DOI 10.1515/pterid-2013-0036 — Pteridines 2013; 24(3-4): 245–250

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Modulation of phenylalanine and tyrosine concentrations by ischemia and guanosine in neuronal PC12 cells

Abstract: Survival of neurons is dependent on the presence of trophic and non-trophic factors. We have previously observed the protective capacity of purine nucleosides in hypoxic neuronal cell cultures. Guanosine appeared especially interesting with respect to its remarkable neurite-stimulating aptitude. Here we report the effect of ischemic stress and guanosine on the concentration of the essential amino acids phenylalanine and tyrosine, in culture supernatants of PC12 cells. In ischemic neuronal cultures, a substantial rise of phenylalanine and tyrosine levels was observed, indicating inhibition of cellular metabolism due to neuronal stress. Elevated phenylalanine and tyrosine concentrations were reduced by the addition of guanosine. Guanosine even down-regulated the ratio of phenylalanine to tyrosine. Results may support current efforts to propagate guanosine as a neuroprotective and neuroregenerative substance.

Keywords: guanosine; ischemia; phenylalanine; phenylalanine-4-hydroxylase; PC12 cells; purine nucleosides; tyrosine.

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Introduction

Multiple signaling pathways regulate the critical balance between cell death and survival in ischemia reperfusion. Following ischemia, dying, injured, and hypoxic cells release soluble nucleotide and nucleoside pools [1], which are then further metabolized to other purine derivatives [2] that remain elevated for days after the insult [3]. We have previously studied neuronal signaling after chemical and physiological hypoxia and observed the protective capacity of purine nucleosides in both rat PC12 cells [4–6] and primary cerebellar granule neurons [7, 8] (see our review, reference [9]). Among purine nucleosides, guanosine gained our special attention due to its outstanding neurite-stimulating capacity, and we have recently reported on its neuroprotection of serum-stressed PC12 cells [10]. Guanosine derives from guanosine triphosphate (GTP) by stepwise dephosphorylation to guanosine diphosphate and guanosine monophosphate by ecto-nucleotidases [11–14]. However, GTP is also a source for the biochemical conversion by the interferon-γ -inducible enzyme GTP cyclohydrolase I [15] to 7,8-dihydroneopterin triphosphate, which is further metabolized by dephosphorylation and oxidation to neopterin, 7,8-dihydroneopterin, and biopterin derivatives [16–18] (see Figure 3). Interestingly, the protective effects clearly distinguish guanosine from its relative, 7,8-dihydroneopterin. Earlier data [19, 20] showed that increased concentrations of 7,8-dihydroneopterin might lead to enhanced apoptosis and disturbance of the redox balance of human leukemic Jurkat T cells. Raised neopterin concentrations were also detected in cerebrospinal fluids from HIV-infected patients with neurological/psychiatric symptoms [21]. Alternatively, 7,8-dihydroneopterin triphosphate is further metabolized by 6-pyruvoyl tetrahydropterin synthase to 6-pyruvoyl-tetrahydropterin, which is converted by two NADPH-dependent reductions to 5,6,7,8-tetrahydrobiopterin (BH4), the essential cofactor of phenylalanine 4-hydroxylase (PAH), which converts phenylalanine to tyrosine, and of all aromatic amino acid monoxygenases [22, 23]. Tyrosine is then further hydroxylated, in dependence of BH4 as a cofactor, to L-3, 4-dihydroxyphenylalanine, the precursor of the neurotransmitters dopamine, norepinephrine, and epinephrine [24, 25]. Increased blood levels of phenylalanine were reported in patients with various pathologies linked to inflammation and immune activation [26–29], and phenylalanine concentrations were associated with increased concentrations of the immune activation marker neopterin [30–35].

Phenylalanine is enzymatically converted by PAH to tyrosine, whereby an increase of the phenylalanine-to-tyrosine ratio may serve as an estimate of PAH activity

[36]. Increased levels of phenylalanine but also higher phenylalanine-to-tyrosine ratios have been described in patients after burns with reduced survival [27, 37] (Figure 3). Based on this coherence, we were inclined to study the effect of guanosine on phenylalanine and tyrosine concentrations in normoxic and in ischemic neuronal PC12 cell cultures.

