Original Article

Insulin Restores Metabolic Function in Cultured Cortical Neurons Subjected to Oxidative Stress

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We previously demonstrated that insulin has a neuroprotective role against oxidative stress, a deleterious condition associated with diabetes, ischemia, and age-related neurodegenerative diseases. In this study, we investigated the effect of insulin on neuronal glucose uptake and metabolism after oxidative stress in rat primary cortical neurons. On oxidative stress, insulin stimulates neuronal glucose uptake and subsequent metabolism into pyruvate, restoring intracellular ATP and phosphocreatine. Insulin also increases intracellular and decreases extracellular adenosine, counteracting the effect of oxidative stress. Insulin effects are apparently mediated by phosphatidylinositol 3-K and extracellular signal–regulated kinase signaling pathways. Extracellular adenosine under oxidative stress is largely inhibited after blockade of ecto-5′-nucleotidase, suggesting that extracellular adenosine results preferentially from ATP release and catabolism. Moreover, insulin appears to interfere with the ATP release induced by oxidative stress, regulating extracellular adenosine levels. In conclusion, insulin neuroprotection against oxidative stress–mediated damage involves *1*) stimulation of glucose uptake and metabolism, increasing energy levels and intracellular adenosine and, ultimately, uric acid formation and *2*) a decrease in extracellular adenosine, which may reduce the facilitatory activity of adenosine receptors.
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Insulin is known to stimulate glucose uptake and intracellular metabolism in peripheral tissues. Although the role of insulin in the brain is less defined, it has been described to have neurotrophic, neuromodulator, and neuroendocrine effects (1). Transport of insulin synthesized in pancreatic β-cells into the brain can occur indirectly, through a receptor-mediated process across the blood-brain barrier, or directly via the area postrema, a circumventricular region that lacks the blood-brain barrier (2). Insulin can be also synthesized de novo in the hippocampus, prefrontal cortex, enthorhinal cortex, and olfactory bulb neurons (3). The presence of preproinsulin I and II mRNA and insulin receptor mRNA was previously demonstrated in the brain (4). Insulin receptor is highly expressed in neurons (5) in the olfactory bulb, hypothalamus, cerebral cortex, and hippocampus (3).

The brain is one of the most metabolically active tissues, relying on continuous glucose supply for synaptic transmission and dendritic and axonal trafficking, thus helping to maintain brain cognitive function (6,7). The main neuronal glucose transporter, GLUT3, is highly expressed at the neutrophil in regions of the central nervous system (CNS) with high synaptic density and metabolic activity, at the hippocampus and frontal and motor cortex (8). Interestingly, evidence suggest that insulin influences cerebral glucose metabolism at the entorhinal cortex and the hippocampus (9).

The brain is continuously exposed to free radicals, which may underlie the pathophysiology of neurodegenerative diseases, stroke, and diabetes complications affecting the CNS (10,11). Lipid and protein oxidation alter the conformation and structure of membrane proteins, including GLUT3, thus decreasing neuronal glucose uptake and intracellular ATP (12). In plasma from diabetic subjects (13) and in brains of diabetic mice (14), high glucose levels also produce permanent chemical alterations in proteins and increase lipid peroxidation. Hyperglycemia associated with stroke exacerbates ischemic brain damage in both humans and animals (15,16). Under such conditions, brain metabolism is affected (17), leading to lactate accumulation and tissue acidosis (18), as well as mitochondrial dysfunction and subsequent decrease in high-energy phosphates, ultimately resulting in oxidative stress and neuronal death (18). Thus, the brain requires neuroendocrine counterregulatory mechanisms to maintain blood glucose within a narrow range.

We have previously demonstrated that oxidative stress induces neuronal apoptosis and necrosis, as well as lipid and protein oxidation, increasing the oxidation of GLUT3 by 4-hydroxynonenal (4-HNE) (19). Treatment with insulin prevented oxidative stress and neuronal death by stimulating uric acid levels and the glutathione redox cycle, in a process mediated by phosphatidylinositol 3-kinase (PI-3K)/Akt and extracellular signal–regulated kinase (ERK) signaling pathways (19). The aim of the current work was to investigate whether insulin neuroprotection during oxidative stress involved the restoration of neuronal glucose energy metabolism. We demonstrate that insulin prevents oxidative stress–induced impairment of glucose uptake

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AMPCP, α,β-methyleneadenosine-5′-diphosphate; CNS, central nervous system; 2-DG, 2-deoxyglucose; ERK, extracellular signal–regulated kinase; 2-[^{3}H]DG, 2-deoxy-D-[1-^{3}H]glucose; 4-HNE, 4-hydroxynonenal; MEK, mitogen-activated protein kinase; NBTI, S-(4-nitrobenzyl)-6-thioinosine; PI-3K, phosphatidylinositol 3-kinase.

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and subsequent decrease in energy metabolism, thereby regulating endogenous ATP and its hydrolysis to adenosine.

RESEARCH DESIGN AND METHODS

α,β-Methyleneadenosine-5′-diphosphate (AMPCP) sodium salt, 2-deoxyglucose (2-DG), ascorbic acid, DNase, $FeSO_4$, insulin from porcine pancreas, penicillin/streptomycin, S-(4-nitrobenzyl)-6-thioinosine (NBTI), soybean trypsin inhibitor, and trypsin were purchased from Sigma (St. Louis, MO). Neurobasal medium and B-27 supplement were from Gibco (Paisley, Scotland). 2-Deoxy-D-[1-^{3}H]glucose (2-[^{3}H]DG; 12.0 Ci/mmol) was from Amersham International (Little Chalfont, U.K.). Scintillation Universol cocktail was from ICN (Irvine, CA). PD98059 was from Calbiochem (Darmstadt, Germany). Lichrospher 100 RP-18 (5 μm) high-performance liquid chromatography column was from Merck (Darmstadt, Germany). Wortmannin and LY294002 were kindly provided by Alomone Labs (Jerusalem, Israel). All other reagents were of the highest grade of purity commercially available.

