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Research Paper

Improvement of neuronal differentiation by carbon monoxide: Role of pentose phosphate pathway

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ABSTRACT

Over the last decades, the silent-killer carbon monoxide (CO) has been shown to also be an endogenous cytoprotective molecule able to inhibit cell death and modulate mitochondrial metabolism. Neuronal metabolism is mostly oxidative and neurons also use glucose for maintaining their anti-oxidant status by generation of reduced glutathione (GSH) via the pentose-phosphate pathway (PPP). It is established that neuronal differentiation depends on reactive oxygen species (ROS) generation and signalling, however there is a lack of information about modulation of the PPP during adult neurogenesis. Thus, the main goal of this study was to unravel the role of CO on cell metabolism during neuronal differentiation, particularly by targeting PPP flux and GSH levels as anti-oxidant system.

A human neuroblastoma SH-S5Y5 cell line was used, which differentiates into post-mitotic neurons by treatment with retinoic acid (RA), supplemented or not with CO-releasing molecule-A1 (CORM-A1). SH-SY5Y cell differentiation supplemented with CORM-A1 prompted an increase in neuronal yield production. It did, however, not alter glycolytic metabolism, but increased the PPP. In fact, CORM-A1 treatment stimulated (i) mRNA expression of 6-phosphogluconate dehydrogenase (PGDH) and transketolase (TKT), which are enzymes for oxidative and non-oxidative phases of the PPP, respectively and (ii) protein expression and activity of glucose 6-phosphate dehydrogenase (G6PD) the rate-limiting enzyme of the PPP. Likewise, whenever G6PD was knocked-down CO-induced improvement on neuronal differentiation was reverted, while pharmacological inhibition of GSH synthesis did not change CO's effect on the improvement of neuronal differentiation. Both results indicate the key role of PPP in CO-modulation of neuronal differentiation. Furthermore, at the end of SH-SY5Y neuronal differentiation process, CORM-A1 supplementation increased the ratio of reduced and oxidized glutathione (GSH/GSSG) without alteration of GSH metabolism. These data corroborate with PPP stimulation. In conclusion, CO improves neuronal differentiation of SH-S5Y5 cells by stimulating the PPP and modulating the GSH system.

1. Introduction

Stem cell fate can be regulated by various factors, namely cellular energy metabolism, which is capable of modulating stem cell decision between self-renewing or differentiation [1–3]. Thus, manipulation of cell metabolism can be a key tool for neurogenesis modulation. In fact, stimulation of endogenous neurogenesis can be particularly important for replacement of impaired neurons in central nervous system (CNS), in future potential applications against neurodegenerative diseases including ischemic stroke and psychiatric disorders.

Abbreviations: G6PD, glucose 6-phosphate dehydrogenase; CNS, central nervous system; CO, carbon monoxide; CORM-A1, Carbon monoxide-releasing molecule A1; COX, cytochrome c oxidase; DMEM/F12, Dulbecco's minimum essential medium with nutrient mixture F12; FBS, foetal bovine serum; Cys, cysteine; CysGly, cysteine-glycine; GC-MS, gas chromatography coupled with mass spectrometry analysis; G6PD, glucose 6-phosphate dehydrogenase; GSH, reduced glutathione; GSSG, oxidized glutathione; HO-1, Haem-oxygenase 1; HPLC, High performance liquid chromatography; LDH, lactate dehydrogenase; NAC, N-acetylcysteine; NSC, neural stem cell; PBS, phosphate buffered saline; PDH, pyruvate dehydrogenase; Pen/Strep, penicillin/streptomycin solution; PGD, phosphogluconate dehydrogenase; PPP, Pentose phosphate pathway; RA, retinoic acid; ROS, reactive oxygen species; RT-Q-PCR, Reverse transcriptase quantitative polymerase chain reaction; R.T., room temperature; TCA, Tricarboxilic acid cycle; TKT, transketolase; UV, ultra violet radiation

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The Pentose Phosphate Pathway (PPP) is an important route of glucose oxidation, divided into two branches, the oxidative and non-oxidative phase, where, by multiple reactions, sugar phosphates are interconverted. The oxidative phase of PPP is linked to glycolysis at the level of glucose-6-phosphate and catalyses its conversion into ribulose-5-phosphate and CO_2. Also in this phase, $NADP^+$ is reduced into NADPH, the major reducing compound, which is required for re-generation of reduced glutathione (GSH). On the other hand, the non-oxidative phase converts pentose phosphates into phosphorylated aldoses and ketones. This branch is linked to glycolysis by their common intermediates glyceraldehyde-3-phosphate and fructose-6-phosphate and it also produces ribose-5-phosphates, which are precursors for nucleotide synthesis [4,5]. The activity of this non-oxidative phase of PPP supports cellular proliferation during neurogenesis by the production of building blocks [6]. In addition, during neuronal differentiation process, there is an enhancement of mitochondrial and oxidative cell metabolism [3,7], which, in turn, increases ROS production. Thus, one can speculate oxidative phase of PPP can be critical during neuronal differentiation for reinforcing cellular anti-oxidant defence. Indeed, PPP generates reducing molecules NADPH, which facilitates recycling of oxidized GSSG into reduced GSH, the first line of cell antioxidant defence [8].

