sensing in vivo involves the mROS signaling pathway.

RESEARCH DESIGN AND METHODS

Male Wistar rats, weighing 250–300 g, were maintained in an animal quarter with a constant temperature (21–23°C) and a 12:12-h light/dark cycle. Food and water were available ad libitum. All rats were treated in accordance with the European community guidelines, and our local institution approved the experimentation. Pharmacological agents were all purchased from Sigma-Aldrich, except carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which was obtained from Calbiochem.

Ex vivo hypothalamic experiments and reactive oxygen species determinations. Brains were rapidly removed from anesthetized animals, the hypothalamic level was separated by transverse section, and hypothalami were dissected on an ice-cooled glass plate and immersed in oxygenated (95% O_2/5% CO_2) saline solution (300 mOsm, pH 7.4) containing 118 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 3 mmol/l KCl, 1 mmol/l $MgCl_2$, 1.2 mmol/l NaH_2PO_4, 15 mmol/l sucrose, 1.5 $CaCl_2$, 5 mmol/l HEPES, and 5 mmol/l glucose. After a 20-min recovery period, hypothalamus slices were rinsed two times and incubated on required milieu, as indicated, for 5 min at 37°C in a final 1-ml volume. At the end of the incubation, they were rapidly frozen and stored at −80°C. Tissue treatment for reactive oxygen species (ROS) determination was performed according to Szabados et al. (12), and hypothalamic pieces

From the [1]Laboratoire de Neurobiologie, Plasticité Tissulaire et Métabolisme, Institut L. Bugnard, Toulouse, France; and the [2]Laboratory of the Physiopathology of Nutrition, Universite Paris, Paris, France.

Address correspondence and reprint requests to Corinne Leloup, UMR 5018-CNRS UPS, Institut L. Bugnard, IFR31, BP 84432, 31 432 Toulouse cedex 4, France. E-mail: coleloup@toulouse.inserm.fr.

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CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; ETC, electron transport chain; K_{ATP} channel, ATP-sensitive K^+ channel; mROS, mitochondrial reactive oxygen species; ROS, reactive oxygen species.

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were loaded with the fluorescent probe dichlorodihydro-fluorescein diacetate (H_2DCFDA; Molecular Probes), 4 μmol/l H_2CFDA in 1 ml, and incubated for 30 min at 37°C. ROS measurements (on 200 μl supernatant) were performed in a Fluorescent Plate Reader (Perkin Elmer). Intensity of fluorescence was expressed as arbitrary units per milligram of protein.

Intracarotid load of glucose or pharmacological agents toward the brain. Tests were performed on anesthetized animals (pentobarbital, 50 mg/kg, intraperitoneal route [Roche et Dessinge, Vincennes, France]). A polyethylene catheter was inserted into the carotid artery, pushed 5 mm in the cranial direction, and secured in place with sutures. Glucose alone (9 mg/kg) and/or pharmacological agents in 200 μl physiological saline were injected through the catheter over 1 min. The concentrations used for the agents were 1 mmol/l trolox, 4,000 units/ml catalase, 1 μmol/l CCCP, 20 μmol/l antimycin, and 2 μmol/l rotenone. Solutions were all adjusted for their osmolarity (300 mOsm). Some of the agents used required DMSO or ethanol vehicle for dissolution. When this was the case, appropriate controls were performed. All of the vehicles used have an effect on insulin secretion on their own (data not shown). Plasma glucose and insulin were collected from the tail vessels (before and 1, 3, 5, and 10 min after injection).

Glucose tolerance test. The glucose tolerance test was carried out according to the method described previously (13). The glucose load (1 g/kg body wt) was injected intraperitoneally.

Neuronal activity recordings in vivo. Each rat was initially prepared to receive the intracarotid load of metabolites or pharmacological agents as described above. They were then placed in a stereotaxic apparatus. A midline incision was made. The skull was drilled at 3.1 mm posterior to the bregma on the midline. The dura was removed to permit electrode insertion. Arcuate nucleus stereotaxic coordinates were obtained according to Paxinos stereotaxic atlas (−3.1 mm posterior to bregma and −8.7 mm under the brain surface). Multiunit recordings were made of arcuate nucleus using monopolarly platinium electrode. Action potentials were displayed and saved on a computer after initial amplification through a low-noise amplifier (BIO amplifier; AD Instrument, Rabalot, France). Data were digitized with digitizer PowerLab/4sp. Signals were monitored with the computer program Chart 4. Multiunit recordings were made in response to a single injection (200 μl) of either saline or tested substances as described above. Baseline unit activity was recorded for 5 min before infusion of a compound. Neuronal activity was normalized to reduce variability resulting from differences in background activity. Specifically, raw data were collected for each recording as the number of discharges per second. Saline and catalase (results similar to that of saline, not shown) recording sessions were used as control data and reflected spontaneous activity.