Culture of cortical neurons. Pregnant female Wistar rats were obtained from our local colony (Animal Facilities, Faculty of Medicine, University of Coimbra). Animals were kept under controlled light and humidity conditions. Cortical neurons were isolated from brains of 15- to 16-day-old Wistar rat embryos, using a previously described procedure (19,20). Adhering to procedures approved by the institutional animal care and use committee, pregnant rat females were killed by cervical displacement and decapitation. The brain cortices were removed from embryos and placed in sterile Krebs medium containing (in mmol/l): 120.9 NaCl, 4.8 KCl, 1.22 KH_2PO_4, 25.5 $NaHCO_3$, 13.0 glucose, and 10.0 HEPES, pH 7.4, supplemented with 0.3% BSA, 0.05% trypsin, and 0.04 mg/ml DNase for 10 min at 37°C. The reaction was stopped by addition of Krebs medium (1:1 dilution) containing 0.075% soybean trypsin inhibitor and 0.04 mg/ml DNase. The supernatant was discarded after centrifugation at 1,000*g* for 5 min in a Sigma 3K10 centrifuge, and the cells were dissociated in new Krebs medium. The neurons were resuspended in neurobasal medium supplemented with 2% B-27, 0.2 mmol/l glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin, and then they were cultured for 6 days in 95% air/5% CO_2 at 37°C. Cortical neurons were plated at different densities on poly-L-lysine (0.1 mg/ml)–covered plates: 0.16×10^6 cells/cm^2 for measurement of 2-[^{3}H]DG; uptake or 0.26×10^6 cells/cm^2 for analysis of pyruvate, lactate, adenine nucleotides, and adenosine. Previous studies in our laboratory showed that cortical cultures contained few glial cells (<10%), as assessed by immunocytofluorescence using anti-MAP_2 (microtubule-associated protein) and anti-GFAP (glial fibrillary acidic protein) (S. Almeida and A.C.R., unpublished observations).

Incubation with insulin and induction of oxidative stress. By following previously established experimental conditions (19), 0.1 or 10 μmol/l insulin was added to neurobasal medium at 48 h (at 4 days in vitro, renewed every 24 h) or immediately (at 6 days in vitro) before induction of oxidative stress with 1.5 mmol/l ascorbic acid and 7.5 μmol/l $FeSO_4$ for 15 min at 37°C in sodium saline solution containing (in mmol/l): 132.0 NaCl, 4.0 KCl, 1.2 Na_2HPO_4, 1.4 $MgCl_2$, 1.0 $CaCl_2$, 6.0 glucose, and 10.0 HEPES, pH 7.4. The cells were further incubated for 5 h in neurobasal medium in the absence of the oxidizing agents. Insulin was present throughout the experiments. To analyze the involvement of insulin receptor–mediated signaling pathways, the neurons were incubated with 50 nmol/l Wortmannin, 5 μmol/l LY294002 (inhibitors of PI-3K/Akt pathway), or 30 μmol/l PD98059 (an inhibitor of ERK pathway) 30 min before insulin incubation and maintained throughout the experiments. When present, 1 μmol/l NBTI (an inhibitor of adenosine transporter) or 50 μmol/l AMPCP (an inhibitor of ecto-5′-nucleotidase) were incubated with the neurons for 5 min before insulin and maintained throughout the experiments.

Analysis of 2-[^{3}H]DG uptake. Glucose accumulation was analyzed by measuring the uptake of 1 μCi/ml (12.0 Ci/mmol) of 2-[^{3}H]DG, a nonmetabolizable analog of glucose, according to a method described elsewhere (6). After incubation, the neurons were washed with sodium saline solution containing 6 mmol/l glucose, then washed with glucose-free sodium solution, and further incubated with glucose-free solution containing 2-[^{3}H]DG (1 μCi/ml) and nontritiated 2-DG (1 mmol/l) at 37°C. 2-[^{3}H]DG uptake was stopped at 10 min by rinsing the cells with ice-cold sodium solution. All experiments were performed in the absence of glucose. After solubilization with 1 mol/l NaOH at 4°C, the radioactivity was counted in a Packard Tri-Carb 2500 TR liquid scintillation analyzer. Results were expressed as nanomoles of 2-[^{3}H]DG uptake per 10^6 cells.

Assessment of intracellular pyruvate and lactate. Neurons were washed with PBS (0–4°C), scrapped, and extracted with 0.6 mol/l perchloric acid supplemented with 25 mmol/l EDTA-Na^+. Cell extracts were centrifuged at 14,000*g* for 2 min in an Eppendorf Centrifuge 5415C at 4°C to remove cell debris. Lactate and pyruvate were determined in the supernatant by using kits from Randox Laboratories (Antrim, U.K.) and Ben SRL (Milan, Italy), respectively. Results were expressed in percent of control (with 0.14 considered as 100%) in the absence of insulin and ascorbate/Fe^{2+}.

