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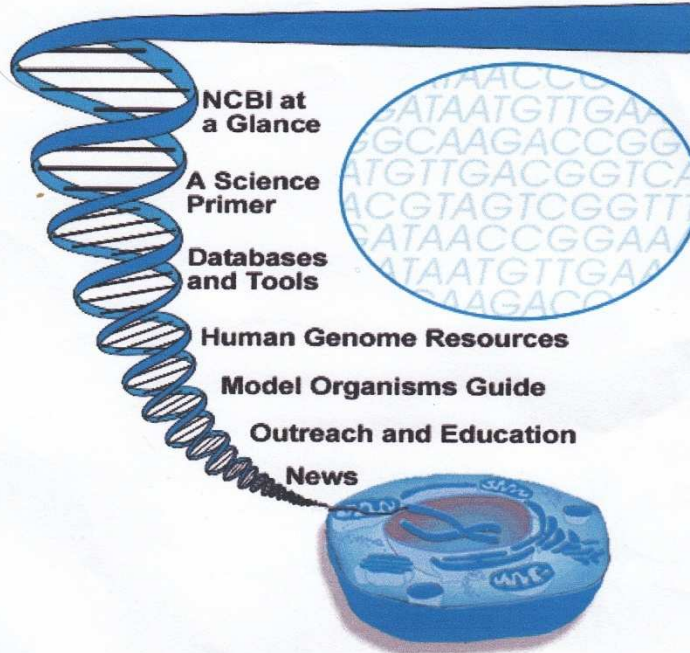
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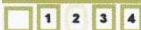
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A teratoproteomics analysis: heat shock protein 70 is upregulated in mouse forelimb bud by methoxyacetic acid treatment.

Ruyani A, Sudarwati S, Sutasurya LA, Sumarsono SH, Kim DJ, Chung JH.

Program Studi Pendidikan Biologi, Universitas Bengkulu, Jalan Raya Kandang Limun, Bengkulu, Indonesia.

Abstract

BACKGROUND: Methoxyacetic acid (MAA) causes fetal limb abnormalities when the substance is administrated on gestation day (GD) 11 in mice. Limb abnormalities are caused mainly by extensive cell death in the mesoderm of the limb plate. This investigation focused on identifying a protein that is linked with mouse limb teratogenicity.

METHODS: A single dose of MAA at 10 mmol/kg body weight was administered by gavage on GD 11; controls were administered vehicle only. Dams were killed by cervical dislocation 4 hr after treatment and forelimb buds were isolated from both the control and treated embryos. Proteins in forelimb buds GD 11 + 4 hr were precipitated out using 40-60% ammonium sulfate and were then analyzed by 2D SDS-PAGE. Excised protein spots were identified by mass spectrometry and amino acid internal sequence analysis. Identified protein was further confirmed by Western blotting.

RESULTS: Two-dimensional gel analysis indicated that 1 protein spot of 81.7 kDa/pI 7.3 was overexpressed, and the protein matched heat shock protein 70 (HSP70; accession no. P08109, SwissProt).

CONCLUSIONS: The results suggest that MAA, when administered to pregnant mice, upregulates HSP70 in the forelimb buds.

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A teratoproteomics analysis: Heat shock protein 70 is upregulated in mouse forelimb bud by methoxyacetic acid treatment

Aceng Ruyani[1,*], Sri Sudarwati[2], Lien A. Sutasurya[2], Sony H. Sumarsono[2], Dong-Jo Kim[3], Jun Ho Chung[3]

Issue

Birth Defects Research Part A: Clinical and Molecular Teratology

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Keywords: methoxyacetic acid; 2D SDS PAGE; HSP70; limb teratogenicity

Abstract

BACKGROUND

Methoxyacetic acid (MAA) causes fetal limb abnormalities when the substance is administered on gestation day (GD) 11 in mice. Limb abnormalities are caused mainly by extensive cell death in the mesoderm of the limb plate. This investigation focused on identifying a protein that is linked with mouse limb teratogenicity.

