Neuroscience 151 (2008) 758–770

SELECTIVE REGULATION OF NEUROSTEROID BIOSYNTHESIS IN HUMAN NEUROBLASTOMA CELLS UNDER HYDROGEN PEROXIDE–INDUCED OXIDATIVE STRESS CONDITION

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Abstract—Neurosteroid biosynthesis is demonstrated in many species but key factors interacting with neurosteroidogenesis under pathophysiological conditions are unknown. Hydrogen peroxide (H_2O_2)–induced oxidative stress is an etiological factor involved in several disorders. We hypothesized that, if neurosteroidogenesis is a pivotal mechanism for nerve cell protection or viability, it might be selectively regulated under oxidative stress condition. To check our hypothesis, we investigated H_2O_2 effects on neurosteroidogenesis in human neuroblastoma SH-SY5Y cells. Pulse-chase, high performance liquid chromatography and flow-scintillation analyses showed that, along neurosteroidogenic pathways converting pregnenolone into various neurosteroids, only estradiol synthesis selectively decreased in SH-SY5Y cells after H_2O_2-treatment. Testosterone conversion into estradiol was also inhibited by H_2O_2. Real-time reverse transcription–polymerase chain reaction revealed aromatase gene repression in SH-SY5Y cells 12 h after the oxidative stress onset. Consistently, viability assays showed that chronic inhibition of aromatase activity by letrozole killed neuroblastoma cells. A 12-h pretreatment of SH-SY5Y cells with estradiol was protective against H_2O_2-induced death. In addition, estradiol was also capable of rescuing markedly neuroblastoma cells from letrozole-evoked death. Altogether, these results suggest that endogenous estradiol formation is pivotal for SH-SY5Y cell viability. Serum deprivation-evoked stress, which also killed SH-SY5Y cells, unaffected neurosteroidogenesis, indicating that inhibitory effect on neuroprotective-neurosteroid estradiol biosynthesis is specific for H_2O_2-induced stress. Selective targeting of neurosteroidogenic pathways may therefore constitute an interesting strategy against H_2O_2-evoked neurodegenerative processes.

Key words: aromatase gene expression, neurosteroidogenesis regulator, steroid and nervous system, neural disorder, nerve cell viability, real time PCR.

Investigations aiming to establish a link between neural diseases and steroid hormones were launched several years ago since steroids, which exert pleiotropic effects in the body, regulate numerous biological mechanisms (Sunderland et al., 1989; Leblhuber et al., 1993; Carlson et al., 1999). Many attempts were made to correlate plasma levels of neuroactive steroids such as cortisol, dehydroepiandrosterone (DHEA) or its sulfated derivative (dehydroepiandrosterone sulfate, DHEAS) with severity of symptoms in Alzheimer's disease (AD) patients but these attempts generated contradictory results hardly exploitable (Davis et al., 1985; Weiner et al., 1993; Legrain et al., 1995; Näsman et al., 1995; Murialdo et al., 2000; Rasmuson et al., 2002). Controversial data were also provided by studies which investigated the possible relationship between plasma concentrations of DHEA or cortisol and the development of schizophrenic symptoms or the gait dynamics in Parkinson's disease (Bellomo et al., 1991; Charlett et al., 1998; Harris et al., 2001; Shirayama et al., 2002; Ritsner et al., 2004, 2006; di Michele et al., 2005; Gallagher et al., 2007). Therefore, it appeared that plasma levels of steroid hormones produced by peripheral glands may not be a good index reflecting accurately the potential risk or the degree of activation of endogenous processes leading to the development of neuropathological disorders. To answer this question, a series of studies performed over the last decade have measured the levels of DHEA, DHEAS, pregnenolone (PREG) and pregnenolone sulfate (PREGS) in the cerebrospinal fluid of controls, AD or vascular dementia patients and in postmortem brain (Weill-Engerer et al., 2002; Kim et al., 2003). These studies, which observed a good correlation between decreased DHEAS or PREGS levels and AD or vascular dementia, suggested that the concentrations of neurosteroids (steroids directly synthesized in the nervous system) might be more suitable than the levels of peripheral steroids to establish a causal link with neuropathological diseases. In support of this hypothesis, we have recently shown that the overexpression of key proteins involved in AD interferes with neurosteroidogenesis in human neuroblastoma SH-SY5Y cells, suggesting that various pathogenic factors may induce neurodegenera-