Measurement of adenine nucleotides, phosphocreatine, and adenosine. Neurons were extracted with 0.6 mol/l perchloric acid, supplemented with 25 mmol/l EDTA-Na^+, and centrifuged at 14,000*g* for 2 min at 4°C in an Eppendorf 5415C centrifuge, according to previously described methods, with some modifications (21). The resulting pellet was solubilized with 1 mol/l NaOH for total protein analysis by the Sedmak method (22). After neutralization with 3 mol/l KOH in 1.5 mol/l Tris, the samples were centrifuged at 14,000*g* for 2 min (0–4°C). The resulting supernatants were assayed for ATP, ADP, and AMP or adenosine by separation in a reverse-phase high-performance liquid chromatograph, with detection at 254 nm. These samples were also used to determine intracellular phosphocreatine, at 340 nm, according to a previously described method (23). To determine extracellular accumulation of adenine nucleotides or adenosine, neurobasal medium was recovered and prepared using a similar procedure. Because measurable levels of extracellular adenosine were detected, experiments were performed without adenosine deaminase inhibitor. Results were expressed in percent of control, in the absence of insulin and ascorbate/Fe^{2+}, considering 2.1, 0.3, 0.9, and 6.3 nmol/mg protein as 100% for intracellular ATP, ADP, AMP, and adenosine, respectively; 2.6, 2.3, and 7.4 pmol/mg protein as 100% for extracellular ATP, ADP, and AMP, respectively; and 1.8 nmol/mg protein as 100% for extracellular adenosine.

Data analysis and statistics. Results are the means ± SEM of the indicated number of independent experiments, run in duplicate or triplicate. Statistical significance was analyzed using the one-way ANOVA for multiple comparisons, with the Bonferroni post test. A P value <0.05 was considered significant.

RESULTS

Insulin stimulates glucose uptake and pyruvate/lactate formation on oxidizing conditions. Previously, we showed that insulin prevents ascorbate/Fe^{2+}-induced neuronal death (19). Neuroprotective effects of insulin were analyzed after incubation for 48 h (0.1 μmol/l insulin) or immediately before (10 μmol/l insulin) exposure to ascorbate/Fe^{2+}, under experimental conditions characterized by no significant changes induced by insulin alone (19). Insulin also prevented oxidation of GLUT3 by 4-HNE (19), strongly suggesting changes in neuronal glucose accumulation, the main brain metabolic substrate. Under physiological conditions, glucose is metabolized to pyruvate via glycolysis. A minor proportion of lactate can also result from the reduction of pyruvate.

In this study, we analyzed the effect of insulin on glucose transport, by measuring 2-[^{3}H]DG uptake. 2-DG is a synthetic glucose analog phosphorylated to 2-deoxy-D-glucose-6-phosphate by hexokinase. Ascorbate/Fe^{2+}-induced oxidative stress (1.9 ± 0.6 nmol thiobarbituric acid reactive substances/mg protein) (19) caused a decrease in neuronal 2-[^{3}H]DG uptake by ~57% (Fig. 1*A*). This decrease was completely prevented by 0.1 and 10 μmol/l insulin. Insulin per se did not significantly affect 2-[^{3}H]DG uptake (Fig. 1*A*).

Because these results suggested that glycolysis was stimulated by insulin, we determined the endogenous levels of pyruvate and lactate, two end products of glycolysis (Fig. 1*B*). Although insulin per se did not affect pyruvate/lactate levels, it completely prevented the decrease in pyruvate/lactate induced by oxidative stress (Fig. 1*B*) by increasing pyruvate formation (data not shown). Insulin-mediated recovery of neuronal 2-[^{3}H]DG uptake and pyruvate/lactate after oxidative stress highly suggest the stimulation of the glycolytic pathway.

Insulin restores intra- and extracellular adenine nucleotides on oxidative stress. One of the main consequences of insulin-mediated metabolic stimulation observed in Fig. 1 is the increase in high-energy compounds, namely ATP and phosphocreatine; the latter con-

TABLE 1
Involvement of PI-3K/Akt and ERK signaling in insulin-induced changes in intracellular adenine nucleotides under oxidative stress

	No insulin			0.1 μmol/l insulin			10 μmol/l insulin		
	ATP	ADP	AMP	ATP	ADP	AMP	ATP	ADP	AMP
No inhibitor (% of control)									
Control	100.0 ± 10.3	100.0 ± 11.2	100.0 ± 10.4	101.0 ± 10.8	107.8 ± 10.6	102.6 ± 12.4	91.3 ± 8.1	96.3 ± 9.9	100.1 ± 11.7
Asc/Fe^{2+}	56.5 ± 8.8*	101.3 ± 6.5	60.8 ± 8.0*	103.5 ± 10.7†	60.5 ± 6.1*‡	100.2 ± 7.6†	115.4 ± 11.2‡	60.4 ± 6.0*‡	107.7 ± 9.3†
Wortmannin (% of control)									
Control	61.5 ± 11.1*	146.7 ± 15.8*	144.4 ± 16.7*	71.6 ± 9.0*	157.8 ± 17.1*	129.8 ± 9.9	74.3 ± 8.6	151.6 ± 10.6*	138.1 ± 11.3*
Asc/Fe^{2+}	57.0 ± 10.9*	154.9 ± 7.6*	177.0 ± 13.8§	64.1 ± 4.1*	154.9 ± 12.5*	159.9 ± 13.7*	74.1 ± 9.8	156.6 ± 8.2*	162.0 ± 19‖
LY294002 (% of control)									
Control	61.4 ± 8.2*	154.4 ± 8.5*	138.9 ± 13.7*	53.3 ± 8.7*	158.7 ± 10.3*	132.2 ± 13.8*	66.1 ± 10.2	145.7 ± 14.7*	140.5 ± 16.9*
Asc/Fe^{2+}	52.3 ± 8.6*	152.7 ± 8.6‖	158.7 ± 15.5*	62.7 ± 10.0*	144.3 ± 19.9*	156.8 ± 17.3*	55.2 ± 9.9*	149.3 ± 17*	161.4 ± 18.0*
PD98059 (% of control)									
Control	50.7 ± 8.2‖	134.4 ± 10.9*	143.6 ± 11.8*	52.0 ± 6.9*	148.6 ± 14.6*	156.2 ± 16.8*	32.6 ± 7.7‖	153.0 ± 9.1*	159.2 ± 10.0*
Asc/Fe^{2+}	50.3 ± 9.4*	151.7 ± 16.3*	148.2 ± 11.0*	67.8 ± 9.7*	165.2 ± 17.1*	176.5 ± 22.9‖	54.4 ± 6.4*	153.4 ± 10.7*	185.5 ± 17.8‖