Lipid peroxidation determinations. Hydroperoxides in biological samples were estimated using the Lipid Hydroperoxide Assay kit (Cayman; Alexis Biochemicals). This method is based on lipid extraction into chloroform, eliminating any interference caused by hydrogen peroxide or endogenous ferric ions.

Glucose and insulin concentrations. These were measured using a glucose analyzer (OneTouch II; LifeScan) and a radioimmunoassay kit (Diasorin, Antony, France), respectively.

ATP determination. ATP contents were determined using a bioluminescent kit (Sigma-Aldrich).

Statistical analysis. Values are expressed as means ± SE. The statistical significance of differences between groups was determined by Student's *t* test after testing the normality and homoscedasticity of the groups.

RESULTS

ROS production by hypothalamus. We first aimed to determine whether transient exposure to increases in glucose might trigger ROS production in hypothalamic slices. To that end, hypothalamic slices were subjected to a 20-mmol/l glucose challenge for 5 min. This stimulating glucose concentration was chosen since arcuate glucose-excited neurons responding to a 5- to 20-mmol/l step have been characterized (7). Under this condition, glucose was found to significantly increase (by 73%) ROS production as measured by the oxidation of H_2DCFDA in hypothalamic extracts (Fig. 1*B*). Adding catalase (a biologically antioxidant active enzyme that converts H_2O_2 into H_2O and O_2) (14) to the stimulating 20-mmol/l glucose concentration completely abolished the ROS increase from hypothalamic slices (Fig. 1*B*). Consequently, these results indicate that ROS are produced from hypothalamic cells under transient glucose stimulation. Hypothalamic slices were secondly exposed to specific mitochondrial inhibitors or uncouplers in order to mimic, or reverse, the ROS production triggered by glucose increase. First, rotenone, a classical complex I blocker (Fig. 1*A*) leads to anion superoxide production (18), which is reflected by mROS increase (Fig. 1*B*). This production was completely abolished when trolox, a vitamin E hydrophilic analog that scavenges ROS (Fig. 1*A*), was added to the milieu (Fig. 1*B*). Then, glucose was coinjected with the mitochondrial uncoupler CCCP, which acts as a channel through the inner membrane to dissipate the H^+ gradient and which accelerates respiration (Fig. 1*A*), leading to diminished superoxide anion generation (10). A significant decrease of mROS production, compared with glucose alone, was observed (Fig. 1*B*). Finally, antimycin (a complex III inhibitor [Fig. 1*A*] that blocks the electron transport chain [ETC]) led to mROS production. An identical response to that induced by glucose alone was observed (Fig. 1*B*). Altogether, these results show that a transient glucose increase elevates mROS production, and this is reproduced by manipulating the ETC.

ROS required for cerebral glucose sensing. To evaluate whether ROS production in brain could play a role in glucose-sensing signaling, we injected a glucose load (9 mg/kg, 30-s injection) toward the brain via the carotid artery. With this protocol, glucose induced an activation of hypothalamic arcuate cells, evidenced by immunodetection of the immediate early gene c-*fos* protein (15). This glucose injection does not change the peripheral glucose level but induces a peak of plasma insulin 1–3 min later (16), which is shown to be due to a parasympathetic activation (17). Animals were submitted to the intracarotid glucose load, as described above, in combination, or not, with antioxidant molecules (either catalase or trolox). Control rats received a bolus of NaCl. The antioxidant doses were carefully chosen to induce no effect themselves on the neuronal stimulation of insulin secretion as well as on glycemia (data not shown). The direct effect of catalase or trolox toward the pancreas has been excluded. Thus, when they were infused to the brain during an intraperitoneal glucose tolerance test (1 g/kg), identical responses between control and treated rats were found (results not shown). As previously demonstrated, the intracarotid glucose load was associated with no change in peripheral glycemia but stimulated the insulin secretion (Fig. 2*A* and *B*). The coadministration of antioxidant agents (catalase or trolox) prevented this secretion without change of the glycemia (Fig. 2*A* and *B*). This demonstrates that scavenging the ROS generation in brain significantly disturbed the insulin secretion induced by the cerebral glucose load.

