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The significant effect of chronic intermittent hypoxia on prostaglandin D_2 biosynthesis in rat brain

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ABSTRACT

Obstructive sleep apnea (OSA) is a common disorder characterized by chronic intermittent hypoxia (CIH). Excessive daytime sleepiness (EDS) is one of severe complications frequently associated with OSA. Lipocalin-type prostaglandin synthase (L-PGDS) is potentially responsible for the production of prostaglandin D_2 (PGD_2) which is an endogenous sleep inducer. To date, whether the content of PGD_2 and PGDS is related to intermittent hypoxia has never been reported. The aim of this study was to compare the content of PGD_2 and L-PGDS in rats' brains with and without intermittent hypoxia. Adult male Wistar rats (n = 48; 8–10 weeks) were averagely divided into two groups. One was control group, and the other group was exposed to IH (12 h/day for 6 weeks). In each group there are four time-points including 0, 2, 4 and 6 weeks, and six rats were killed and studied at each time-point. At the end of 0, 2, 4 and 6 weeks, the concentrations of PGD_2 in brains were measured by LC-MS/MS. In addition, the expressions of L-PGDS protein and mRNA in brains were investigated by western blotting and real-time polymerase chain reaction (RT-PCR), respectively. The results showed the concentrations of PGD_2 in CIH rat brains were higher than those in control groups from the second week. At the end of 6 weeks, the concentrations of PGD_2 in CIH and control groups were 11.1 and 5.9 ng/g, respectively. The levels of L-PGDS protein and mRNA followed the same trend during the whole 6 weeks. The results will provide a new idea to explore that patients with OSA are always accompanied by excessive daytime sleepiness.

1. Introduction

Obstructive sleep apnea (OSA) is a common disorder characterized by recurrent collapse of the upper airway at night leading to repetitive episodes of asphyxia and chronic intermittent hypoxia (CIH) [1]. Intermittent hypoxia is a kind of hypoxia which is often related to cancer and cardiovascular disease [2,3]. Intermittent hypoxia rat is often used for studying sleep apnea [4,5]. OSA significantly increases risk of cardiovascular diseases [6,7], and induces diverse neuropsychological symptoms, such as cognitive [8], psychological functioning [9] and vigilance decrements [10]. In addition, patients with OSA exhibit frequent awakenings, disrupted sleep and consequent excessive daytime sleepiness (EDS) and fatigue [11]. EDS and sleep disruption are common in a large cohort of Chinese OSAHS patients and most moderate and severe OSAHS patients are associated with symptoms of EDS [12]. As we know, EDS have a major impact on quality of life and is often associated with accidents and reduced productivity [13]. Prostaglandins (PGs) play important roles in diverse physiological processes in the central nervous system. Prostaglandin D_2 (PGD_2) is the most abundant PG in the brain and shows a crucial role in sleep regulation [14–16]. Lipocalin-type prostaglandin D synthase (L-PGDS)

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catalyzes the biosynthesis of PGD_2.

Reliable LC–MS/MS methods for measuring PGD_2 have been reported previously [17–20]. For EDS is one of the most frequent complaints in patients suffering from OSA, many studies have focused on analyzing the influence factors in OSA patients with EDS. Especially, the influence of PGD_2 system on OSA is of interest. For example, OSAS patients with EDS were found the increased levels of circulating L-PGDS suggesting a possible role of the prostaglandin D system in the formation of daytime sleepiness [21]. Further, L-PGDS has been proven to be higher in the subjects with severe obstructive sleep apnoea than in control subjects [22]. To date, what causes elevated PGD_2 and L-PGDS levels has not been reported.

In this study, we used LC-MS/MS to measure the content of PGD_2 in brains from CIH and control rats. The western blotting and RT-PCR were used to analyze and compare the expression of L-PGDS between CIH and control rats. The study will provide a new idea to explain why OSA patients often accompany with EDS.

2. Materials and methods

2.1. Chemicals and reagents

Monoclonal antibodies against L-PGDS were purchased from Abcam (Cambridge, UK), and β-actin was purchased from Cell Signaling Technology Inc (Beverly, MA, USA). PGD_2, PGE_2 and PGD_2-4d were purchased from Cayman Chemical (Ann Arbor, MI, USA). Methoxyamine hydrochloride, acetonitrile, ammonium hydroxide and ethyl acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents and other reagents were HPLC grade.

2.2. Studies in rats

We used 48 male Wistar rats (200–240 g of weight) for the research, which were supplied by Jinzhou Medical University. The rats were housed in Animal Care Center under a 12-h light-dark cycle with 24 °C and allowed free access to standard chow and water. This study was approved by the Animal Ethic Committee of Jinzhou Medical University.

The rats were divided into two groups (n = 24 per group): control group and CIH group. In each group there are four time-points including 0, 2, 4 and 6 weeks, and six rats were killed and studied at each time-point. The protocol for the CIH exposure has been reported previously [23]. Briefly, the hypoxic rats with free access to water and chow were housed in gas chamber with a gas control delivery system (Oxycycler model A84XOV; BioSpherix, NY) and were exposed to CIH stimuli for 12 h per day. During IH procedure, the oxygen concentration in the gas chamber was controlled in a 180 s cycle including hypoxia (10% O_2 in N_2 for 90 s) and normoxia (21% O_2 in N_2 for 90s). Control group received the gas-flow exposure as CIH group, but using only room air.