Data are the means ± SE of six experiments. Neurons were preincubated with 50 nmol/l wortmannin, 5 μmol/l LY294002, or 30 μmol/l PD98059. Adenine nucleotides were determined by reverse-phase high-performance liquid chromatography. *$P < 0.05$ compared with the control in the absence of inhibitors; †$P < 0.05$, ‡$P < 0.01$ vs. ascorbate/Fe^{2+} without insulin; §$P < 0.001$, ‖$P < 0.01$ vs. control in the absence of inhibitors.

FIG. 1. Insulin prevents impairment of glucose metabolism under oxidative stress. Glucose metabolism was evaluated by determining glucose accumulation, using 2-[^{3}H]-deoxyglucose uptake (*A*) and intracellular pyruvate/lactate (*B*). Data are the means ± SE of five (*A*) or eight (*B*) experiments. **P* < 0.05 vs. control; £*P* < 0.05, ££*P* < 0.01, £££*P* < 0.001 vs. ascorbate/Fe^{2+} without insulin. □, control; ■, ascorbate/Fe^{2+}.

stitutes a reservoir for rapid ATP regeneration. Thus, we determined the effect of insulin and insulin receptor–mediated signaling pathways on intracellular ATP, ADP, AMP, and phosphocreatine levels (Tables 1 and 2). Oxidative stress significantly decreased intracellular ATP by ~44%, which was completely prevented by 0.1 or 10 μmol/l insulin (Table 1). Insulin per se did not significantly affect these parameters. Although oxidative stress did not modify intracellular ADP, ascorbate/Fe^{2+} plus insulin decreased ADP levels by 41%, following the rise in intracellular ATP (Table 1). Thus, under oxidized conditions the ATP-to-ADP ratio was largely restored by insulin. Additionally, insulin (0.1 and 10 μmol/l) almost completely regenerated the ascorbate/Fe^{2+}-induced decrease in intracellular phosphocreatine (Table 2). A decrease in phosphocreatine/creatine and an increase in lactate/pyruvate may constitute early signs of mitochondrial defect (24). Moreover, uncoupling of oxidative phosphorylation was associated with a decrease in ATP and phosphocreatine on kainate injection in rat brain (25).

Pretreatment with the PI 3-K inhibitors wortmannin (50 nmol/l) or LY294002 (5 μmol/l) and the mitogen-activated protein kinase (MEK) inhibitor PD98059 (30 μmol/l) significantly decreased intracellular ATP and the ATP-to-ADP ratio under control and oxidized conditions, and it increased intracellular ADP under control conditions (Table 1). Nevertheless, insulin was unable to rescue oxidative stress–mediated changes in intracellular ATP or ADP in the presence of the inhibitors of PI 3-K or MEK (Table 1), suggesting that insulin-induced recovery of intraneuronal

TABLE 2
Insulin restores intracellular phosphocreatine on oxidative stress

	No insulin	0.1 μmol/l insulin	10 μmol/l insulin
Control (pmol/mg protein)	9.19 ± 0.88	7.97 ± 0.75	8.65 ± 0.76
Asc/Fe^{2+} (pmol/mg protein)	5.27 ± 0.47*	7.56 ± 0.6†	8.07 ± 0.74†

Data are means ± SE of six experiments. Neurons were incubated with insulin before ascorbate/Fe^{2+} treatment. Intracellular phosphocreatine was determined as described in RESEARCH DESIGN AND METHODS. *$P < 0.01$ vs. control; †$P < 0.05$ vs. ascorbate/Fe^{2+} without insulin.

ATP from ADP under oxidative stress may involve both PI 3-K and MEK signaling pathways.

The effect of insulin on intracellular AMP was also analyzed (Table 1). Insulin restored the decrease in intracellular AMP on oxidation. Treatment with wortmannin, LY294002, or PD98059 increased intracellular AMP per se (Table 1). However, neither ascorbate/Fe^{2+} nor insulin significantly affected intracellular AMP in the presence of PI 3-K and MEK inhibitors (Table 1).

To further explain insulin-induced changes in intracellular adenine nucleotides, namely ATP, in oxidized neurons, we examined the levels of extracellular ATP, ADP, and AMP. Ascorbate/Fe^{2+}-induced increase in extracellular ATP was completely abolished by insulin (Table 3). Conversely, insulin prevented the decrease (by ~26%) in extracellular ADP induced by oxidative stress (Table 3). Neither ascorbate/Fe^{2+} nor insulin significantly affected extracellular accumulation of AMP (Table 3).

These data suggest that decreased intracellular ATP after oxidative stress was caused by inhibition of glucose metabolism and increased extracellular ATP. Furthermore, insulin-induced recovery of intracellular ATP from ADP on oxidative stress resulted from *1*) stimulation of glucose accumulation and metabolism through glycolysis and possibly oxidative phosphorylation and/or at the expense of phosphocreatine for rapid ATP regeneration and/or *2*) blockade of ATP release.