Mitochondrial origin of ROS produced in cerebral glucose sensing. To demonstrate that ROS production involved in glucose sensing was of mitochondrial origin, we conducted several complementary experiments in the same model using specific mitochondrial inhibitors or an uncoupler. First, glucose was coinjected with the mitochondrial uncoupler CCCP, which diminishes superoxide anion generation (10) (Fig. 1*A*). A significant decrease of the insulin peak, compared with glucose alone, was observed (Fig. 2*C*). Then, antimycin (Fig. 1*A*), which leads only to mROS production, triggered an identical response to that observed with glucose alone (Fig. 2*C*). These results strongly suggest that mROS are the effectors of the

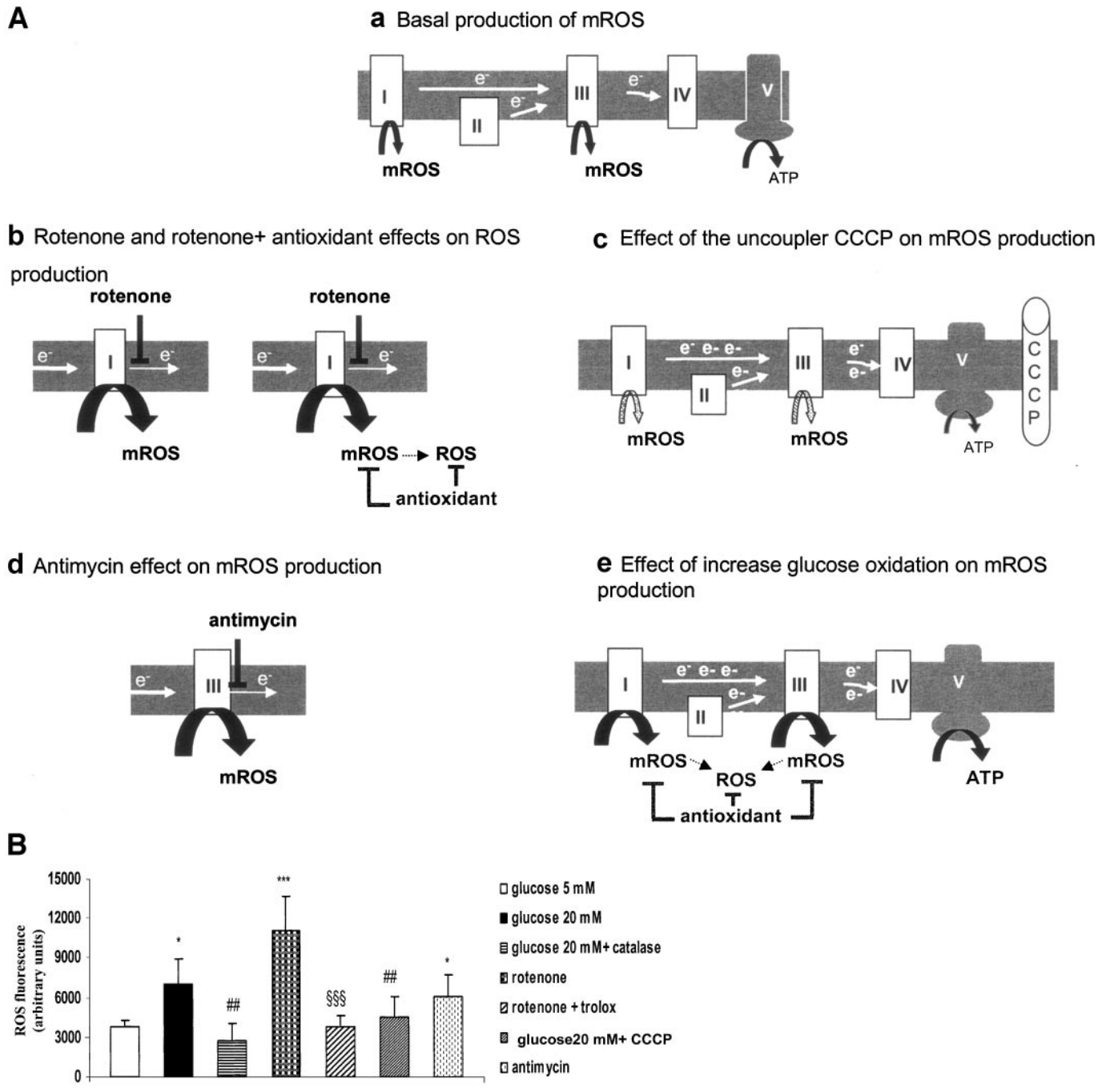

FIG. 1. ROS are produced in hypothalamic slices after a glucose challenge. *A*: Effects of pharmacological agents used to modulate mROS generation. Approximately 1–5% of the O_2 consumed by the ETC is incompletely metabolized and reduced into anion superoxide (O_2^-) at the complexes I and III (*a*). O_2^- is then diverted into other ROS as H_2O_2. Rotenone inhibits complex I and electrons accumulate and react with O_2, leading to enhanced mROS generation (*b*). Adding antioxidants scavenges the free radicals generation. The mitochondrial uncoupler CCCP increases electron flux into the ETC, which are less "exposed" in time to oxygen (*c*). Antimycin inhibits complex III and electrons accumulate and react with O_2, leading to enhanced mROS generation (*d*). Glucose