2.3. HPLC-MS/MS conditions

The rats were anesthetized with 1–3% halothane and decapitated at the end of 0, 2, 4 and 6 weeks, respectively. Brains were rapidly removed and frozen in liquid nitrogen and pulverized under liquid nitrogen temperatures to come to being a fine homogeneous powder. The pulverized tissue was homogenized in 3 mL of acetone/saline (2:1) containing 0.005% of butylated hydroxytoluene (BHT) and deuterated PGD_2 (PGD_2-4d) was added into each samples as internal standard. Samples were acidified to pH 4.0 using HCl and placed immediately in solid phase extraction cartridges (Waters, Milford, MA, USA) to purify the samples. The cartridges were washed with 2 mL of 0.1% formic acid (v/v) and 1 mL of methanol/water (0.5:9.5, v/v), and the loaded samples were eluted with 2 mL of ethyl acetate-hexane solution (1:1 v/v). The supernatant fluids were evaporated under nitrogen and redissolved in 100 μL of mobile phase.

The analysis was performed using an ABSciex Eksigent Microflow LC system (Redwood City, California, USA) coupled to an ABSciex 4500 QTtrap linear ion trap quadrupole mass spectrometer (ABSciex, Darmstadt/Germany). The MS was controlled by Analyst 1.6.2 software. The HPLC column was a Welch C18 column (1.7 mm × 100 mm, 1.7 m, Milford, USA). Acetonitrile/water mixture (20/80, v/v) containing 0.01% formic acid was used as a mobile phase and was pumped at a flow rate of 0.4 $mL \cdot min^{-1}$. Total run time was 15 min for each sample injection. The column temperature was kept at 40 °C. Detection of the analyte ions was performed in a multiple reaction monitoring (MRM) mode. Transition of m/z 351.2 → 189.3 was used for PGD_2 and PGE_2 and transition of m/z 355 → 275 was used for PGD_2-4d. Quantification of the analytes was performed in negative ion mode (ESI^-). The ion spray voltage was 5500v and ion-source temperature was set 500 °C. Ion spray gas 1 and 2 was 50 psi and 60 psi, respectively.

2.4. Western blots

Western blotting analysis was performed on samples of whole brain from CIH and control rats. Samples were lysed in RIPA Lysis Buffer (Beyotime, Jiangsu, China) with protease inhibitor phenylmethanesulfonyl fluoride (PMSF, Beyotime, Jiangsu, China). 20 μg of total protein from each sample was separated on 10% polyacrylamide gels (Beyotime, Jiangsu, China). After electrophoresis, separated proteins were transferred onto polyvinyllidenediflouride (PVDF) membranes (Roche Applied Science). Membranes were then blocked for 1 h at room temperature with 5% BSA in TBST. The PVDF membranes were incubated over night with the polyclonal anti-PGD2 synthase. The mixture was washed and then incubated for 1 h with horseradish peroxidase (HRP) including secondary antibody (Beyotime, Jiangsu, China). The blots were then assayed with an enhanced chemiluminescence detection kit (Beyotime, Jiangsu, China) and visualized on a commercial X-ray film.

2.5. Real-time PCR

The mRNA levels of L-PGDS were measured by RT-PCR. Firstly, we extracted total RNA from brain tissues of IH and control rats using TRIZOL Reagent (Invitrogen, CA) according to the manufacturer's instructions. The RNA was quantified for concentration and checked for purity by spectrophotometry at 260 and 280 nm, and the A260/A280 ratio from 1.8 to 2.0 was denoted an acceptable rang. Reverse transcription of 1 μg RNA from each sample was performed using a TaKaRa PCR Kit (TaKaRa, Tokyo, Japan). RT reactions were performed in triplicate with an ABI 7500 Prism Sequence Detection System (Applied Biosystems, Foster City, CA). The reaction conditions were as follows: 95 °C for 30 s, followed by 40 cycles (95 °C for 10 s and 62 °C for 31 s). β-actin expression was used as an endogenous internal standard control. PCR primers for L-PGDS and β-actin were as follows: (L-PGDS) forward: 5′- ATG GCT GCT CTT CCA ATG CTG TG-3′ and reverse: 5′- TTA CTC TTG AAT GCA CTT ATC CGG T -3′; (β-actin) forward: 5′- ATG GAT GAC GAT ATC GCT GCG CTC -3′ and reverse: 5′- CTA GAA GCA TTT GCG GTG CAC GAT -3′. Data were expressed as a ratio: relative quantity of L-PGDS mRNA/relative quantity of β-actin mRNA.

2.6. Statistical analysis

Statistical analysis was applied on SPSS software (version 16.0). PGD_2 and L-PGDS content in brains is expressed as mean ± SD.

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