Insulin modifies intra- and extracellular levels of adenosine on oxidative stress. On oxidative stress, reduction of intracellular ATP was followed by a decrement in AMP, both of which were prevented by insulin. Adenosine resulting from the catabolism of purines may constitute a very sensitive signal of increased metabolic rate or metabolic stress. Moreover, subsequent adenosine catabolism results in uric acid, an important neuronal antioxidant. Extracellularly, adenosine modulates the activity of inhibitory A_1 and A_3 receptors, or facilitatory A_{2A} and A_{2B} receptors (26), which mediate cell survival or cell death, respectively. Taking into account the dual role of adenosine in neuronal degeneration, we further explored the effect of insulin on intra- and extracellular adenosine in neurons subjected to oxidative stress. To our knowledge, this is the first report that evaluates the effect of insulin on adenosine levels.

Similarly to ATP and AMP (Table 1), ascorbate/Fe^{2+}-induced decrease in intracellular adenosine (~51%) was significantly prevented by insulin (Fig. 2*A*). Pretreatment with the PI 3-K inhibitors wortmannin (Fig. 2*B*) or LY294002 (Fig. 2*C*) and the MEK inhibitor PD98059 (Fig. 2*D*), per se, significantly decreased intracellular adenosine. Nevertheless, insulin did not rescue the decrease in intracellular adenosine induced by oxidative stress in the presence of inhibitors of PI 3-K (Fig. 2*B* and *C*) or MEK (Fig. 2*D*). Moreover, insulin differently affected the extracellular accumulation of adenosine (Fig. 3*A* and *B*). Ascorbate/Fe^{2+}-induced increase in extracellular adenosine (~51%) was only significantly attenuated by 10 μmol/l insulin, whereas 0.1 μmol/l insulin completely decreased extracellular adenosine (data not shown).

TABLE 3
Insulin affects extracellular adenine nucleotides under oxidative stress

	Control	Asc/Fe^{2+}
No insulin (% of control)		
ATPext	100.0 ± 5	135.3 ± 10.2*
ADPext	100.0 ± 1.1	73.6 ± 5.1*
AMPext	100.0 ± 8.7	105.7 ± 8.1
0.1 μmol/l insulin (% of control)		
ATPext	90.7 ± 6.7	90.8 ± 8.5†
ADPext	103.6 ± 8.8	104.4 ± 6.1†
AMPext	100.3 ± 14.0	102.5 ± 8.6
10 μmol/l insulin (% of control)		
ATPext	92.6 ± 8.8	88.6 ± 5.3‡
ADPext	96.9 ± 9.3	110.8 ± 8.3‡
AMPext	102.5 ± 8.1	97.6 ± 9.6

Data are means ± SE of six experiments. Neurons were incubated with insulin before ascorbate/Fe^{2+} treatment. Intracellular ATP, ADP, and AMP were determined by reverse-phase high-performance liquid chromatography. *$P < 0.05$ vs. control; †$P < 0.05$, ‡$P < 0.01$ vs. ascorbate/Fe^{2+} without insulin.

Because extracellular adenosine can result either from intracellular AMP degradation and subsequent release via bidirectional adenosine transporters or from extracellular catabolism of released nucleotides, catalyzed by ectonucleotidases (27), we also analyzed the origin of extracellular adenosine. Cortical neurons were pretreated with inhibitors of adenosine transporter (NBTI) and ecto-5′-nucleotidase (AMPCP), which were previously shown to prevent the increase in extracellular adenosine induced by oxidative stress (21,28).

NBTI (1 μmol/l) per se did not significantly affect intracellular adenosine (Fig. 2*E* compared with Fig. 2*A*), but it increased extracellular adenosine by ~29% (Fig. 3*A*), demonstrating the role of this transporter under control conditions. On oxidative stress in the presence of NBTI, intracellular adenosine increased by ~26% (Fig. 2*E* compared with Fig. 2*A*), whereas extracellular adenosine decreased by ~13% (Fig. 3*A*) compared with ascorbate/Fe^{2+} alone. These data suggest that the nucleoside transporter partially contributed to the increase in extracellular adenosine under oxidative stress. Treatment of oxidized neurons with NBTI plus insulin (10 μmol/l) completely restored intra- and extracellular adenosine (Figs. 2*E* and 3*A*).

To analyze the contribution of the catabolism of released ATP to extracellular adenosine, cortical neurons were pretreated with AMPCP, which blocks the hydrolysis of 5′-AMP into adenosine (29). AMPCP (50 μmol/l) per se reduced extracellular adenosine by ~25% (Fig. 3*B*). Incubation with AMPCP under oxidative stress conditions largely decreased extracellular adenosine (~91%) compared with ascorbate/Fe^{2+} alone, suggesting that this pathway highly contributes to the increase in extracellular adenosine. On oxidative stress, insulin (10 μmol/l) plus AMPCP decreased (by ~19% compared with ascorbate/Fe^{2+} plus insulin) extracellular adenosine accumulation to