oxidation leads to increased NADH and $FADH_2$ formation which enter ETC at complexes I and II, respectively (*e*). They then activate the ETC, resulting in ATP generation. It was recently proposed that this activation increases mROS formation. Adding antioxidants scavenges free radical generation. *B*: Relative ROS fluorescence (DCFDA intensity, arbitrary units, adjusted per milligram of protein) from hypothalamic pieces in 5 or 20 mmol/l glucose, or 20 mmol/l glucose + catalase (4,000 units/ml) and after treatment with rotenone (2 μmol/l), rotenone + trolox (1 mmol/l), 20 mmol/l glucose + CCCP (1 μmol/l), or antimycin (20 μmol/l). In the experiments with rotenone and antimycin, 5 mmol/l glucose was in the milieu. n = 8–10 hypothalami per group, 3-min incubation. *$P \leq 0.05$ vs. 5 mmol/l glucose, ##$P \leq 0.01$ vs. 20 mmol/l glucose, ***$P \leq 0.001$ rotenone vs. 5 mmol/l glucose, and §§§$P \leq 0.001$ rotenone + trolox vs. rotenone alone.

response. Finally, rotenone, which leads also to anion superoxide production (18) (Fig. 1*A*), produced a peak secretion similar to that measured with glucose, as shown with antimycin (Fig. 2*C*). This insulin peak was reversed when trolox was coinjected (Fig. 2*C*), reinforcing the hypothesis that mROS act as signaling molecules in the parasympathetic-controlled insulin secretion. Any modification of the glycemia was present whatever the injection performed (Fig. 2*D*). Using inhibitors of oxidative phosphorylation might block ATP generation. This was evalu-

