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# GUANOSINE IS NEUROPROTECTIVE AGAINST OXYGEN/GLUCOSE DEPRIVATION IN HIPPOCAMPAL SLICES VIA LARGE CONDUCTANCE $CA^{2+}$-ACTIVATED $K^+$ CHANNELS, PHOSPHATIDILINOSITOL-3 KINASE/ PROTEIN KINASE B PATHWAY ACTIVATION AND GLUTAMATE UPTAKE

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**Abstract—Guanine derivatives (GD) have been implicated in many relevant brain extracellular roles, such as modulation of glutamate transmission and neuronal protection against excitotoxic damage. GD are spontaneously released to the extracellular space from cultured astrocytes and during oxygen/glucose deprivation (OGD). The aim of this study has been to evaluate the potassium channels and phosphatidilinositol-3 kinase (PI3K) pathway involvement in the mechanisms related to the neuroprotective role of guanosine in rat hippocampal slices subjected to OGD. The addition of guanosine (100 $\mu$M) to hippocampal slices subjected to 15 min of OGD and followed by 2 h of re-oxygenation is neuroprotective. The presence of $K^+$ channel blockers, glibenclamide (20 $\mu$M) or apamin (300 nM), revealed that neuroprotective effect of guanosine was not dependent on ATP-sensitive $K^+$ channels or small conductance $Ca^{2+}$-activated $K^+$ channels. The presence of charybdotoxin (100 nM), a large conductance $Ca^{2+}$-activated $K^+$ channel (BK) blocker, inhibited the neuroprotective effect of guanosine. Hippocampal slices subjected to OGD and re-oxygenation showed a significant reduction of glutamate uptake. Addition of guanosine in the re-oxygenation period has blocked the reduction of glutamate uptake. This guanosine effect was inhibited when hippocampal slices were pre-incubated with charybdotoxin or wortmanin (a PI3K inhibitor, 1 $\mu$M) in the re-oxygenation period. Guanosine promoted an increase in Akt protein phosphorylation. However, the presence of charybdotoxin blocked such effect. In conclusion, the neuroprotective effect of guanosine involves augmentation of glutamate uptake, which is modulated by BK channels and the activation of PI3K pathway. Moreover, neuroprotection caused by guanosine depends on the increased expression of phospho-Akt protein.** 

**Key words: guanosine, oxygen/glucose deprivation, glutamate uptake, Akt phosphorylation, BK activation, hippocampal slices.**

Stroke is a major cause of disability and death, worldwide (Mattson, 2000). Ischemic stroke accounts for, approximately, 80% of all strokes and it results from a thrombotic or embolic occlusion of a major cerebral artery (Durukan and Tatlisumak, 2007). Experimental *in vitro* models for ischemia comprise advantages for the study of cellular pathophysiological responses. Oxygen and/or glucose deprivation in brain tissue preparations reproduce several pathological states induced by brain energy failure. Energy deprivation leads to the production of reactive oxygen species (ROS), (White et al., 2000) which causes the release of neurotransmitters such as glutamate (Bonde et al., 2003).

Excessive extracellular glutamate leads to overstimulation of glutamate receptors and consequent influx of $Na^+$, $Cl^-$ and $Ca^{2+}$ ions, through the channels gated by those receptors. The increase of $Ca^{2+}$ levels inside the cell results in the activation of $Ca^{2+}$-dependent enzymes which lead to processes such as proteolysis, hydrolysis, lipid peroxidation and ROS production (Choi, 1988). Glutamate uptake is a crucial process to maintain extracellular glutamate concentrations below toxic levels. This effect is achieved through specific high-affinity sodium-dependent excitatory amino acid transporters that are mainly present in astrocytes. Glutamate transporters are modulated by the cell redox status (Anderson and Swanson, 2000), thus increased ROS production may result in impairment of glutamate uptake (Trotti et al., 1998).