CO is mostly known as a silent-killer due to its great affinity to haem-proteins, such as haemoglobin or cytochrome c oxidase (COX). Thus, high levels of CO can compromise systemic oxygen delivery or cellular mitochondrial function, promoting high levels of intoxication and even death. Nevertheless, CO is an endogenously produced gasotransmitter generated by the cleavage of haem group via the enzymatic activity of haem-oxygenase (HO) [9]. HO is a stress-related enzyme, whose expression or activity increases in response to several stressful stimuli including oxidative stress, hypoxia, hyperoxia, hyperthermia, inflammation, UV and misfolded protein response [9–11]. Furthermore, it has been demonstrated that low levels of exogenous CO promote cytoprotection, limit inflammation, prevent cell death and improve neuronal differentiation [9,10,12–18]. The molecular mechanisms underlying CO-induced cytoprotection are associated with improvement of mitochondrial function and are dependent on generation of low amounts of ROS, as signalling molecules [12,19–22]. Likewise, low concentrations of CO promote mitochondrial biogenesis [23,24], increase COX activity [13,25–28], improve oxidative metabolism [23,29] and induce mild mitochondrial uncoupling that protects mitochondria from oxidative stress [30,31]. For further reading, there are reviews [23,32,33]. Because of the great potential use of CO as a therapeutic gas, several strategies to deliver CO under biological context have been developed. CO-releasing molecules (CORM) are small organic or organometallic molecules able to release CO under a more physiologically relevant way than CO gas applications [34]. CORM-A1 (carbon-monoxide releasing molecule A1) is a boron-based molecule that has been often studied because it slowly releases CO under a controlled manner. CO-releasing is dependent on temperature and pH, with optimal release at pH of 7.4 and 37 °C and it presents a half-life of approximately 21 min to transfer CO to myoglobin in vitro [35,36].

Recently, it has been demonstrated that CO promotes neuronal differentiation [16] and increases dopaminergic differentiation [18]. The underlying molecular mechanisms of neuronal differentiation involve CO-induced improvement of mitochondrial metabolism [17]. Likewise, CO modulates cellular GSH levels in astrocytes [12] and GSSG/GSH recycling is dependent on PPP. Thus, it is hypothesized that CO can stimulate PPP flux, which in turn, facilitates the cellular machinery rearrangement needed during neuronal differentiation. For assessing PPP modulation by CO, a human neuroblastoma SH-S5Y5 cell line was used as cell model. This is a simple model to study neuronal differentiation process [37], allowing the assessment of the associated cellular mechanisms. SH-SY5Y cells are derived from neural crest [38,39] and present the ability to differentiate into neuron-like cells that fulfil the morphological, biochemical and functional neuronal criteria [37,39,40], constituting a valuable model also for neuronal toxicity studies [41–44].

The main goal of this study was to assess the metabolic regulation of neuronal differentiation achieved by CO, in particular, the role of PPP. CORM-A1 supplementation increased the yield of neuronal production following SH-SY5Y neuronal differentiation, along with an increase of ROS generation, PPP flux and a change in GSH availability. Likewise, whenever the limiting PPP enzyme glucose 6-phosphate dehydrogenase (G6PD) was knocked down, the CO-induced increase of neuronal yield was reverted, while pharmacological inhibition of GSH synthesis had no effect on neuronal differentiation. In conclusion, in the SH-SY5Y model of adult neurogenesis, CO improves neuronal differentiation in a PPP dependent manner.

2. Material and methods

2.1. Materials

All chemicals were of analytical grade and were obtained from Sigma unless stated otherwise. Plastic tissue culture dishes were from Sarstedt (Germany); foetal bovine serum, penicillin/streptomycin solution, and Dulbecco's minimum essential medium (high glucose, L-glutamine and pyruvate) were obtained from Invitrogen (United Kingdom).

The mass spectrometry derivatization reagents MTBSTFA (*N*-methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide), MSTFA (*n*-methyl-*n*-(trimethylsilyl) trifluoroacetamide) and the t-BDMS-Cl (tert-butyldimethylchlorosilane) were purchased from Regis Technologies, Inc. (Morton Grove, IL, USA). All other chemicals were of the purest grade available from regular commercial sources.

For liquid chromatography, the reduction reagent TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) and derivatization reagent SBD-F (7-fluorobenzofurazan-4-sulfonic acid ammonium salt) and the standards used, namely Cys (cysteine), CysGly (cysteinylglycine), GluCys (glutamylcysteine) and GSH (γ-glutamyl-cysteinylglycine) were purchased from Sigma-Aldrich.

2.2. SH-SY5Y human neuroblastoma cell line

2.2.1. Maintenance of undifferentiated cells

The SH-SY5Y cell line was cultured in DMEM/F-12 supplemented with 10% (v/v) FBS and 2% (v/v) Pen/Strep (growth medium). Cells were maintained in a humidified atmosphere of 5% (v/v) CO_2 at 37 °C. Undifferentiated cells were grown in 75 cm^2 T-flasks and sub-cultured with fresh growth medium, whenever cell confluence achieved (about 80–90% cell confluence). Cells were detached by trypsinization at room temperature (R.T.) and slight shaking and hitting to drain down cells with trypsin and resuspended in growth medium in a 1:4 cell passage. Growth medium was changed twice a week.

2.2.2. Neuronal differentiation protocol

Following trypsinization and resuspension in growth medium, cells were plated on 75 cm^2 T-flasks in a 1:2 cell passage. Neuronal differentiation was induced 24 h after plating undifferentiated cells to ensure settle and attachment to flask surface and attain appropriate density, approximately about 50% cell confluence in all 75 cm^2 T-flasks.

Neuronal differentiation was stimulated using DMEM/F-12 medium, reduced serum to 1% (v/v) FBS, 2% (v/v) Pen/Strep and supplemented with 10 μM of *all-trans* RA (differentiation medium). CO effect was studied by using the same composition of differentiation medium supplemented with 25 μM CORM-A1. Whenever necessary, buthionine sulfoximine (BSO) at 50 μM was added in differentiation medium for preventing GSH synthesis. Differentiation medium was replaced twice (day 1 and day 4) during 7 days of treatment (Fig. 1A). On day 7, cells were collected for analysis.