METHODS

A single dose of MAA at 10 mmol/kg body weight was administered by gavage on GD 11; controls were administered vehicle only. Dams were killed by cervical dislocation 4 hr after treatment and forelimb buds were isolated from both the control and treated embryos. Proteins in forelimb buds GD 11 + 4 hr were precipitated out using 40–60% ammonium sulfate and were then analyzed by 2D SDS-PAGE. Excised protein spots were identified by mass spectrometry and amino acid internal sequence analysis. Identified protein was further confirmed by Western blotting.

RESULTS

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CONCLUSIONS

The results suggest that MAA, when administered to pregnant mice, upregulates HSP70 in the forelimb buds. Birth Defects Research (Part A), 2005. © 2005 Wiley-Liss, Inc.

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A Teratoproteomics Analysis: Heat Shock Protein 70 is Upregulated in Mouse Forelimb Bud by Methoxyacetic Acid Treatment

Aceng Ruyani,[1]* Sri Sudarwati,[2] Lien A. Sutasurya,[2] Sony H. Sumarsono,[2] Dong-Jo Kim,[3] and Jun Ho Chung[3]

[1]Program Studi Pendidikan Biologi, Universitas Bengkulu, Jalan Raya Kandang Limun, Bengkulu, Indonesia
[2]Departemen Biologi, Institut Teknologi Bandung, Bandung, Indonesia
[3]Department of Biochemistry, Cancer Research Institute, Seoul National University Collage of Medicine, Seoul, Korea

BACKGROUND: Methoxyacetic acid (MAA) causes fetal limb abnormalities when the substance is administrated on gestation day (GD) 11 in mice. Limb abnormalities are caused mainly by extensive cell death in the mesoderm of the limb plate. This investigation focused on identifying a protein that is linked with mouse limb teratogenicity. **METHODS:** A single dose of MAA at 10 mmol/kg body weight was administered by gavage on GD 11; controls were administered vehicle only. Dams were killed by cervical dislocation 4 hr after treatment and forelimb buds were isolated from both the control and treated embryos. Proteins in forelimb buds GD 11 + 4 hr were precipitated out using 40–60% ammonium sulfate and were then analyzed by 2D SDS-PAGE. Excised protein spots were identified by mass spectrometry and amino acid internal sequence analysis. Identified protein was further confirmed by Western blotting. **RESULTS:** Two-dimensional gel analysis indicated that 1 protein spot of 81.7 kDa/pI 7.3 was overexpressed, and the protein matched heat shock protein 70 (HSP70; accession no. P08109, SwissProt). **CONCLUSIONS:** The results suggest that MAA, when administered to pregnant mice, upregulates HSP70 in the forelimb buds. *Birth Defects Research (Part A) 73:517–521, 2005.* © 2005 Wiley-Liss, Inc.

Keywords: methoxyacetic acid; 2D SDS PAGE; HSP70; limb teratogenicity

INTRODUCTION

2-Methoxyethanol (2-ME) is intensively used in the manufacture of semiconductors and other industrial applications. The teratogenic effects of 2-ME have been attributed to its primary metabolite, methoxyacetic acid (MAA), which is produced by alcohol dehydrogenase and aldehyde dehydrogenase in the rat dam's liver (Cheever et al., 2001). MAA and 2-ME have similar biological activities in mice; therefore, their dose-response characteristics for producing developmental toxicity are nearly indistinguishable (Sleet et al., 1988). MAA treatment may interfere with the availability of purine and pyrimidine bases, which are believed to affect DNA and/or RNA synthesis, in turn influencing normal cellular proliferation and differentiation in the developing mouse embryo (Mebus and Welsch, 1989). The inhibition of RNA or protein synthesis in certain cells can increase apoptotic cell death (Umansky, 1996). It has been clarified that MAA treatment causes an increase in intracellular free calcium, which will activate certain endonuclease enzymes to cut DNA into 180–200-bp frag-

ments in rat testis (Li et al., 1997), and which is a prelude to apoptotic cell death (Takagi et al., 2002).

