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Hexahydropyridoindoles as potential inducers of cellular antioxidant and anti-inflammatory response

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Introduction

Among the various synthesized hexahydro-pyridoindole derivatives, the compound (±)-cis-8-methoxy-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indole-2-carboxylic acid ethyl ester (SMe1EC2) [1] has been subjected to several preclinical studies. In particular, it showed significant neuroprotective and antioxidant effects in the murine model of acute head trauma [2], rat hippocampal slices exposed to reversible hypoxia/low glucose [2, 3], brain cortex homogenates of young rats treated with Fe^{2+}/ascorbic acid pro-oxidative system [4] and HT22 hippocampal neuronal cells subjected to high glucose [5]. Moreover, in our *in vivo* study, 8-week supplementation with SMe1EC2 showed a moderately enhancing effect on cognitive function in aged rats [6]. SMe1EC2 also improved cardiometabolic parameters and reduced oxidative stress and inflammatory markers in our experimental model of metabolic syndrome [7].

In the present study, we assessed the potential effect of SMe1EC2 on the expression levels of antioxidant and anti-inflammatory mediators and pro-inflammatory markers in activated murine BV-2 microglial cells.

Materials and Methods

Cells were plated in a 6-well plate at the density of 60,000 cells/cm^2, grown for 24 h followed by 16h incubation in DMEM with LPS (2μg/ml) with or without SMe1EC2 (200μmol/l). Next, the cells were lysed in Cell Lysis Buffer (1X, Cell Signalling Technologies, Inc.) with 1mM PMSF and homogenized by passing 15 times through a 25G-needle followed by 20 min incubation on ice and centrifugation (12 000 rpm, 4°C, 15 min). Then, equal amounts (30μg) of proteins were denatured and separated by SDS-polyacrylamide gel electrophoresis. Next, proteins were transferred by Western blotting to the nitrocellulose membrane. Membranes were afterward blocked with 3 % BSA or 5 % non-fat milk in PBS-T (PBS with 0.1 % (v/v) Tween-20) for 2h and incubated with primary antibodies against inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), heme oxygenase-1 (HOX-1) and β-actin (Cell Signalling Technologies, Inc.) overnight at 4°C. After four washes with PBS-T, membranes were incubated with a secondary anti-rabbit horseradish peroxidase-conjugated antibody and detected using a western blotting luminol reagent. Densitometric analyses were performed by using ImageJ software. The levels of 15-deoxy-Δ12,14-prostaglandin J2 (15-d-PGJ2) in the culture medium were analyzed using the commercial kit (Enzo

Life Sciences) following the manufacturer's protocol. Experiments were repeated minimally three times. All the values were expressed as mean ± standard error of the mean (S.E.M.). For multiple comparisons, *p* values were calculated using a one-way analysis of variances (ANOVA) with Tukey's post hoc analysis, where homogeneity of variances was met. Otherwise, Games-Howell post hoc analysis was used.

Results and Discussion

In our study, SMe1EC2 downregulated LPS-elicited proinflammatory iNOS expression (**Fig. 1A, E**) along with a significant increase in 15d-PGJ2 production (**Fig. 1B**) and mild enhancement of the levels of HO-1, an Nrf2-regulated phase II detoxifying enzyme with antioxidant and anti-inflammatory roles (**Fig. 1A, C**). Unpredictably, SMe1EC2 also enhanced the COX-2 levels induced by LPS treatment (**Fig. 1A, D**).

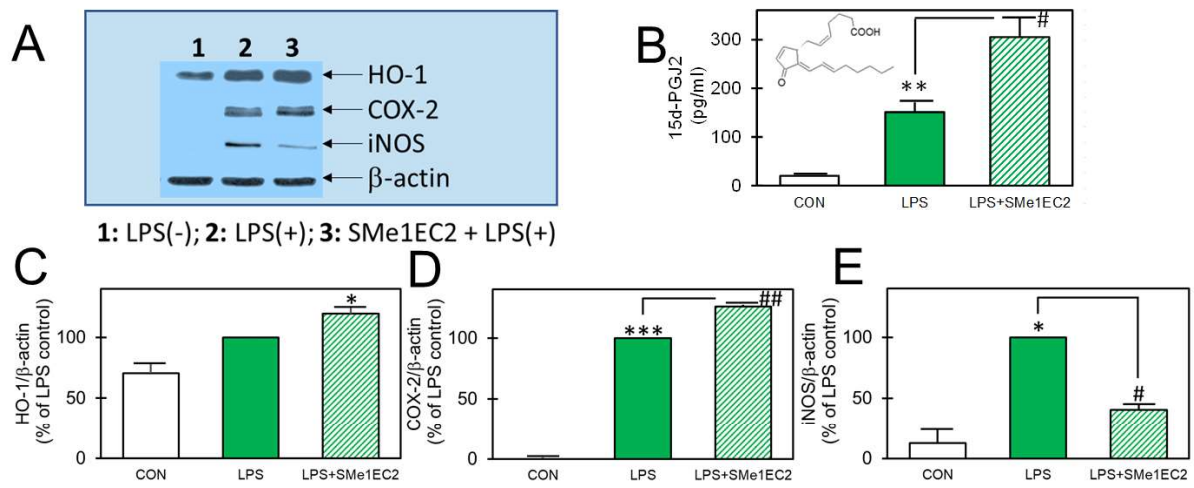

Figure 1. The effect of SMe1EC2 on the expression levels of antioxidant and anti-inflammatory mediators and pro-inflammatory markers in LPS-activated murine BV-2 microglial cells. **A:** Representative Western blots of proteins HO-1, COX-2, and iNOS; **B:** 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15-d-PGJ2) levels measured by ELISA in culture medium. **C, D, E:** Densitometric analysis of proteins. Protein levels were normalized to β-actin. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ *vs*. control cells, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ *vs*. LPS-stimulated cells; LPS – lipopolysaccharide; CON – control.

In a paradox, COX-2 is also a well-accepted mediator of anti-inflammatory processes since it also plays a role in the resolution of inflammation and establishment of the acute inflammatory response [8]. COX-2 has been recognized as a pro-inflammatory enzyme promoting the synthesis of prostaglandin E2 (PGE2), an essential component of the inflammatory cascade [9]. Nevertheless, COX-2 was found to mediate the intracellular accumulation of 15d-PGJ2, a cyclopentenone PG, which employs its anti-inflammatory activity through activation of

peroxisome proliferator-activated receptor-γ (PPAR-γ) [10, 11] or by directly inhibiting NFκB activation by binding covalently to the IκB kinase [12]. Furthermore, COX-2 was shown to activate Nrf2, which in turn regulates the expression of antioxidant enzymes in activated inflammatory macrophages [13, 14]. Moreover, the results of Luo et al., 2015 pointed to a novel role of Nrf2 in inducing COX-2 expression through binding to promoter ARE in the absence of increased ROS in rat preglomerular vascular smooth muscle cells [15].

15d-PGJ2 exerts protective properties in diverse cell systems [16]. It was reported to suppress the p22phox expression to protect against apoptosis of neurons in a PPAR-γ-dependent manner [17]. Also, 15d-PGJ2 induced negative regulator of ROS (NRROS) expression mediated through a PI3K/Akt-dependent FoxO1 and Sp1 phosphorylation and Nrf2 cascade suppressing ROS generation in astrocytes [18].

Thus, the enhancing effect of SMe1EC2 and potentially of other pyridoindole congeners on 15d-PGJ2 release can contribute to restoring intracellular redox homeostasis and suppressing inflammatory processes mediated by activated microglia in the CNS.

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