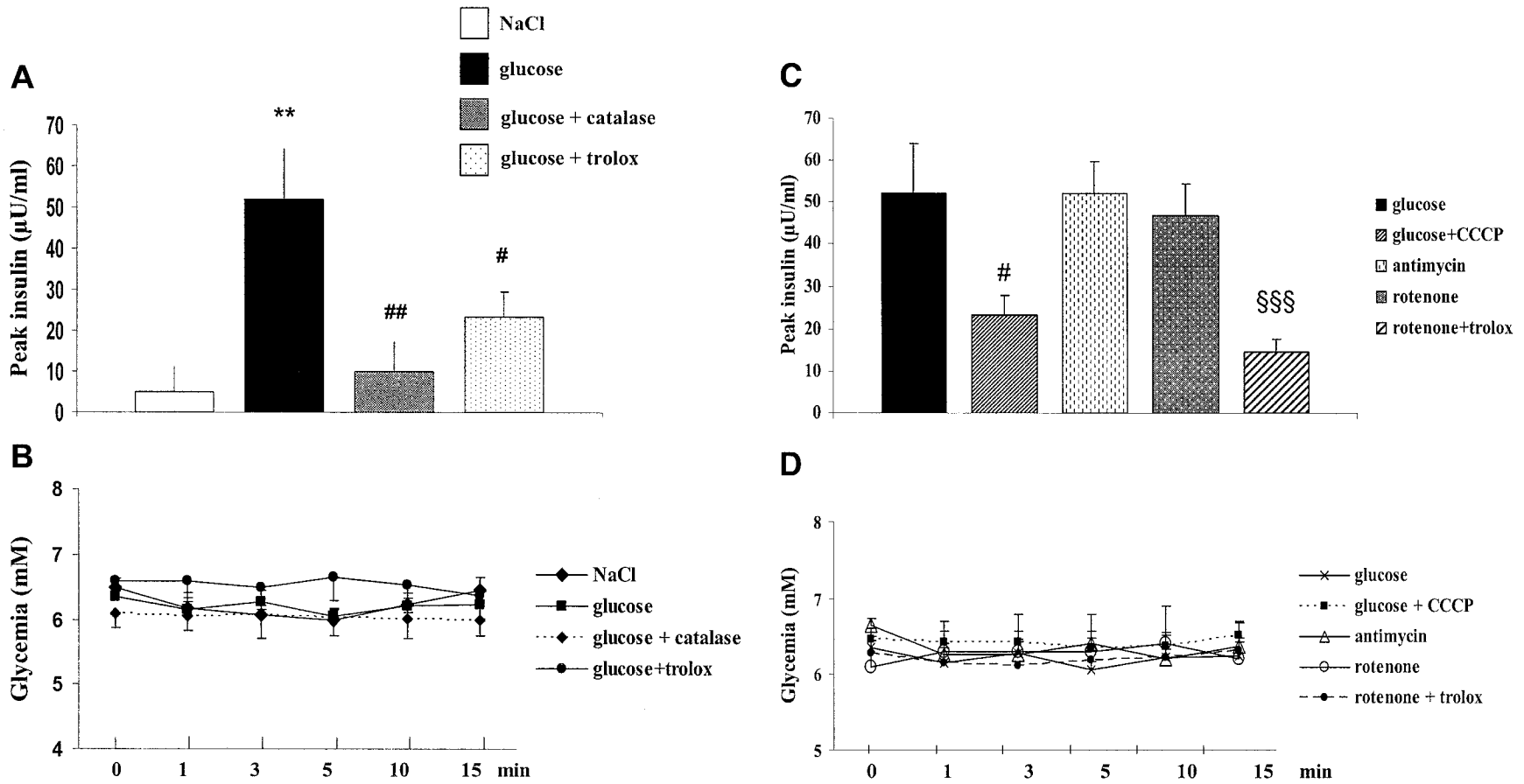

FIG. 2. mROS are required for cerebral glucose sensing. ***A***: Insulin secretion 1–3 min after the carotid load (200 µl) toward the brain of saline, glucose (9 mg/kg), glucose coinjected with trolox (10^{-3} mol/l), or glucose coinjected with catalase (4,000 units). Osmolarity has been adjusted (300 mosM). n = 6–9 male Wistar rats per group. $^{**}P \leq 0.01$ vs. NaCl, $\#\#P \leq 0.01$ vs. glucose alone, and $\#P \leq 0.05$ vs. glucose alone. ***B***: Respective glycemia for each group; any modification of the glycemia was present whatever the brain treatment. ***C***: Insulin secretion as described in ***A***, except the agents injected were glucose (9 mg/kg), glucose coinjected with CCCP (10^{-6} mol/l), antimycin alone (20 µmol/l), rotenone alone (2 µmol/l), and rotenone coinjected with trolox (10^{-3} mol/l). n = 6–9 male Wistar rats per group. $\#P \leq 0.05$ vs. glucose and $\S\S\S P \leq 0.001$ vs. rotenone. ***D***: Respective glycemia for each group described in ***C***; any modification of the glycemia was present whatever the brain treatment.