Cell injuries, like hypoxia and hypoglycemia, are thought to cause remarkable outflow of purines (Ciccarelli et al., 1999; Jurányi et al., 1999). Nucleotides are rapidly catabolized extracellularly and monophosphate nucleotides and nucleosides accumulate within the traumatic tissue, reaching high concentrations in the extracellular space (Zimmermann et al., 1998; Ciccarelli et al., 2001; Caciagli et al., 2000). Guanine derivatives (GD) are released in amounts three-fold greater than their adenine derivatives counterparts (Ciccarelli et al., 1999).

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*Abbreviations:* Akt, protein kinase B; BK, large (big) conductance $Ca^{2+}$-activated $K^+$ channels; GD, guanine derivatives; GDP, guanosine 5′-diphosphate; GMP, guanosine 5′-monophosphate; GTP, guanosine 5′-triphosphate; GUO, guanosine; HBSS, Hank's balanced salt solution; $K_{ATP}$, ATP-sensitive $K^+$ channels; $K_{Ca}$, $Ca^{2+}$-activated $K^+$ channels; KRB, Krebs-Ringer bicarbonate buffer; MEK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, N-methyl-D-aspartate; OGD, oxygen/glucose deprivation; PI3K, phosphatidilinositol-3kinase; ROS, reactive oxygen species; TEA, tetraethylammonium.

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The nucleoside guanosine (GUO) and guanine nucleotides have been implicated in neuroprotection by counteracting glutamate excitotoxicity, which was observed in diverse models of glutamate toxicity. It has been shown GD protect from seizures induced by quinolinic acid *in vivo* (Schmidt et al., 2000, 2005; Oliveira et al., 2004). Quinolinic acid is a known N-methyl-D-aspartate (NMDA) receptor agonist and it has also been revealed as a modulator of astrocytic and neuronal glutamate transport, causing an excessive stimulation of the glutamatergic system (Tavares et al., 2000, 2002). Thus, the neuroprotective effects of GD on quinolinic acid-induced damage may involve the modulation of glutamate receptors or transporters.

Studies from our laboratory have demonstrated that guanosine-5′-monophosphate (GMP) is able to reduce neuronal damage in hippocampal slices submitted to an *in vitro* model of ischemia (Oliveira et al., 2002), or to a hypoglycemia-like insult induced by a glucose-deprived incubation medium (Molz et al., 2005). GMP has also prevented the reduction in cell viability and apoptotic DNA fragmentation induced by NMDA (Molz et al., 2008a), although it might be toxic for neurons at elevated concentrations (Molz et al., 2009). In cultured hippocampal neurons, guanine nucleotides, namely guanosine 5′-triphosphate (GTP), guanosine 5′-diphosphate (GDP) and GMP, inhibited NMDA- or kainate-mediated toxicity, whereas guanosine did not shown any protective effect (Morciano et al., 2004). However, it was also previously shown that guanosine was able to increase glutamate uptake in cortical slices maintained at basal conditions or submitted to oxygen/glucose deprivation (OGD) (Frizzo et al., 2002). Moreover, we have shown that guanosine promotes neuroprotection depending on extracellular $Ca^{2+}$ levels and modulation of protein kinase A, protein kinase C, mitogen-activated protein kinase (MEK) and phosphatidilinositol-3 kinase (PI3K) pathways in slices subjected to OGD (Oleskovicz et al., 2008).

Increasing evidence show that guanosine displays a relevant neuroprotective effect within *in vivo* and *in vitro* neurotoxicity models (for review, see Schmidt et al., 2007). However, the exact extracellular site of interaction (putative receptor) and mechanisms of action for this nucleoside have not yet been fully characterized. Nevertheless, we showed that guanosine neuroprotection depends on $K^+$ channels activation in hippocampal slices subjected to OGD (Oleskovicz et al., 2008), and Benfenati et al. (2006) showed that guanosine promotes an increased expression of functional $K^+$ rectifying channels in astrocytes culture.