Materials and methods

Cell culture

PC12 cells (LGC Promochem ATCC, Manassas, VA, USA) were grown in Dulbecco's Modified Eagle's Medium (DMEM) high-glucose medium supplemented with 1% Pen/Strep, 1% L-glutamine, and 10% fetal calf serum (all from PAA Laboratories, Vienna, Austria) at 37°C with 5% CO_2 and 21% O_2 on collagen-S type I-coated culture dishes (Becton Dickinson, Canaan, CT, USA). Cells were subcultured at a density of about 80% or 2 days before onset of an experiment.

Induction of ischemic conditions

In PC12 cells, ischemic stress was induced by simultaneous deprivation of oxygen and glucose (OGD). OGD was achieved using an incubator designed for maintaining hypoxia (HERAcell 240, Thermo Electron Corporation, Vienna, Austria) that was set to continuous conditions of 1% O_2 balanced with N_2 and controlled with O_2 and CO_2 sensors and additional exchange of DMEM high-glucose to glucose-free DMEM.

Cell viability assay

PC12 cells (5×10^4) were cultured for 1 day on collagen-S type I-coated chamber slides (NUNC, Rochester, NY, USA). After this time, PC12 cells were stimulated under normoxic (21% O_2, DMEM high-glucose) or ischemic (1% O_2, DMEM glucose-free) conditions. Cells were additionally treated with guanosine (500 µmol/L; Sigma-Aldrich, Vienna, Austria). After 24 h, cell viability was measured by staining the cells with fluorescent dyes (Hoechst 33342; 10 µg/mL; Life Technologies, Vienna, Austria) for 10 min and with propidium iodide (PI; 5 µg/mL; Sigma-Aldrich, Vienna, Austria) for an additional 5 min at 37°C. The Hoechst dye is membrane permeable and stains the nuclei of both