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Abbreviations: AD, Alzheimer's disease; DCM, dichloromethane; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DMEM, Dulbecco's modified Eagle medium; Flo/One, continuous flow scintillation detection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high performance liquid chromatography; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; OD, optical density; PREG, pregnenolone; PREGS, pregnenolone sulfate; PROG, progesterone; ROS, reactive oxygen species; rt-RT-PCR, reverse transcription followed by real-time polymerase chain reaction; T, testosterone; [^{3}H]PREG, 7-^{3}H(N)-pregnenolone; [^{3}H]PROG, 1,2,6,7-^{3}H(N)-progesterone; [^{3}H]T, 1,2,6,7-^{3}H(N)-testosterone; [^{3}H]3αDIOL, 9,11-^{3}H(N)-3α-androstanediol; [^{3}H]17OHPROG, 17α-hydroxy-[1,2,6,7-^{3}H]-progesterone; 3αDIOL, 3α-androstanediol.

0306-4522/08$32.00+0.00 © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2007.11.032

tive disorders through a down-regulation of neuroprotective steroid biosynthesis in nerve cells (Schaeffer et al., 2006).

Among the pivotal pathogenic processes involved in the etiology of neural diseases is oxidative stress induced by excessive amounts of reactive oxygen species (ROS) such as hydrogen peroxide or H_2O_2 (Beal, 1995; Mizuno et al., 1998; Giasson et al., 2002; Andersen, 2004). Postmortem brain tissues from patients with Parkinson's disease, AD and amyotrophic lateral sclerosis clearly display increased indices of ROS in affected brain regions (Dexter et al., 1989; Yoritaka et al., 1996; Smith et al., 1997; Good et al., 1998; Hensley et al., 1998; Butterfield et al., 2002; Andersen, 2004). Oxidative cell injury is also involved in the pathophysiology of schizophrenia (Lohr et al., 1990; Mahadik and Mukherjee, 1996; Reddy and Yao, 1996; Khan et al., 2002). In the ROS family, H_2O_2 is well-known to mediate brain damages caused by the accumulation of β-amyloid peptide in AD (Behl et al., 1994; Schubert et al., 1995; Yatin et al., 1999; Miranda et al., 2000; Tamagno et al., 2003; Tabner et al., 2005). Moreover, H_2O_2-evoked oxidative stress has also been identified as a pivotal mechanism mediating cytotoxic action of neuropathologic factors such as α-synuclein, 6-hydroxydopamine and the prion protein (Soto-Otero et al., 2000; Turnbull et al., 2001, 2003; Andersen, 2004). Therefore, we made the hypothesis that, if the process of neurosteroid biosynthesis is a pivotal mechanism intervening in the protection or viability of nerve cells, it might be regulated or significantly affected under oxidative stress conditions. Indeed, although the process of neurosteroidogenesis has been evidenced in several animal species, little is known about its regulation; in particular, the key factors interacting with neurosteroid biosynthesis under pathophysiological conditions are totally unknown (Baulieu et al., 1999; Mensah-Nyagan et al., 1999; Patte-Mensah et al., 2006). This situation seriously hampers the exploitation of neurosteroids or pharmacological regulators of neurosteroidogenic pathways to develop therapeutic strategies against neural disorders.

To determine whether or not the process of neurosteroidogenesis belongs to cellular mechanisms selectively regulated during oxidative stress-induced neural disorders, we decided to investigate the effect of H_2O_2 on neurosteroid biosynthesis in human neuroblastoma SH-SY5Y cells. This cell line, which displays neuronal phenotype and properties, is a representative model for biochemical investigations of neurodegenerative diseases (Li et al., 1996; Misonou et al., 2000; Olivieri et al., 2002; Jämsä et al., 2004). SH-SY5Y cells were also characterized as neurosteroid-producing cells containing key neurosteroidogenic enzymes (Melcangi et al., 1993; Wozniak et al., 1998; Guarneri et al., 2000). To tackle the question in an effective manner, we combined several approaches including MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) reduction assays to assess cell viability, reverse transcription followed by real-time polymerase chain reaction (rt-RT-PCR) to detect the impact of H_2O_2 on neurosteroidogenic enzyme gene expression, pulse-chase experiments, high performance liquid chromatography (HPLC) and flow scintillation detection (Flo/One) to characterize neurosteroid newly-synthesized in neuroblastoma cells under physiological and H_2O_2-induced oxidative stress conditions.