Mouse limb development has been used as a model in investigating embryotoxicity and teratogenicity of 2-ME (Sleet et al., 1988) and MAA (Rasjad et al., 1991) in mice. A single dose of MAA at 10 mmol/kg body weight (bw) causes fetal limb abnormalities such as ectrodactyly, brachydactyly, and syndactyly when the substance is administered on GD 11 in mice. Limb abnormalities are caused mainly by extensive cell death in the mesoderm of the limb plate (Sudarwati et al., 1995). The same dose of MAA, administered orally by gavage on GD 11 to Swiss

Webster mice, reduced limb weight and its total protein and DNA content at GD 11 +6, +12, +24, and +48 hr (Surjono et al., 1999). A similar experimental design showed that a protein band of 66.3–97.4 kDa intensifies at GD 11 + 4 hr in treated mouse forelimb buds versus controls (Ruyani et al., 2001b). Therefore, we used a proteomics approach in this limb teratogenicity study to further investigate the identity of such regulated proteins.

MATERIALS AND METHODS
Experimental Animals

Swiss Webster mice were used as experimental animals. Animals were reared in a room at 23–27°C and 83% humidity. Food and water were given ad libitum. When female mice achieved their sexual maturity (10–12 weeks old) they were mated with a male (1:1). The presence of a vaginal plug detected the following morning, defined GD 0 (Sudarwati et al., 1995).

Materials, Dosage, and Sample Collection

MAA (CH_3CH_2COOH), a liquid, was produced by Wako Pure Chemical Industries, Osaka, Japan (CAS Number: 135-07762). MAA diluted with sterilized distilled water was administered by gavage at a single dose of 10 mmol/kg bw on GD 11. The control animals received sterilized distilled water (Sudarwati et al., 1995).

Pregnant mice were killed by cervical dislocation 4 hr after MAA treatment (Ruyani et al., 2001a). Forelimb buds were isolated and stored at −85°C until required for further analysis.

Protein Precipitation

Forelimb bud samples were homogenized at 4°C in 3 volumes of 40 mM sodium phosphate, pH 8. Cellular debris was removed by centrifugation at 4350g, 4°C, for 20 min (Beckman J2-HS, Midland, Canada). The supernatants were isolated as crude protein extract.

A part of the crude protein extract was precipitated out over 30 min using 40–60% ammonium sulfate at 4°C. Precipitates were collected by centrifugation at 17,400g, 4°C, for 20 min (Beckman J2-HS); the protein fraction was solubilized completely using 40 mM sodium phosphate, pH 8, dialyzed on cellulose membranes (Klotz, 1989), and stored at −20°C prior to 2D electrophoresis.

2D Electrophoresis

2D SDS-PAGE was performed in miniformat. The separation of proteins in the first dimension was performed according to their pIs, using 2 kinds of ampholytes (pH 3.5–10 and pH 5–7), and separation the second dimension according to their molecular weights, using SDS-PAGE (Gestern, 1996). Control and treated protein samples, each 75 μg (Sarto et al., 1999), were separated in accordance with the instructional manual (BioRad, Hercules, CA).

Before running the second dimension, the first-dimensional gels were equilibrated in SDS buffer for 15 min and then transferred onto a separating gel in miniformat with 10% pI. A broad range protein marker (6.5–200 kDa) was used as a standard. The gels were run at 120 V and finally stained with Coomassie Brilliant Blue R-250 (CBB R-250) (Gestern, 1996).

Mass Spectrometry and Internal Sequence Analysis

Excised spots from 2D SDS-PAGE gels were digested with trypsin and extracted according to the procedures described by Fernandez et al. (1998). Extracted peptides were dried and solubilized in 10 μl of 0.1% trifluoroacetic acid (TFA). One microliter of the 10-μl preparation was used for mass spectra, and the remaining (9 μl) was used for further analysis.

Mass spectra were obtained by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF-MS). A total of 1 μl of the solution was deposited on the sample probe of a Voyager Elite mass spectrometer (PerSeptive Biosystems, Framingham, MA) operated in the delayed extraction and reflection modes. The spectra were internally calibrated using the matrix dimmer (379.09), and one of the trypsin autolytic peaks (2163.05; 2273.15) (Fernandez et al., 1998). Peptide masses were used for tentative protein identification by a peptide mass database searching (ProFound; http://129.85.19.192/profound_bin/WebProFound.exe?FORM=1).

The remaining 9 μl of solution was purified by HPLC. The amino acid (AA) internal sequence of the HPLC-purified peptide was determined by Edman sequence analysis (Fernandez et al., 1998) and was used for definitive protein identification by primary sequence database searching (ScanProsite; http://www.ca.expasy.org/tools/scanprosite).