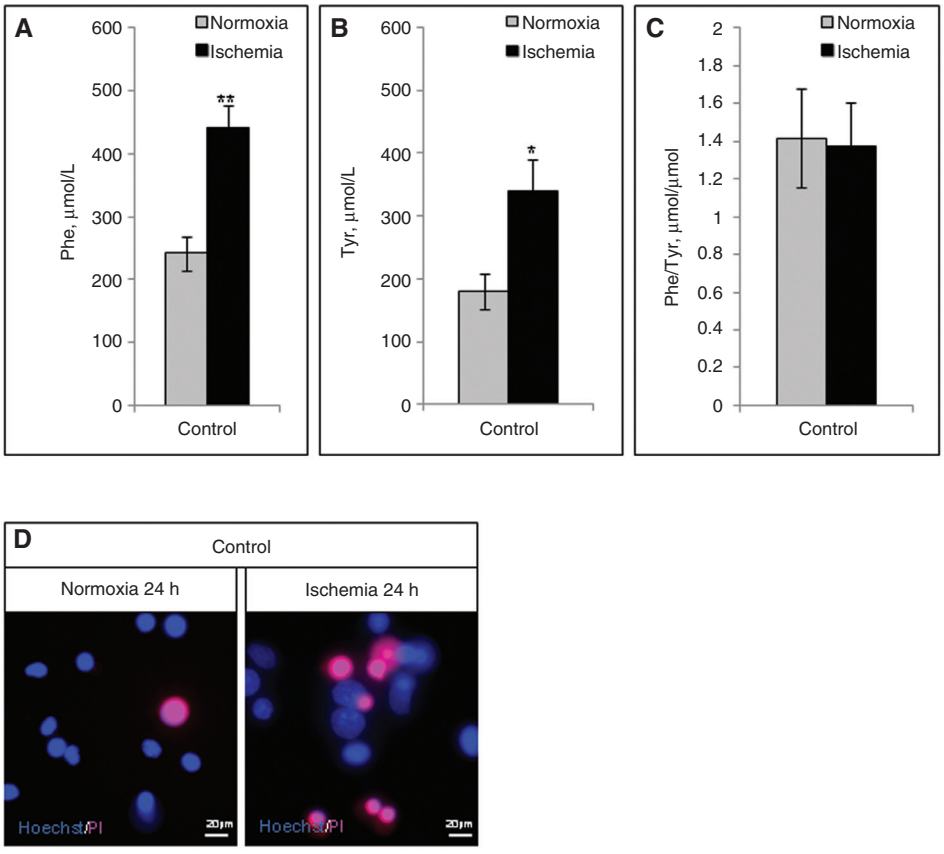


Figure 1 Phenylalanine and tyrosine concentrations in normoxic and ischemic PC12 cell cultures. Neuronal PC12 cells were cultivated in normoxic and ischemic conditions in a hypoxia incubator. Ischemic culture supernatants showed a substantial rise in phenylalanine and also, to a minor extent, in tyrosine concentrations (A and B), but phenylalanine-to-tyrosine ratios showed no significant difference in normoxic as compared to ischemic samples (C). Cell death was studied by double fluorescence analysis of the Hoechst dye (total) and propidium iodide (dead cells) in normoxic and ischemic cell cultures (D). Values represent the mean±SEM, n=3. Differences were analyzed using unpaired one-tailed t-test: *p<0.05, **p<0.01.

living and dead cells (total cell number). However, PI is membrane impermeable and stains only the DNA of cells with disrupted plasma membranes. Cells were visualized on a fluorescence microscope (Zeiss Axioplan2, Zeiss, Vienna, Austria) equipped with a spot camera (RT-slider 2.3.1, Visitron Systems, Puchheim, Germany), using filters for Hoechst (excitation: 400 nm; emission: 420 nm) and PI (excitation: 570 nm; emission: 590 nm). Pictures of both fluorescence excitations were merged in Adobe Photoshop 6.01 (Adobe Systems Inc., San Jose, USA), and double-stained cells (pink) represent dead and blue viable ones.

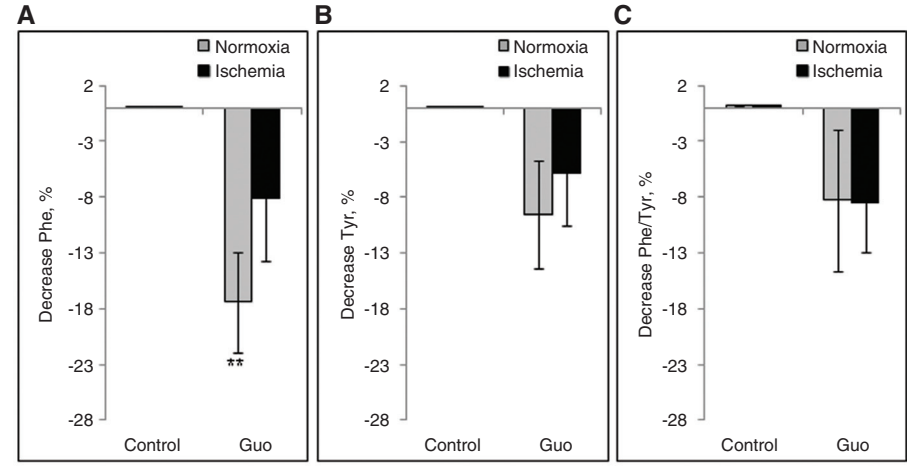
Phenylalanine and tyrosine analysis

PC12 cells (1×10^7) were cultured on collagen-S type I-coated dishes and stimulated under normoxic (21% O_2, DMEM high-glucose) or ischemic (1% O_2, DMEM glucose-free) conditions. Cells were additionally treated with guanosine (500 µmol/L) for 24 h. After this time point, supernatants were collected and measurements of phenylalanine and tyrosine concentrations were performed as previously described [38].

