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Decrease in acrolein toxicity based on the decline of polyamine oxidases

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ABSTRACT

We have shown recently that acrolein is strongly involved in cell damage during brain infarction and chronic renal failure. To study the mechanism of acrolein detoxification, we tried to isolate Neuro2a cells with reduced sensitivity to acrolein toxicity (Neuro2a-ATD cells). In one cell line, Neuro2a-ATD1, the level of glutathione (GSH) was increased. We recently isolated a second cell line, Neuro2a-ATD2, and found that acrolein-producing enzymes [polyamine oxidases (PAO); i.e. acetylpolyamine oxidase (AcPAO), and spermine oxidase (SMO)] are reduced in this cell line due to changes at the level of transcription. In the Neuro2a-ATD2 cells, the IC_{50} of acrolein increased from 4.2 to 6.8 μM, and the levels of FosB and C/EBPβ — transcription factors involved in the transcription of *AcPAO* and *SMO* genes — were reduced. Transfection of siRNAs for FosB and C/EBPβ reduced the levels of AcPAO and SMO, respectively. In addition, the synthesis of FosB and AcPAO was also decreased by siRNA for C/EBPβ, because C/EBPβ is one of the transcription factors for the *FosB* gene. It was also found that transfection of siRNA for C/EBPβ decreased SMO promoter activity in Neuro2a cells but not in ATD2 cells confirming that a decrease in C/EBPβ is involved in the reduced SMO activity in Neuro2a-ATD2 cells. Furthermore, transfection of the cDNA for AcPAO or SMO into Neuro2a cells increased the toxicity of acrolein. These results suggest that acrolein is mainly produced from polyamines by PAO.

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1. Introduction

Polyamines (putrescine, spermidine and spermine) are present at millimolar concentrations in prokaryotic and eukaryotic cells and play regulatory roles in cell growth and viability (Igarashi and Kashiwagi, 2010, 2015; Pegg, 2009). However, when cells are damaged, the toxic compounds acrolein ($CH_2{=}CHCHO$) and hydrogen peroxide (H_2O_2) are produced from polyamines, in particular from spermine, by polyamine oxidases (PAO), consisting of acetylpolyamine oxidase (AcPAO) and spermine oxidase (SMO) (Igarashi and Kashiwagi, 2011; Park and Igarashi, 2013; Pegg, 2013; Tomitori et al., 2005). When the toxicities of acrolein and H_2O_2 were compared in a cell culture system, acrolein was more toxic than H_2O_2 (Sharmin et al., 2001; Yoshida et al., 2009).

We found that plasma levels of protein-conjugated acrolein (PC-Acro) and PAO were correlated with the severity of chronic renal failure and stroke (Igarashi et al., 2006; Sakata et al., 2003; Tomitori et al., 2005). The size of stroke was nearly paralleled with the multiplied value of PC-Acro by PAO (Tomitori et al., 2005). We also found that brain infarction in a mouse model of stroke was correlated with an increase in PC-Acro rather than reactive oxygen species (ROS), both at the locus of infarction and in plasma (Nakamura et al., 2016; Saiki et al., 2009; Saiki et al., 2011).

Abbreviations: AcPAO, acetylpolyamine oxidase; ATD, acrolein toxicity-decreasing; GSH, glutathione; ODC, ornithine decarboxylase; PC-Acro, protein-conjugated acrolein; SAMDC, *S*-adenosylmethione decarboxylase; SMO, spermine oxidase; SSAT, spermidine/spermine N^1-acetyltransferase.

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However, it is not clear how acrolein is detoxified in cells. To study the mechanism of the detoxification of acrolein, we isolated Neuro2a cells with reduced sensitivity to acrolein toxicity (Neuro2a-ATD1) and found that an increase in glutathione (GSH) in these cells could account for the decrease in acrolein toxicity (Tomitori et al., 2012). In this study, we isolated another Neuro2a cell line with reduced sensitivity to acrolein (Neuro2a-ATD2). In this cell line, there is a decrease in acrolein-producing enzymes, i.e. PAO (AcPAO and SMO). The mechanism underlying the reduction in PAO was also studied.

2. Materials and methods

2.1. Culture of Neuro2a cells and isolation from them of acrolein toxicity-decreasing (Neuro2a-ATD2) cells

Mouse neuroblastoma Neuro2a cells were cultured in D-MEM (low-glucose) supplemented with 10% heat inactivated fetal bovine serum (FBS) and non-essential amino acids (Sigma) at 37 °C in an atmosphere of 5% CO_2 in air. The viable cell number was counted in the presence of 0.05% trypan blue. For isolation of cells with reduced sensitivity to acrolein, cells at the logarithmic growth phase (10^5 cells/cm^2) were trypsinized and diluted to 2.5×10^5 cells/mL culture medium in a 50 mm dish. They were mutagenized by treatment with 0.1% ethylmethanesulfonate (Sigma-Aldrich) for 3 h, washed three times with the medium, and cultured for 4 days. Cells were then cultured in the medium containing acrolein (Tokyo Chemical Industry Co., Ltd.). The concentrations of acrolein in the medium were gradually increased in a stepwise manner from 10 to 35 μM over 6 months, and Neuro2a-ATD2 cells were isolated from cells which were able to grow in the presence of 35 μM acrolein.