Western Blotting

An equal amount of control and treated crude protein extract were separated by 1-dimensional (1D) SDS-PAGE and then transferred onto a PVDF membrane. Nonspecific binding of the PVDF membrane was blocked with 2% gelatin TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20) at room temperature for 1 hr. The blot was then incubated with the monoclonal antibody against HSP70 (Kim et al., 2000) at dilution 1:1000 as primary antibody. Antigen-antibody reaction was determined by anti-immunoglobulin G (anti-IgG) mouse at dilution 1:5000 as the secondary antibody (gift from Jason C. Young, Max Planck Institute, Germany). Furthermore, the secondary antibody was detected using 5-bromo-4-chloro-3-idolyl phosphate (BCIP) and Nitroblue Tetrazolium (NBT; Sigma Chemical Co., St. Louis, MO) as substrate and staining, respectively.

RESULTS

Both control and treated forelimb buds were collected from fetuses of 65 pregnant mice. The 2D gel analyses (9 replicates) indicated that 1 protein of 81.7 kDa/pI 7.3 was upregulated in the forelimb buds by MAA treatment (Fig. 1). This significant difference in protein pattern between the control and treated groups suggests that the protein level was influenced by MAA treatment. Further protein characterization was performed using MALDI TOF-MS and AA internal sequence analysis.

MALDI TOF-MS spectra were obtained from tryptic digests of the 81.7 kDa/pI 7.3 protein (data not shown). A total of 17 peptide masses were used for tentative protein identification by peptide mass database searching with ProFound. The search results reveal that the AA sequence of the matched peptide masses with minimal coverage 21% of the protein, a molecular chaperone hsc 73-mouse (accession no. JC4853, National Center for Biotechnology

A. Control   B. Treated
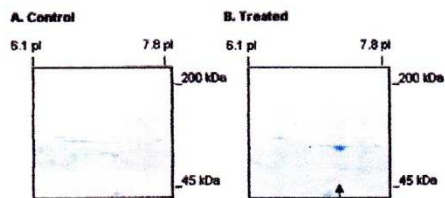
Figure 1. Two-dimensional SDS-PAGE of the protein fraction of 40–60% trifluoroacetic acid–ammonium sulfate extracted from Swiss Webster mouse forelimb buds GD 11 + 4 hr. Detection was done by CBB R-250 staining. The arrow indicates a 81.7 kDa/pI 7.3 protein with increased expression in the forelimb buds after MAA treatment. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]
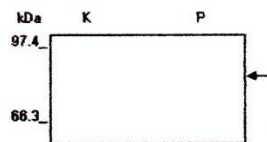
Figure 2. Western blotting with HSP70 monoclonal antibody. The arrow shows that MAA administration to pregnant mice upregulates the HSP70 (82 kDa) in the forelimb buds. K: control; P: treated.

Information [NCBI]; http://www.nebi.nim.nih.gov/Database/; Table 1).

The digested protein mixture was purified using HPLC, and one fragment of the HPLC- purified peptide was sequenced using an Edman micro sequencer (Applied Biosciences, Foster City, CA) at 9 cycles, and showed the presence of an ITTNDKGR internal sequence (sequence position 501–502). This internal sequence was applied for definitive protein identification via a primary sequence database search using the ScanProsite program. The results obtained indicated that the 81.7 kDa/pI 7.3 protein matched HSP70 (accession no. P08109, SwissProt).

JC4853 and P08109 are highly homologous proteins. It is known that HSP70 (P08109) is encoded by Hspa8 gene at 24.0 cM of chromosome-9 in the mouse (MGI:105384; http://www.informatics.jax.org). Its identity was further confirmed by Western blotting in triplicate with HSP70-monoclonal antibody (Fig. 2). The results obtained indicated that MAA administration to pregnant mice upregulates HSP70 in the forelimb buds.

