Neurotoxicology and Teratology 48 (2015) 49–55

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

Activation of the AGE/RAGE system in the brains of rats and in SH-SY5Y cells exposed to high level of fluoride might connect to oxidative stress

Kai-Lin Zhang [a], Di-Dong Lou [a], Zhi-Zhong Guan [a,b,*]

[a] Department of Pathology in the Affiliated Hospital at Guiyang Medical University, Guiyang 550004, PR China
[b] Key Laboratory of Medical Molecular Biology at Guiyang Medical University, Guiyang 550004, PR China

ARTICLE INFO

Article history:
Received 2 July 2014
Received in revised form 6 January 2015
Accepted 28 January 2015
Available online 7 February 2015

Keywords:
AGE/RAGE
Fluorosis
MDA
NOX_2
ROS

ABSTRACT

To explore the mechanisms by which chronic fluorosis damages the brain, we determined the levels of the advanced glycation end-products (AGEs), the receptor for AGE (RAGE), NADPH oxidase-2 (NOX_2), reactive oxygen species (ROS) and malondialdehyde (MDA) in the brains of rats and/or SH-SY5Y cells exposed to different levels of sodium fluoride (5 or 50 ppm in the drinking water for 3 or 6 months and in the incubation medium for as long as 48 h, respectively). The levels of AGEs, RAGE and NOX_2 protein and mRNA were measured by an Elisa assay, Western blotting and real-time PCR, respectively. The ROS content was assessed by fluorescein staining and MDA by thiobarbituric acid-reactive substance assay. In comparison to the unexposed controls, the protein and mRNA levels of AGEs, RAGE and NOX_2 in the brains of rats after 6 months of exposure and in SH-SY5Y cells following high-dose exposure to fluoride were elevated. In contrast, no significant changes in these parameters were detected in the rats exposed for 3 months. In addition, the levels of ROS and MDA in the SH-SY5Y cells exposed to high-dose of fluoride were elevated in a manner that correlated positively with the levels of AGE/RAGE. In conclusion, our present results indicate that excessive fluoride can activate the AGE/RAGE pathway, which might in turn enhance oxidative stress.

1. Introduction

Endemic or chronic fluorosis exerts a significant impact on human health in many countries, causing harm to many tissues and organs, including the central nervous system (CNS). The brains of experimental animals exposed to high doses of fluoride for a prolonged period exhibit alterations in both morphology and function (El-lethey et al., 2010; Guan et al., 1998; Liu et al., 2011; Sharma et al., 2009). In addition, the reduced capacity for learning and memory due to fluorosis has attracted increasing attention in recent years (X. Liu et al., 2010a; Y.J. Liu et al., 2010b; Rocha-Amador et al., 2007). However, the molecular mechanisms underlying the brain damage induced by excessive fluoride have not yet been elucidated.

Advanced glycation end-products (AGEs) affect cells by interacting with specific receptors, in particular the receptor for AGE (RAGE) (Goldin et al., 2006; Matsumoto et al., 2008), and extensive accumulation of such products in the brain can cause direct injury (Misur et al., 2004; Wada and Yagihashi, 2005). Furthermore, injury is promoted by of activation of the AGE/RAGE system, including NADPH oxidase-2 (NOX_2), nuclear factor κB (NF-κB), mitogen-activated protein kinases (MAPK) and protein kinase C (PKC) (Origlia et al., 2008; Wang et al., 2013; Zhu et al., 2012). Though alteration of the expression of multiple genes, AGE/RAGE can be involved in apoptosis, differentiation and oxidative stress (Anan et al., 2010; Kuhla et al., 2011; Kume et al., 2005; Tomino et al., 2011). Interestingly, a high level of oxidative stress has been proposed to be a key factor in the pathogenesis of chronic fluorosis (Feng et al., 2011; Guan et al., 1989, 2000).