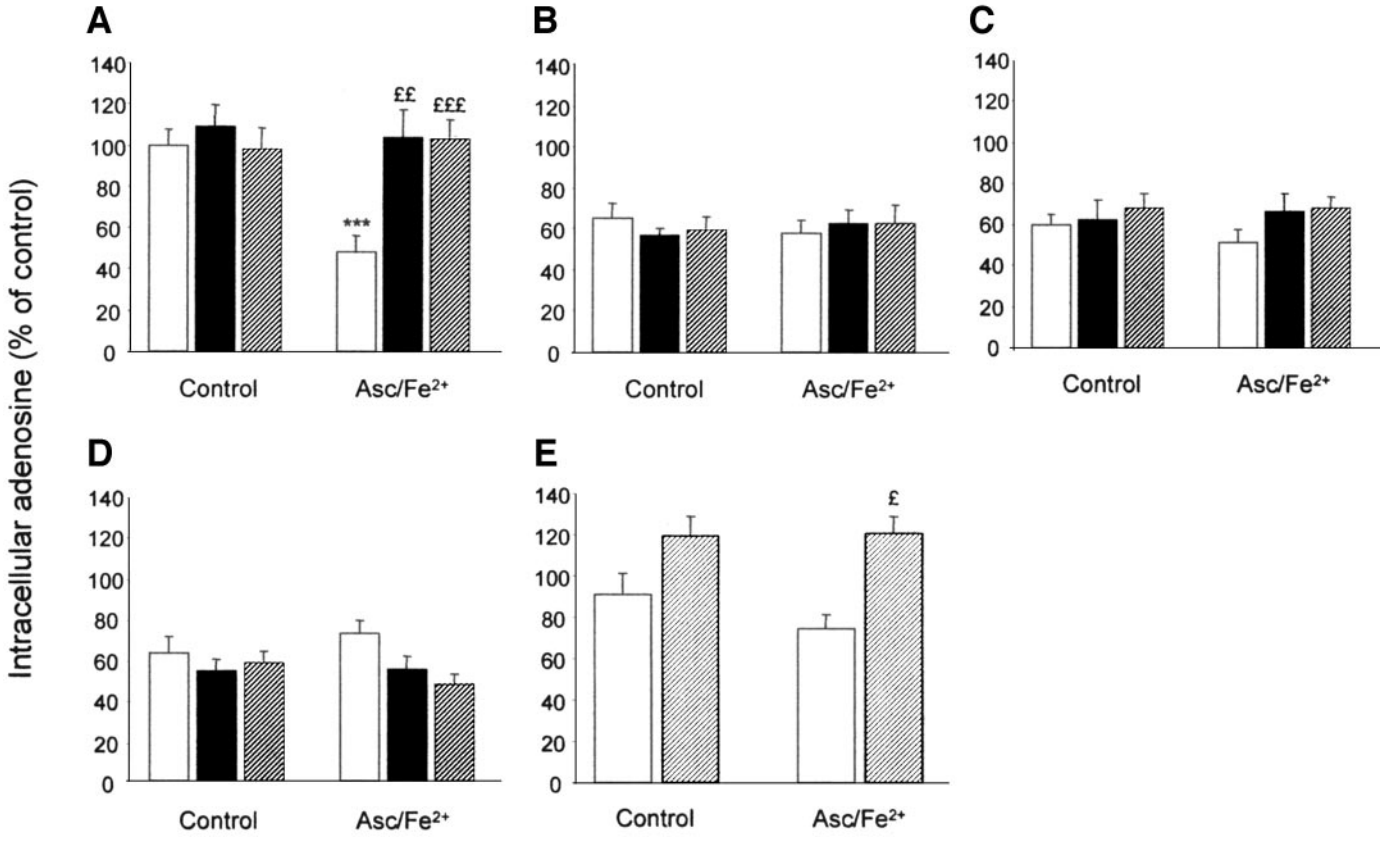

FIG. 2. Insulin-mediated changes in intracellular adenosine on oxidative stress. Neurons were preincubated in the absence (*A*) or in the presence of 50 nmol/l wortmannin (*B*), 5 μmol/l LY294002 (*C*), 30 μmol/l PD98059 (*D*), or 1 μmol/l NBTI (*E*). Adenosine was determined by reverse-phase high-performance liquid chromatography. Data are the means ± SE of six experiments. ***$P < 0.001$ vs. control without insulin; £$P < 0.05$, ££$P < 0.01$, £££$P < 0.001$ vs. ascorbate/Fe^{2+} without insulin. □, no insulin; ■, 0.1 μmol/l insulin; ▨, 10 μmol/l insulin. Asc, ascorbate.

the same extent as the decrease observed in the presence of NBTI.

Thus, similar to intracellular adenine nucleotides, PI 3-K and ERK signaling pathways appear to be involved in the regulation of intracellular adenosine by insulin. Furthermore, blockade of ectonucleotidases largely inhibited extracellular adenosine accumulation under oxidized conditions, suggesting that extracellular adenosine occurs mainly through the release of adenine nucleotides to the extracellular space and their rapid degradation into adenosine, as described previously (21,28). However, we cannot exclude a contribution of the reversal of adenosine transport under these conditions.

Insulin partially prevented extracellular adenosine accumulation in oxidized neurons mainly by interfering with the release of ATP, limiting its subsequent metabolization into adenosine. This can be explained by two observations: *1*) insulin plus NBTI produced an additive effect, suggesting the occurrence of two independent mechanisms, and *2*) AMPCP slightly influenced the decrease in extracellular adenosine caused by insulin under oxidative stress, suggesting that it occurred, at least partially, independently of ecto-5′-nucleotidase inhibition. Thus, reduction of extracellular adenosine most likely occurred because of the inhibition of adenine nucleotide (ATP) release, preceding ecto-5′-nucleotidase.

DISCUSSION

In the current study, we demonstrate that insulin protects cortical neurons against oxidative stress–induced impairment of glucose accumulation and metabolism through the stimulation of glycolysis and high-phosphate metabolism, including phosphocreatine and ATP generation. This supports the replenishment of intracellular adenosine, which can be metabolized to uric acid, as we have previously observed (19). Moreover, the neuroprotective role of insulin in oxidative stress may involve the inhibition of ATP release and its subsequent extracellular metabolization, restoring the levels of extracellular adenosine. Furthermore, activation of PI 3-K/Akt and ERK signaling pathways appears to mediate the neuroprotective role of insulin against metabolic impairment under oxidative stress. Thus, the current results are complementary to those previously observed in primary cortical neurons regarding insulin neuroprotection against oxidative stress (19).