In brief, 100 µL of supernatant, 100 µL of internal standard, 500 µmol/L of 3-nitro-L-tyrosine, and 25 µL of 2 mol/L of trichloroacetic acid were vortexed and centrifuged to precipitate proteins. Supernatants were diluted 1:25 with 15 mmol/L of potassium dihydrogen phosphate. An albumin-based mixture with 100 µmol/L of phenylalanine and tyrosine was treated in the same way as supernatants and served as an external calibrator. Separation of analytes was achieved on reversed-phase high-performance liquid chromatography using 15 mmol/L of aqueous potassium dihydrogen phosphate as an elution buffer. Phenylalanine and tyrosine were detected by their natural fluorescence at an excitation wavelength of 210 nm and an emission wavelength of 302 nm [38], employing a fluorescence detection device (ProStar 360, Varian, Palo Alto, CA, USA), and the phenylalanine-to-tyrosine ratio was calculated.

Statistical analysis

All data represent the means±standard error of the mean of the indicated number of independent experiments. Statistical analysis was performed using SPSS software (version 19.0.0; IBM, Chicago, USA). With an unpaired one-tailed t-test, the significance of differences between two experimental groups was assessed. The acceptance level of statistical significance was $p<0.05$.



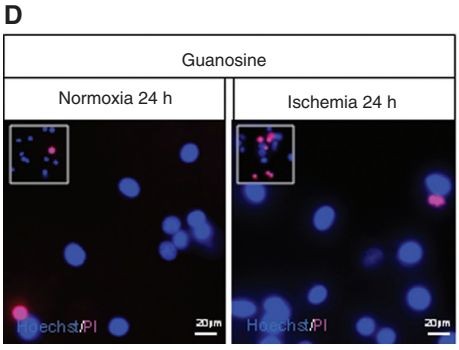


Figure 2 Effect of guanosine on phenylalanine and tyrosine concentrations in normoxic and ischemic PC12 cell cultures. Neuronal PC12 cells were cultivated in normoxic and ischemic conditions in a hypoxia incubator with and without the addition of purine nucleoside guanosine. Guanosine-treated cell culture supernatants showed a substantial decrease in phenylalanine and also, to a minor extent, in tyrosine concentrations (A and B), accompanied by decreased phenylalanine-to-tyrosine ratios (C). Cell death was studied by double fluorescence analysis of the Hoechst dye (total) and propidium iodide (dead cells) in normoxic and ischemic cell cultures treated with and without guanosine and reflects its neuroprotective effect (D). Values represent the mean±SEM, n=3. Differences were analyzed using unpaired one-tailed t-test: **$p<0.01$.

Results and discussion

Following cultivation of neuronal PC12 cells in normoxic and ischemic conditions in a hypoxia incubator, ischemic culture supernatants showed a substantial rise in phenylalanine and also, to a minor extent, in tyrosine concentrations (Figure 1A and B).

Increased blood levels of phenylalanine were reported in patients with various pathologies linked to inflammation and immune activation [26–29], whereby an increase of the essential amino acid phenylalanine is likely to result from an impaired conversion of phenylalanine to tyrosine by PAH. Disturbances of BH4 levels are, for example, critical in individuals suffering from neurodegenerative diseases [33, 35]. An impaired PAH activity could be due to insufficient supply of the cofactor BH4, since BH4 was shown to be very sensitive to oxidative stress [39]. Its depletion may lead to a suppression of BH4-dependent enzymes like PAH or tyrosine hydroxylase [40]. This fact may explain why tyrosine concentrations were also increased in our supernatants from ischemic cultures as compared to control values. Hence, phenylalanine-to-tyrosine ratios, which allow an estimate of the activity of PAH [25, 36, 41], showed no significant difference in normoxic as compared to hypoxic samples and potentially reflect an inhibition of cellular metabolism due to ischemic starvation rather than to a specific effect on enzymes related to phenylalanine metabolism (Figure 1C).

In addition, cell death was studied by double fluorescence analysis of the Hoechst dye and propidium iodide. In agreement with earlier studies by our group [4, 42, 43], ischemia led to a substantial increase in cell death (Figure 1D) and thus support the hypothesis that up-regulated phenylalanine and tyrosine concentrations are a direct consequence of ischemic starvation.