The activation of $K^+$ channels in nerve cells is linked to the control of membrane potential. Activation of some $K^+$ channels types, such as $Ca^{+2}$-activated $K^+$ channels, occur in response to increased $Ca^{+2}$ ions concentrations inside the cells and cell membrane depolarization (Burg et al., 2006). The ATP-sensitive $K^+$ channels ($K_{ATP}$) are regulated by the intracellular ATP concentration. The modulation of large conductance $Ca^{2+}$-activated $K^+$ channels (BK) and $K_{ATP}$ channels activity has been also described in the literature as a mechanism of neuroprotection after an ischemic damage (Gribkoff et al., 2001; Yamada and Inagaki, 2005).

Accumulating evidences suggest PI3K/protein kinase B (Akt) pathway plays a crucial role in neuronal survival after cerebral ischemia. Immediately after global ischemia, a drastic decrease in the levels of phosphorylated-Akt (p-Akt) occurs, and this event precedes the release of cytochrome c and caspases activation (Ouyang et al., 1999). However, the activation of PI3K/Akt signaling pathway may mediate survival of vulnerable hippocampal CA1 neurons after transient global cerebral ischemia (Endo et al., 2006). The mechanism of neuroprotection promoted by guanosine against OGD was shown to involve the activation of PI3K/Akt pathway (Oleskovicz et al., 2008).

Therefore, the aim of this study has been to evaluate the involvement of different subtypes of potassium channels and PI3K/Akt pathway in the mechanisms of neuroprotection promoted by guanosine. Herein, we show that the addition of guanosine to hippocampal slices subjected to 15 min of OGD and followed by 2 h of re-oxygenation has been neuroprotective. The mechanism of neuroprotection promoted by guanosine involves the activation of BK channels and augmentation of glutamate uptake, which is modulated by BK channels and PI3K pathway activation. Guanosine promoted increased phosphorylated levels of Akt that resulted in neuroprotection against the damage induced by the OGD. Some of the data present here have appeared in the form of an abstract (Dal-Cim et al., 2010).

## EXPERIMENTAL PROCEDURES

### Animals

Male adult Wistar rats (60–90 days postnatal) maintained on a 12 h light–12 h dark schedule at 25 °C, with food and water *ad libitum*, have been obtained from our local breeding colony. Experiments have followed the “Principles of laboratory animal care” (NIH publication No. 85-23, revised in 1985) and they have been approved by the local Ethical Committee for Animal Research.

### Preparation and incubation of hippocampal slices

Rats were killed by decapitation and hippocampi were rapidly removed and placed in an ice-cold Krebs-Ringer bicarbonate buffer (KRB) of the following composition (in mM): 122 NaCl, 3 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 0.4 $KH_2PO_4$, 25 $NaHCO_3$ and 10 D-glucose. The buffer was bubbled with 95% $O_2$–5% $CO_2$ up to pH 7.4. Slices (0.4 mm) were prepared using a McIlwain Tissue Chopper, separated in KRB at 4 °C, and one slice per tube was allowed to recover for 30 min in KRB at 37 °C.

In the OGD model, hippocampal slices were incubated in an OGD buffer with a similar salt composition as described above. The OGD buffer consists of a Hank’s balanced salt solution (HBSS), composition in mM: 1.3 $CaCl_2$, 137 NaCl, 5 KCl, 0.65 $MgSO_4$, 0.3 $Na_2HPO_4$, 1.1 $KH_2PO_4$, 5 HEPES and 10 2-deoxyglucose (Pocock and Nicholls, 1998), and the buffer was bubbled with nitrogen throughout the incubation period (Strasser and Fischer, 1995). During the re-oxygenation period, the OGD buffer was replaced by physiological KRB and bubbled with 95% $O_2$–5% $CO_2$.

The hippocampal slices were subjected to three different conditions: control—hippocampal slices were maintained in Krebs Ringer buffer for 15 min; OGD—hippocampal slices were only subjected to OGD buffer for 15 min; OGD and re-oxygenation