It has been widely demonstrated that neuronal and synaptosomal treatment with $FeSO_4$ inhibits the glucose transporter either directly, by 4-HNE– or acrolein-induced oxidation of GLUT3, or indirectly via lipid peroxidation (30,31). Glucose transport inhibition can also result from oxidative stress–mediated decrease in endogenous glutathione (19,32). We previously observed the formation of 4-HNE adducts on GLUT3 in ascorbate/Fe^{2+}-treated cortical neurons (19), supporting GLUT3 inhibition and the subsequent impairment of neuronal glucose uptake and metabolism.

Changes in phosphocreatine and pyruvate/lactate levels may constitute one of the early signs of mitochondrial respiratory chain dysfunction (24). In this regard, the observed decrease in pyruvate/lactate and phosphocre-

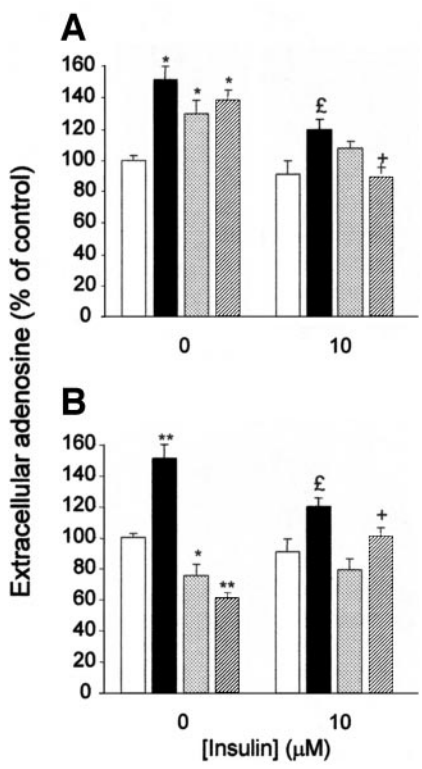

FIG. 3. Regulation of extracellular adenosine induced by insulin on oxidative stress. Neurons were preincubated with 1 μmol/l NBTI (*A*) or 50 μmol/l AMPCP (*B*). Data are the means ± SE of six experiments. *$P < 0.05$, **$P < 0.01$ vs. control (not treated with inhibitors or insulin); £$P < 0.05$ vs. ascorbate/Fe^{2+} without insulin; +$P < 0.05$ vs. ascorbate/Fe^{2+} in the presence of insulin. □, control; ■, ascorbate/Fe^{2+}; ▨, NBTI (*A*) or AMPCP (*B*); ▨, NBTI plus ascorbate/Fe^{2+} (*A*) or AMPCP plus ascorbate/Fe^{2+} (*B*).

atine suggest that oxidative stress affects both glycolysis and oxidative metabolism. ATP depletion may exacerbate free radical generation, culminating in neuronal death (19,33). A decrease in intracellular ATP under oxidative stress may also result from its release and subsequent extracellular metabolization to adenosine by ectonucleotidases. These results are in agreement with our previous data obtained in cultured retinal neurons exposed to ascorbate/Fe^{2+} (21,28) and with an increase in ecto-5′-nucleotidase activity observed after oxidation of brain membranes (34). However, we cannot exclude the contribution of reversal of the nucleoside transporter.

Despite some controversy regarding the role of extracellular adenosine, the type of receptor activated after oxidation appears to be determined by its origin; adenosine resulting from extracellular degradation of AMP preferentially activates facilitatory A_{2A} receptors (27), whereas adenosine resulting from the reversal of nucleoside transporter activates both inhibitory A_1 (28,35) and facilitatory A_{2A} receptors (28). Under our experimental conditions, neuronal oxidation caused ATP release and its subsequent catabolism into extracellular adenosine, which can activate facilitatory adenosine receptors, leading to neuronal death. Nevertheless, oxidative stress was previously shown to decrease ligand binding to synaptosomal A_1 (36) and striatal membrane A_{2A} receptors (37), emphasizing the dual role of adenosine upon oxidation. We also show that oxidative stress decreases intracellular ATP production, which is metabolized into adenosine, ultimately decreasing the levels of uric acid (19), an important endogenous antioxidant. These data suggest that increased extracellular adenosine and decreased intracellular adenosine contribute to neurodegeneration on oxidative stress.

There is some controversy regarding the role of insulin in brain glucose transport. Seaquist et al. (38) hypothesized that glucose uptake and metabolism in the human brain was insulin independent. However, Prasannan (39) and Bingham et al. (40) showed that insulin stimulated glucose uptake and metabolism in rat brain cortical slices and in human brain cortex. These authors hypothesized that GLUT3 mediated glucose uptake evoked by insulin. Accordingly, insulin resistance may also affect GLUT3 activity (41). In this regard, insulin-mediated stimulation of glucose uptake on oxidative stress correlates with the observation of decreased GLUT3 oxidation by 4-HNE, most likely resulting from the stimulation of endogenous antioxidant defenses (19). Alternatively, insulin-induced regulation of glucose uptake may involve the activation of phospholipase Cγ and protein kinase C, leading to phosphorylation of glucose transporters (42). Nevertheless, insulin could not rescue serum-free treated cultured neurons from decreased glucose uptake (43), suggesting that insulin neuroprotection may involve mechanisms other than the regulation of glucose transport/metabolism.