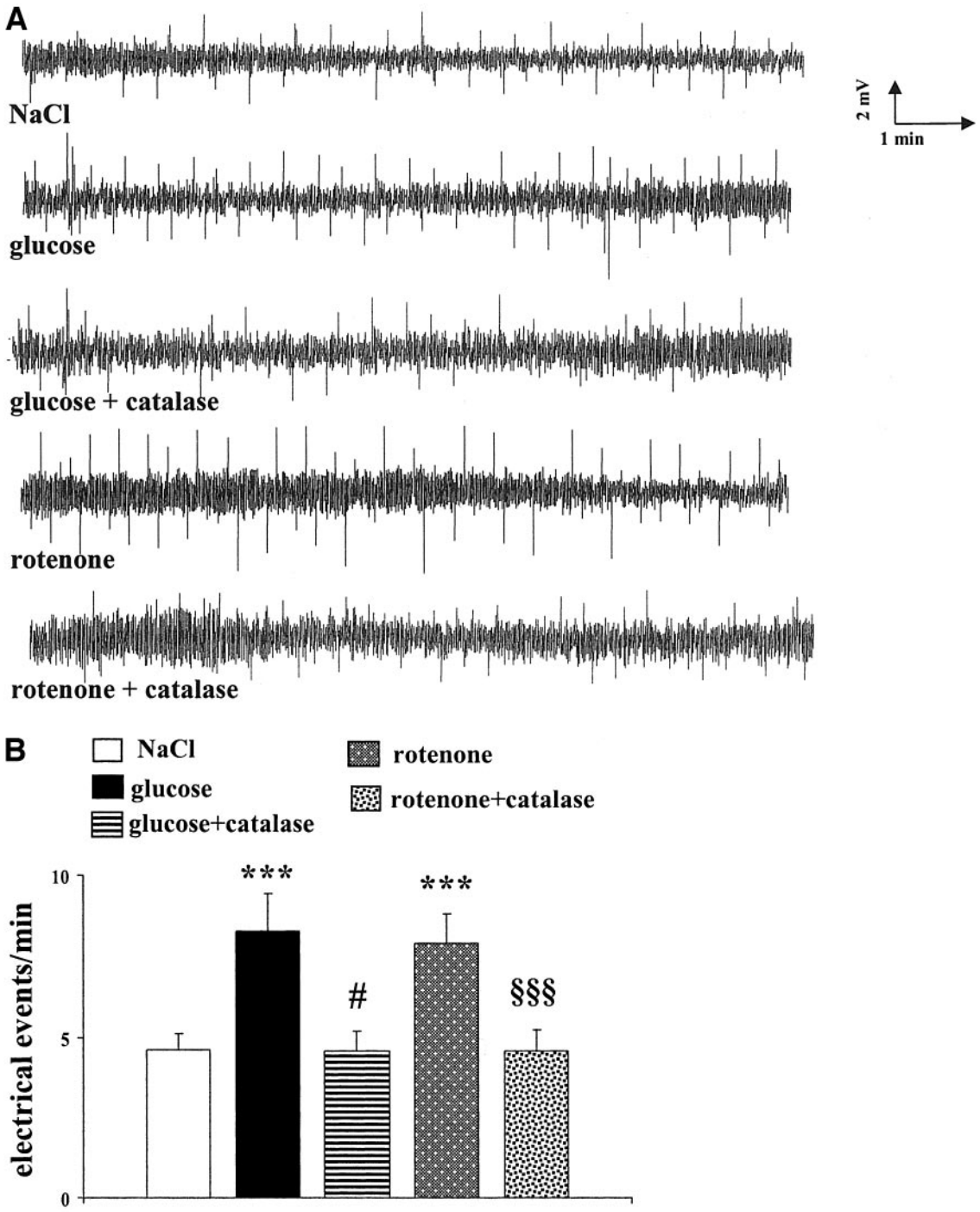

FIG. 3. mROS are required for glucose-stimulated arcuate neuronal activity. *A*: Extracellular recordings of arcuate nucleus neuronal activity in male Wistar rats after the carotid load of saline (NaCl), glucose, glucose + catalase, rotenone, and rotenone + catalase, from *left* to *right*, respectively. *B*: Recordings expressed as electrical events per minute. After injection, the recordings were 10 min in duration. Concentrations of the different solutions injected in the brain through the carotid artery were the same as described in Fig. 2. Data were analyzed by Chart 4 software; $n = 6–8$ each case. ***$P \leq 0.001$ vs. NaCl, #$P \leq 0.05$ vs. glucose, and §§§$P \leq 0.001$ vs. rotenone.

ated for rats injected with the mitochondrial complexes blockers, either rotenone or antimycine. ATP content was measured and compared with NaCl controls and glucose-injected rats. A significant difference was detected between hypothalamic ATP concentrations in NaCl and glucose-injected rats when compared with rotenone- and antimycin-injected rats ($n = 6–8$ for each group, data expressed as 10^{-10} mol/l ATP/μg cytosolic protein: 9.15 ± 1.64 in NaCl, 8.39 ± 1.7 in glucose-injected rats, and 2.83 ± 1.22 in rotenone- and 2.68 ± 0.97 in antimycin-injected rats). These data confirm the inhibitory effect of rotenone and antimycine on oxidative phosphorylation and ATP synthesis.