Accordingly, this investigation was designed to examine whether the oxidative stress induced in the CNS by high level of fluoride is related to AGE/RAGE signaling pathways. To this end, the levels of AGEs, RAGE, NOX_2, reactive oxygen species (ROS) and malondialdehyde (MDA) in the brains of rats and/or SH-SY5Y cells exposed to fluoride were determined.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibodies directed towards AGEs and RAGE, anti-rabbit IgG conjugated with horseradish peroxidase, goat polyclonal antibodies directed towards non-phagocytic NOX_2 and anti-goat IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc., USA); mouse monoclonal anti-β-actin antibody (Sigma-Aldrich, USA); Hyper Performance Chemiluminescence film and ECL (Enhanced Chemiluminescence) Plus reagent (Amersham, Sweden); QPCR SYBR Green Mix (Infinigen Biotechnology Inc., USA); sodium fluoride (NaF,

* Corresponding author at: Department of Pathology in the Affiliated Hospital of Guiyang Medical University, Guiyang 550004, PR China.
E-mail addresses: zhizhongguan@yahoo.com, guan169713@qq.com (Z.-Z. Guan).

http://dx.doi.org/10.1016/j.ntt.2015.01.007
0892-0362/

analytical grade), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and all other general chemicals (Sigma-Aldrich, USA) were purchased from the sources indicated.

2.2. Experimental animals

One hundred twenty Sprague–Dawley (SD) rats (half males and half females) with two months of age and an initial weight of 90–120 g were purchased from the Experimental Animal Center in Guizhou, China, and ethical permission for this study was obtained from the regional ethical committee for animal studies in Guizhou. Prior to treatment, these animals were acclimatized for one week in a housing facility with humidity ranging from 30 to 55% and a temperature of 22–25 °C. The rats were housed in stainless-steel cages suspended in stainless-steel racks and administered the diet and water *ad libitum*.

The animals were divided randomly into 3 groups of 20 animals each: the control group (receiving normal tap water containing less than 0.5 ppm naturally occurring fluoride), the group exposed to a low concentration of fluoride (drinking water containing 5 ppm flouride in the form of added NaF) and those exposed to a high concentration of fluoride (50 ppm) in the drinking water for 3 or 6 months.

2.3. Exposure of cell cultures to fluoride

SH-SY5Y cells, a human neuroblastoma cell line (the German Collection of Microorganisms and Cell Cultures), were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 25 units of penicillin–streptomycin/ml under a humidified atmosphere containing 5% CO_2. These cells were exposed to different concentrations of NaF (from 0.5 to 100 ppm) for 48 h. On the basis of their viability as evaluated by MTT reduction (Liu et al., 1997), 5 and 50 ppm fluoride were selected as the doses for detailed study.

2.4. Quantitation of AGEs

The cerebral cortex or SH-SY5Y cells were homogenized in 10% phosphate buffered saline (PBS, pH 7.4) on ice (Yan et al., 1996); the resulting homogenates were centrifuged at 12,000 ×*g* for 20 min at 4 °C; and the contents of AGE proteins in the supernatants thus obtained were determined with the AGE Elisa kit 96 t (R&D, USA).

2.5. Quantitation of RAGE and NOX_2 proteins by Western blotting

The cortical brain tissues were homogenized in PBS supplemented with 2% Triton X-100 on ice using a Telfon homogenizer (X. Liu et al., 2010a; Y.J. Liu et al., 2010b); the resulting homogenates were centrifuged at 14,000 ×*g* for 40 min at 4 °C; and the protein concentrations of the supernatants thus obtained were determined. The cultured cells were lysed in this same buffer. The proteins in these supernatants and lysates were separated by 10% SDS-PAGE and then blotted onto polyvinylidene difluoride (PVDF) membranes utilizing a transfer unit (Bio-Rad Inc). These PVDF membranes were subsequently incubated with rabbit polyclonal antibodies directed towards RAGE, or goat polyclonal antibodies directed towards NOX_2 (at dilutions of 1:500 and 1:800 respectively) overnight at 4 °C, then washed and incubated with HRP-conjugated anti-rabbit IgG or anti-goat IgG (1:5000), respectively, for 90 min. Finally, these membranes were treated with ECL Plus reagent for 5 min and the signals thus produced were visualized by exposure to Hyper Performance Chemiluminescence film.