It is widely accepted that the major rate-limiting step in neuronal glucose metabolism is its phosphorylation by hexokinase, the first ATP-driven reaction of glycolysis (44). Insulin appears to upregulate hexokinase activity, localization, and expression, thus modulating glucose metabolism to meet the energy demands of sensory neurons (45). In this regard, insulin-induced increases in intracellular pyruvate/lactate, phosphocreatine, and ATP-to-ADP ratio on oxidative stress support the hypothesis that, similar to nonneuronal tissues (3), the peptide stimulates neuronal glycolysis and oxidative metabolism and/or the rapid phosphorylation of ADP into ATP at the expense of phosphocreatine, implicating creatine kinase activation. Brain insulin infusion was previously shown to have an anabolic effect on energy metabolism in the hippocampus, increasing phosphocreatine (46), whereas neuronal insulin receptor inhibition led to severe abnormalities in energy metabolism (3). Metabolic effects of insulin appear to depend on the activation of PI 3-K and/or ERK signaling pathways. In agreement, insulin stimulation of mitochondrial function involved the activation of PI 3-K signaling pathways in cultured adult sensory neurons (45). Furthermore, insulin can modulate hippocampal synaptic plasticity through the activation of *N*-methyl-D-aspartate receptors, a subtype of glutamate receptors, and the PI 3-K pathway (47).

In this study, we clearly show that insulin prevents a decrease in intracellular adenosine and an increase in extracellular adenosine evoked by oxidative stress. Concordant with changes in adenine nucleotides, alterations in intracellular adenosine evoked by insulin seemed to depend on the activation of PI 3-K and ERK signaling pathways. Hence, the insulin signaling cascade may protect neurons against oxidative stress either by stimulating intracellular ATP metabolism to adenosine (Fig. 4) and uric acid (19), thus contributing to the replenishment of intracellular antioxidant defenses, and/or by interfering with ATP release and extracellular degradation into adenosine, decreasing the extracellular adenosine pool (Fig. 4). Insulin protection, determined by a decrease in apoptotic and necrotic neuronal death (19), appears to involve a

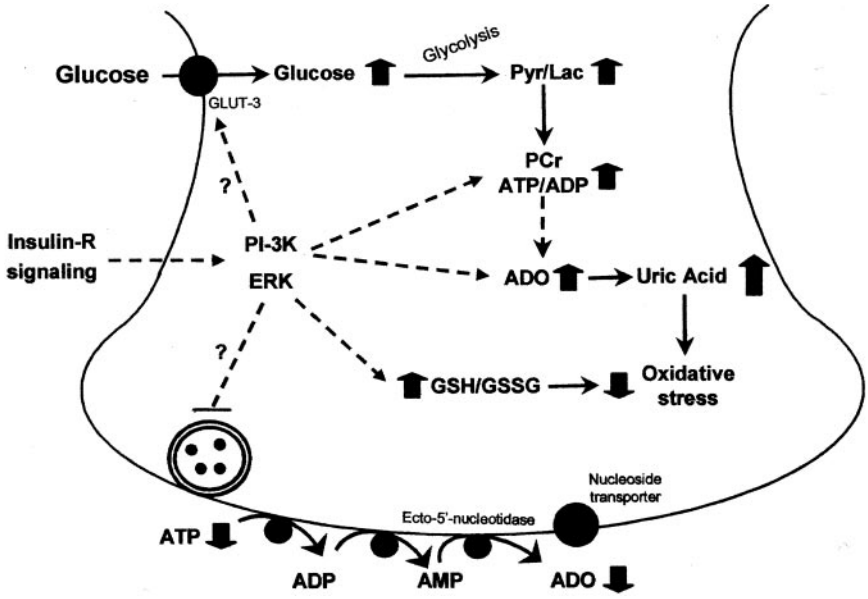

FIG. 4. Stimulation of glucose metabolism and decreased extracellular adenosine mediate insulin neuroprotection against oxidative stress. After ascorbate/Fe^{2+} treatment, insulin-induced activation of PI 3-K and ERK signaling pathways stimulates neuronal glucose uptake and glycolysis, increasing intracellular ATP-to-ADP ratio, phosphocreatine (PCr), and adenosine (ADO). As a consequence, uric acid levels are increased, which, together with the increase in reduced glutathione (GSH)/oxidized glutathione (GSSH), help to counterbalance oxidative stress. Insulin also interferes with ATP release, regulating its metabolization to extracellular adenosine under oxidized conditions. Pyr, pyruvate; Lac, lactate.

decrease in extracellular adenosine, possibly protecting against facilitatory adenosine receptor activation.

After transient global ischemia in CNS parenchyma, insulin administration was shown to prevent neuronal damage and necrosis via activation of insulin receptor and IGF-1 receptor but independently of its effect on glucose levels (48). However, in focal ischemia, insulin protection depended on the regulation of blood glucose levels (49). Under such conditions, insulin infusion combined with glucose may stimulate glucose uptake and glycolysis, producing anaerobic ATP. This would limit the decrease in tissue high-energy phosphates and the increase in free radicals occurring in ischemia (18,50). By showing that insulin prevents neuronal apoptosis and necrosis (19) and restores neuronal glucose metabolism, modulating the extracellular adenosine pool through the activation of intracellular signaling pathways, we provide important evidence toward the potential therapeutic use of insulin against neuronal dysfunction in strokes. However, when considering insulin therapy for the treatment of stroke, blood glucose levels should be maintained in the normal range to avoid biochemical and neuropathological consequences of hyperinsulinemia-induced severe hypoglycemia in the brain (49).

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