Taken together, these coherent and convergent results demonstrate that mROS production is required for brain glucose sensing, and in counterpart, that mROS mimic brain glucose sensing.

Stimulation of hypothalamic arcuate neuronal activity by mROS produced in cerebral glucose sensing. As the arcuate nucleus was described as being critical for

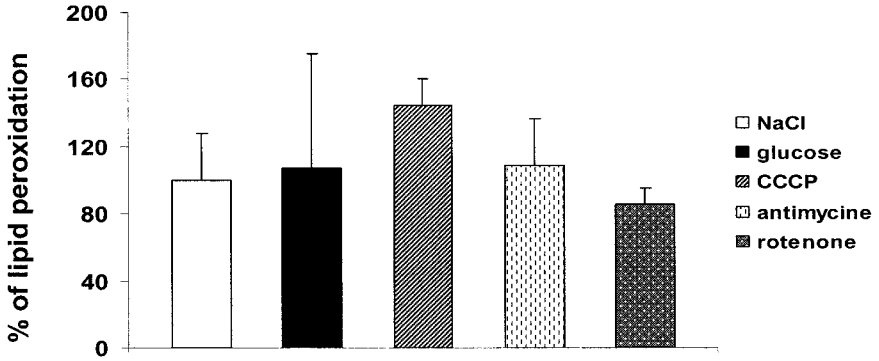

FIG. 4. Hydroperoxide measurements on hypothalami of pharmacological-injected rats. To exclude any damaging and nonspecific oxidative stress of the different mitochondrial agents tested, lipid peroxidation was evaluated. Hydroperoxides were evaluated on hypothalami of rats injected with NaCl, glucose (9 mg/kg), CCCP (10^{-6} mol/l), antimycine (20 μmol/l), or rotenone (2 μmol/l) (as described in Fig. 2*A* and *C*). Hypothalami are dissected 5 min after injection, which is the maximal time for both the increased electrical activity and the peak of plasma insulin. No difference of any of these groups compared with NaCl or glucose was observed.

control of insulin secretion by the central nervous system, we recorded in vivo extracellular activity in this hypothalamic nucleus in some of the key demonstrating experiments. As expected, an intracarotid glucose load triggered a significant increase in electrical events. Strikingly, the coinjection of the antioxidant enzyme catalase completely prevented this response (Fig. 3*A* and *B*). Rotenone injection, which was previously shown to mimic glucose response, reproduced a neuronal activation similar to that observed with glucose (Fig. 3*A* and *B*). As seen with glucose coinjected with catalase, the coinjection of rotenone with catalase also prevented the neuronal activation (Fig. 3*A* and *B*). Altogether, these data demonstrate that mROS behave as pivotal signals in brain glucose sensing by controlling the neuronal activity of arcuate nucleus.

These effects were reversible because extracellular recordings showed a return to basal activity after the stimulation. These activities returned to their minima between 3 (glucose) and 5 (rotenone) min after the injections. Moreover, the stimulating effect could be reproduced in a repeated manner (data not shown). These results reinforce our proposition of a specific effect of rotenone on transient mROS formation, since the concentration used for this pharmacological agent allows a reversible effect, as measured with electrical activity.

Deleterious oxidative stress and physiological responses. Regarding the toxicity related to the pharmacological agents used, they have (to our knowledge) never been used via the carotid route in order to explore brain function. Nanomolar doses were ineffective in our in vivo model. Since the load injected into the carotid was first diluted in the plasma before reaching the brain, it is reasonable to propose that the effective amount acting at the target site was far from the initial concentration. To rule out a putative damaging and nonspecific oxidative stress of the different mitochondrial agents tested, lipid peroxidation was evaluated. Hydroperoxide measurements were conducted on hypothalami of NaCl-, glucose-, and pharmacological-injected rats, dissected 5 min after injection, which is the maximal time for both the increased electrical activity and the peak of plasma insulin. No difference in any of these groups compared with NaCl or with glucose was observed (Fig. 4). This excludes a nonspecific toxic effect.