2.2. Measurement of GSH, ATP, polyamines, PC-Acro and GSH synthetic enzymes

Cells were homogenized with 5% trichloroacetic acid (TCA), and centrifuged at 12,000 × *g* for 10 min. The supernatant was used for the measurement of GSH, ATP and polyamines. GSH was measured using total glutathione assay kit (Northwest Life Science Specialities LLC, USA) according to the manufacturer's instructions. ATP content was determined by ENLITEN® ATP Assay System Bioluminescence Detection Kit for ATP measurement (Promega). Polyamine contents were measured by HPLC as described previously (Igarashi et al., 1986). Protein content in the precipitate was determined by the method of Bradford (Bradford, 1976). PC-Acro was detected by Western blotting using an antibody against [N^{ϵ}-(3-formyl-3,4-dehydropiperidino)-lysine (FDP-lysine)] (NOF corporation) (Uchida et al., 1998). The level of γ-glutamylcysteine ligase catalytic unit (GCLC) and glutathione synthetase (GSHS) was measured by Western blotting (Nielsen et al., 1982) using corresponding antibodies (Santa Cruz Biotechnology, Inc.). The level of these proteins was quantified with a LAS-3000 luminescent image analyzer (Fuji Film).

2.3. Measurement of GSH-Acro-OH

The glutathione fractions were analyzed by nano liquid chromatography (nLC: Easy-nLC 1000, Thermo Fisher Scientific) – tandem mass spectrometry (MS/MS: Q-Exactive, Thermo Fisher Scientific). The samples were separated using nano ESI spray column on an NTCC analytical column (C18, Φ0.075 × 100 mm, 3 μm, Nikkyo Technos, Tokyo, Japan) at a flow rate 300 nL/min using a linear gradient of 0–35% solvent B over 10 min (solvent A: 0.1% trifluoroacetic acid (TFA); solvent B: 100% acetonitrile with 0.1% TFA). The mass spectrometer was operated in the positive-ion mode and the targeted MS/MS mode (at m/z = 308.09, 364.12 and 366.13). The selected ion chromatogram of m/z = 130.05 was extracted for each parent ion.

2.4. Measurement of activities of polyamine biosynthetic and metabolizing enzymes

Ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (SAMDC) were measured as described previously (Suzuki et al., 1993). Measurements of acetylpolyamine oxidase (AcPAO), spermine oxidase (SMO), and spermidine/spermine N^1-acetyltransferase (SSAT) were performed according to the methods published previously (Shappell et al., 1993; Tomitori et al., 2005).

2.5. Measurement of the levels of transcription factors

The levels of 15 kinds of transcription factors were estimated by Western blotting (Nielsen et al., 1982) using 20 μg proteins and antibodies against c-Jun, c-Fos, C/EBPβ, p300, Sp1, E2F-1, NF-κB, GATA-2 and GATA-3 from Santa Cruz Biotechnology, Inc., JunD, Fra1 and Fra2 from Abcam, and FosB, JunB and GATA-1 from Cell Signaling Technology.

2.6. Plasmids and luciferase assay

Plasmids, pcDNA3.1(−)hPAO encoding human AcPAO, pcDNA3.1(−)PAOh1 encoding human SMO, and pGL2-SMO-1117, which contains 1117 base pairs upstream of SMO transcriptional initiation site and the firefly luciferase gene, were cloned as described previously (Wang et al., 2001, 2005a,b). Plasmids were transformed into cells using Lipofectamine 3000 reagent, Invitrogen, according to the manufacturer's protocol. After 2 days culture, cells were washed with PBS and frozen at −80 °C. Luciferase activity was measured using luciferase assay system, Promega, USA.

2.7. Transfection of cells with siRNAs

Neuro2a cells were inoculated in a 6-well plate, Corning, USA, at the density of 10^5 cells/well. Transfection of siRNAs for FosB and C/EBPβ and control sRNA (scrambled RNA), purchased from Invitrogen, was performed using Lipofectamine 3000 reagent, Invitrogen, according to the manufacturer's protocol. The sequences of siRNAs used are as follows:

FosB-1F, 5′-AGAUCGACUUCAGGCGGAAACUGAU-3′
FosB-1R, 5′-AUCAGUUUCCGCCUGAAGUCGAUCU-3′
FosB-2F, 5′-UAGCCCUUCGUACACUUCCUCGUUU-3′
FosB-2R, 5′-AAACGAGGAAGUGUACGAAGGGCUA-3′
FosB-3F, 5′-GACAUGCCAGGAACCAGCUACUCAA-3′
FosB-3R, 5′-UUGAGUAGCUGGUUCCUGGCAUGUC-3′
C/EBPβ-1F, 5′-GCACCCUGCGGAACUUGUUTT-3′
C/EBPβ-1R, 5′-AACAAGUUCCGCAGGGUGCTG-3′
C/EBPβ-2F, 5′-GUUUCGAGCAUUAAAGUGATT-3′
C/EBPβ-2R, 5′-UCACUUUAAUGCUCGAAACGG-3′
C/EBPβ-3F, 5′-AGUAAUCACUUAAAGAUGUTT-3′
C/EBPβ-3R, 5′-ACAUCUUUAAGUGAUUACUCA-3′.

2.8. Semi-quantitative PCR analysis of AcPAO and SMO mRNAs

Total RNA was isolated using a Qiagen RNeasy Mini Kit according to the manufacturer's protocol. Levels of mRNAs for AcPAO and SMO were measured by Qiagen OneStep RT-PCR kit according to the manufacturer's protocol using primer sets of AcPAO-F (5′-TGTCTTCCAGCTGGCTGCAGAATTCGGGCT-3′) and AcPAO-R (5′-AAGCGACCGTATCCTTGGGCAAGGAAGCCA-3′) for AcPAO, and

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