Table 1
Sequences of the primers employed for amplification of the mRNAs encoding RAGE, NOX_2 and β-actin by real-time PCR.

mRNA	Sequences	Length of the products (bp)
RAGE	5′-ACTCACAGCCAATGTCCCTAA-3′ 5′-CTTTGCCATCAGGAATCAGAG-3′	113
NOX_2	5′-GCCTCCATTCTCAAGTCTGTCT-3′ 5′-GGAAGTTGGCATTGTTTCTTTC-3′	172
β-actin	5′-CACCGCGAGTACAACCTTC-3′ 5′-CCCATACCCACCATCACACC-3′	207

Table 2
Dental fluorosis, urinary levels of fluoride and the body weight of rats administered with low or high levels of fluoride in their drinking water for 3 and 6 months.

Groups	No	Degree of dental fluorosis (n)				F content in urine (mg/l)	Body weight (g)
		0°	I°	II°	III°		
Three-month exposure							
Control	20	0	0	0	0	0.59 ± 0.26	371 ± 20
Low-F	20	0	16	0	0	3.78 ± 0.97*	389 ± 19*
High-F	20	0	11	9	0	4.61 ± 1.23*#	360 ± 17*#
Six-month exposure							
Control	20	0	0	0	0	0.64 ± 0.37	450 ± 33
Low-F	20	0	14	6	0	4.17 ± 1.39*	431 ± 37*
High-F	20	0	6	13	1	5.70 ± 1.70*#	424 ± 34*#

Notice: Low-F, 5 ppm fluoride in the drinking water; High-F, 50 ppm fluoride in the drinking water. The values presented are means ± SD.
* $p < 0.05$ in comparison to the control values.
\# $P < 0.05$ in comparison to the values for the Low-F group.

2.6. Quantitation of RAGE and NOX_2 mRNA by real-time PCR

Total RNA was isolated from the cerebral cortex with Trizol reagents (Invitrogen, USA) and 3 μg of this RNA was converted into first-strand cDNA with the first-strand cDNA synthesis kit (Promega, USA) and oligo-dT primers, in accordance with the protocol recommended by the manufacturer. The RT-PCR primers for the target transcripts were designed on the basis of the corresponding complete cDNA sequences deposited in GenBank (accession numbers: L33413.1 for RAGE, NM_023965.1 for NOX_2, NM_031144.2 for β-actin) (Table 1).

By using ABI PRISM 7300 Sequence Detection System (Applied Biosystems, USA) and analyzed with GeneAmp7300 SDS software, the real-time PCR reactions (20 μl) were carried out in the Universal TaqMan 2 × PCR master mix (Infinigen Biotechnology Inc., USA) containing 2 μl of first-strand cDNA, 2 × Hotsybr PCR Reaction Mix and 1 mM of the forward and reverse primers, in accordance with the detailed procedure recommended by the manufacturer. Thermal cycling involved 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Following this amplification melting curve analysis was always employed to confirm specificity. GeneAmp7300 SDS

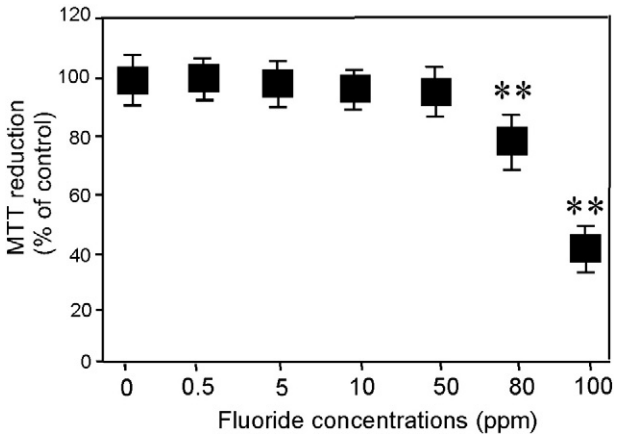

Fig. 1. MTT reduction in SH-SY5Y cells exposed to fluoride. The cells were treated with 0.5–100 ppm fluoride for 48 h and reduction of MTT detected thereafter spectrophotometrically. The values shown are means ± SD ($n = 10$ for each group). **$P < 0.01$ in comparison to the control value.