EXPERIMENTAL PROCEDURES

Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), Glutamax, penicillin/streptomycin, propylene glycol, H_2O_2, 17β-estradiol and MTT were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum and horse serum were from Gibco (Basel, Switzerland). Synthetic steroids including PREG, progesterone (PROG), 17α-hydroxyprogesterone (17OHPROG), testosterone (T), 3α-androstanediol (3αDIOL) were purchased from Steraloids (Newport, RI, USA). Dichloromethane (DCM) was from Acros Organics (Noisy Le Grand, France). Hexane and isopropanol were obtained from Fischer Bioblock Scientific (Illkirch, France). Tritiated steroids such as 7-^{3}H(N)-pregnenolone ([^{3}H]PREG), 1,2,6,7-^{3}H(N)-progesterone ([^{3}H]PROG), 1,2,6,7-^{3}H(N)-testosterone ([^{3}H]T) and 9,11-^{3}H(N)-3α-androstanediol ([^{3}H]3αDIOL) were obtained from PerkinElmer (Boston, MA, USA). 17α-Hydroxy-[1,2,6,7-^{3}H]-progesterone ([^{3}H]17OHPROG) was purchased from Amersham (Piscataway, NJ, USA). Letrozole was a generous gift from Dr. D. Evans of Novartis (Basel, Switzerland).

Cell culture

Human neuroblastoma SH-SY5Y cells were grown at 37 °C under an atmosphere of 5% CO_2 in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 5% (v/v) heat-inactivated horse serum, 2 mM Glutamax and 1% (v/v) penicillin/streptomycin (normal culture medium). Cells were passaged every 3–4 days and were used for pulse-chase experiments when they reached 80–90% confluence.

MTT reduction assay

To assess cell viability, MTT reduction assays were performed. SH-SY5Y cells were seeded at 5×10^4 cells per well into 96-well plates and allowed to attach. After 48 h, SH-SY5Y cells were incubated in the following conditions for evaluation of the effects of H_2O_2-induced oxidative stress, serum deprivation-evoked stress or letrozole (aromatase inhibitor) on cell viability: (i) normal culture medium containing H_2O_2 at various concentrations (for 3 h, 6 h, 12 h, 24 h or 48 h in order to determine effective H_2O_2 concentrations and incubation times inducing the death of a significant percentage of cells); (ii) serum deprived medium for 48 h; (iii) normal culture medium containing letrozole at various concentrations (from 0.1–8 μM) for 4 days; and (iv) normal culture medium alone for different times (controls).

To assess the neuroprotective ability of estradiol, SH-SY5Y cells were pretreated with the normal culture medium alone or the normal medium containing estradiol at 10 or 100 nM. After the pretreatment, each one of these categories of SH-SY5Y cells was incubated for 24 h or 48 h with the normal medium (NT_{24} and NT_{48}), the normal medium containing estradiol alone at 10 nM (groups $[E_210]_{24}$ and $[E_210]_{48}$) or 100 nM (groups $[E_2100]_{24}$ and $[E_2100]_{48}$), the normal medium with only H_2O_2 at 1 mM (groups $[H_2O_2]_{24}$ and $[H_2O_2]_{48}$) or the normal medium containing both H_2O_2 (1 mM) and estradiol at 10 nM (groups $[H_2O_2+E_210]_{24}$ and $[H_2O_2+E_210]_{48}$) or 100 nM (groups $[H_2O_2+E_2100]_{24}$ and $[H_2O_2+E_2100]_{48}$). MTT (10 μl of a 3.6 mM stock solution) was added to all wells and allowed to incubate in the dark at 37 °C for 5 h. After cell lysis, spectrophotometric measurement was performed at 595 nm to determine the cell viability. All MTT assays were repeated four times.

To investigate the protective effects of estradiol against cell death induced by letrozole, SH-SY5Y cells were directly incubated for 4 days with letrozole (4, 6 or 8 μM) in the presence of estradiol