DISCUSSION

Although it is now accepted that small fluctuations in the steady-state concentration of mROS may play a role in intracellular signaling (19,20), their pivotal role in brain glucose sensing had not been considered before. This study is the first to provide evidence that mROS play an essential role in glucose sensing in hypothalamus, one of the main critical systems in maintaining glucose homeostasis.

Most of the metabolic substrates, and glucose in particular, lead to the formation of NADH or $FADH_2$, which are donors of the respiratory chain, activating the ETC and then ATP generation. It was recently proposed that these reduced equivalents predisposed to increased mROS formation through a direct effect on the ETC in a number of cell types (9,21). Taking into account the constraints of the respiratory chain, the transient increase and oxidation of these reduced equivalents lead to a small excess of electrons that react with oxygen, increasing superoxide formation. In this case, mROS increase is dependent on metabolic activation (11). First, we demonstrate that transient glucose challenge at the hypothalamic level has the ability to trigger an increased ROS production, reversed with antioxidant treatment. Second, we demonstrate that both the neuronal arcuate activation and the consequent insulin secretion due to neuronal stimulation are monitored by mROS. In both cases, quenching the ROS formation with antioxidants prevented the glucose response. Third, in contrast, mROS generation by the inhibitors antimycin and rotenone mimics the glucose effect. In each case, a toxic effect of the pharmacological agents was carefully examined and can be excluded. Indeed, no excessive hydroperoxidation was observed, and the effects were reversible. The fact that glucose, as well as an inhibitor of glucose metabolism, produce the same effect on hypothalamic neurons may first appear as a paradox. This might be explained if NADH, not ATP, constitutes the key metabolite mediating the stimulatory effects of 20 vs. 5 mmol/l glucose on hypothalamic neurons, as already suggested (22). Indeed, because complex I is the main source of mROS and the production of mROS increases when NADH is in its more reduced state, blocking complex I also enhances the production of mROS and mimics the effects of glucose. Other studies have also implicated NADH in glucose sensing in the pancreas (23,24). Thus, the mROS mechanism we describe here is consistent with an NADH mechanism mediating glucose signaling, as suggested earlier (22). Moreover, it is noteworthy that the obligatory generation of mROS is dependent on the proton gradient and the electrons fluxes. These fluxes result from the balance between the electron supply to the respiratory chain and the constraints on the ETC exerted by the control of the dissipation of protons gradient via the ATP synthesis or uncoupling mechanism (25). Thus, mROS appear as a signal integrating both the NADH redox state and the phosphate potential, thus reflecting the whole mitochondrial metabolism and then the cell metabolism.

Altogether, these complementary and convergent data clearly support a critical role for mROS as part of the hypothalamic glucose-sensing mechanism. Such a role is consistent with the emerging general view of mROS as a sensor of the nutrient environment (11).

Although the mechanisms involved in glucose sensing have been extensively studied, some of the issues are still controversial. Neurons that respond to glucose challenge, named glucose-stimulated neurons, possess an K_{ATP} channel that inactivates as the ATP level increases during glucose metabolism (26), as already described in β-cells. More recently, the role of intracellular ATP in the closure of K_{ATP} has been discussed, since no detectable increase in cytosolic ATP was present (6). Our results suggest that hypothalamic K_{ATP} should be sensitive to mROS production, which is consistent with a recent study reporting that K_{ATP} channels control transmitter release in dorsal striatum through a H_2O_2-dependent mechanism (27) or that the signaling involving mROS generation is partially, or totally, K_{ATP} independent, as suggested from recent data (7). Thus, further speculation should be made since a variety of ROS-sensitive and nonselective cationic channels have been already described (28,29).

Whether a similar mechanism might be extended to β-cells is of considerable interest. The implication of NADH in glucose sensing in the pancreas has been fully exemplified (23,24), and there are some strong arguments to sustain that glucose-induced insulin secretion and mROS production may be a tightly linked process (21). Our finding opens new perspectives for investigating the putative involvement of mROS in metabolic controls, apart from their already well-known effect as oxidative stressors.

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