The addition of guanosine significantly rescued PC12 cell death induced by ischemia (Figure 2). The data presented here are in line with previous reports on the positive effects of purine nucleosides on hypoxic neuronal cells (for a review, see reference [9]) and support current efforts to propagate purine nucleosides as neuroprotective and neuroregenerative substances [9, 44–50]. In support of these data, results obtained from the analysis of phenylalanine and tyrosine concentrations showed that, upon addition of guanosine, phenylalanine as well as tyrosine levels were diminished (Figure 2A and B). Interestingly, the phenylalanine-to-tyrosine ratio was also reduced by guanosine. However, the difference was only significant for phenylalanine under normoxic conditions (Figure 2A).

Analysis of cell viability showed that guanosine may efficiently protect PC12 cells from ischemic insult (Figure 2D). The data are in line with previous findings of guanosine-mediated protection in serum-starved [10] and

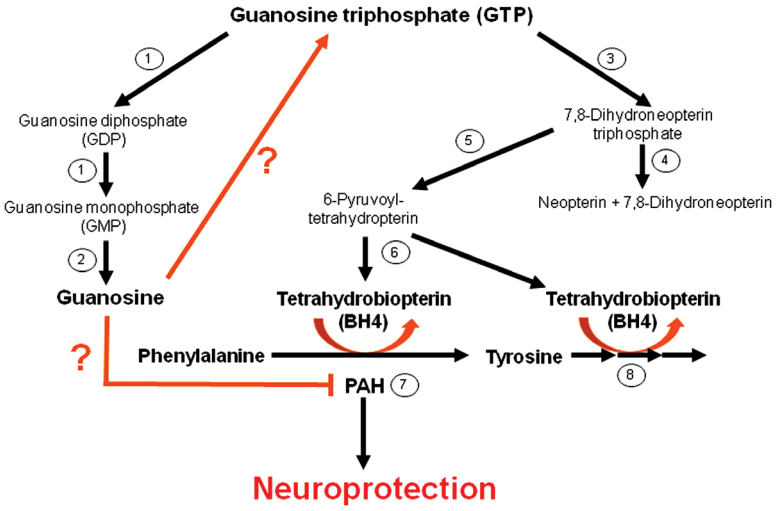


Figure 3 Schematic model of the sources of guanosine and other products derived from GTP. The purine nucleoside guanosine is the product of the stepwise dephosphorylation of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) and guanosine monophosphate (GMP) by ectonucleotide diphosphohydrolases (E-NTPDases, 1) and by ecto-5′-nucleotidase (2). In addition, GTP can be enzymatically cleaved by GTP-cyclohydrolase I (GCH-I, 3) to 7,8-dihydroneopterin triphosphate. Neopterin and 7,8-dihydroneopterin are then metabolized by dephosphorylation and oxidation (4). Alternatively, 7,8-dihydroneopterin triphosphate is further metabolized by 6-pyruvoyl tetrahydropterin synthase to 6-pyruvoyl-tetrahydropterin (5), which is converted by two NADPH-dependent reductions (6) to tetrahydrobiopterin (BH4). BH4 is the essential cofactor of phenylalanine 4-hydroxylase (PAH, 7), converting phenylalanine to tyrosine, and of other monoxygenases (8). Guanosine-mediated downregulation of phenylalanine-to-tyrosine conversion potentially contributes to its neuroprotective effects either by directly controlling PAH activity or by feedback-regulation of GTP and BH4 concentrations.

in hypoxic neuronal cell cultures [42] and support current efforts analyzing guanosine-mediated neuroprotection [9].

This result might imply either a direct specific guanosine-mediated stimulating effect on PAH activity – although alterations of phenylalanine-to-tyrosine ratios were not significant – or a guanosine-mediated elevation of the GTP pool, which potentially leads to elevated levels of BH4 [51] (Figure 3). Based on our data, we assume that the GTP-derivative guanosine mediates the protection of cells in stress situations by regulation of different mechanisms. As discussed earlier, guanosine was shown to regulate specific intrinsic signaling mechanisms [9, 42], and these mechanisms are likely supported by the regulation of the levels of essential amino acids such as phenylalanine and tyrosine (Figure 3). The exact mechanism, however, will be subject to future investigations.

Received October 22, 2013; accepted October 30, 2013

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