DISCUSSION

It is well established that the level of mRNA expression does not represent the amount of active protein in the cell. Therefore, the change of biological functions that are caused by developmental processes or certain pathological and toxicological conditions were studied effectively through protein rather than mRNA profile. Global analyses of cellular proteins are termed as proteomics. Proteomics is rapidly growing in accordance with a series of innovations of 2D SDS-PAGE, mass spectrometry (MS), and bioinformatics databases. Although numerous toxicological studies have used proteomics tools such as 2D SDS-PAGE and MS, the potential of proteomics in routine toxicology has yet to be realized (Anderson et al., 2000). Proteomics has been applied in our developmental toxicology investigations (Ruyani et al., 2001a, 2001b, 2003), and the applications are proposed as "teratoproteomics analysis" (Ruyani and Muktiningsih dan Barlian, 2004). In the present study we used not only 2D SDS-PAGE and MS but also the AA internal sequence analysis (Fernandez et al., 1998) and the established monoclonal antibody (Kim et al., 2000). It is hoped that this brief report could be used as a model for improving molecular teratology research.

This teratoproteomics analysis revealed that HSP70 is overexpressed in the forelimb bud 4 hr after MAA treatment. The finding is in accord with an observation that the MAA concentration peaks 2 hr after MAA treatment when a single dose of 2-ME is administered orally on GD 11 to mice (Terry et al., 1996). Moreover, a MAA dosimetry study indicated that the half-elimination time of MAA in mouse is 6 hr (Welsch et al., 1996). It has been reported that HSPs can be detected at 2–4 hr after stress induction (Goering et al., 1993). Furthermore, the expression level of HSP72 (a member of the HSP70 family)

Table 1
The Amino Acid Sequence of Matched Peptide Fragments from Tryptic Digestion of the 81.7 kDa/pI 7.3 Protein and a Candidate Protein According to the ProFound Search Results*

Measured mass (M)	Avg/ mono	Computed mass	Error (Da)	Residues Start	To	Missed cut	Peptide sequence
1198.753	M	1198.666	0.087	160	171	0	DAGTIAGLNVLR
1250.788	M	1250.611	0.177	237	246	0	MVNHFIAEFK
1479.895	M	1479.746	0.149	300	311	1	ARFEELNADLFR
1486.855	M	1486.693	0.162	37	49	0	TTPSYVAFTDTER
1787.157	M	1786.982	0.175	172	188	1	IINEPTAAAIAYGLDKK
1804.970	M	1804.888	0.082	57	72	1	NQVAMNPTNTVFDAKR
1837.153	M	1837.005	0.148	326	342	1	LDKSQIHDIVLVGGSTR
1951.297	M	1951.052	0.245	452	469	1	DNNLLGKFELTGIPPAPR
1981.167	M	1980.990	0.177	138	155	0	TVTNAVVTVPAYFNDSQR

*Candidate: dnaK-type molecular chaperone hsc 73-mouse (accession no. JC4853, NCBI).

remains elevated at 48 hr after stress induction, whereas the HSP72-mRNA increases after 1–2 hr and remain elevates for 6 hr (Sarto et al., 2000). The half-life of HSPs in human epidermoid cells was determined to be 48 hr after stress induction (Kiang and Tsokos, 1998). Using the ProtParam program (http://ca.expasy.org/tools/protparam.html) can be estimated that the half-life of HSP70 (accession no. P08109, SwissProt) in mammalian reticulocytes is 30 hr. Extensive research indicates that a major function of HSP70 is as a molecular chaperone, which is importantly required for cell division in normal embryos (Sconzo et al., 1999). The protein plays also a role in development, differentiation, and apoptosis (Moreau et al., 1998).

HSP70, a protein upregulated during cellular stress, is a molecular chaperone that facilitates protein folding and antigen presentation within cell. Recently in vitro and in vivo studies have shown that when released by necrotic cells, HSP70 acts as a danger signal and enhances the maturation of dendritic cells, the most potent type of antigen-presenting cells) (Garza et al., 2000; Millar et al., 2003). HSP70 also plays an important role in longevity determination, demonstrated by the extended longevity phenotypes observed in transgenic flies overexpressing it (Aigaki et al., 2002). In addition, HSP70 acts as a strong suppressor of apoptosis, downstream of the cytochrome c release and upstream of the caspase-3 activation (Li et al., 2000). Furthermore, HSP70 has been reported to block apoptosis by binding apoptosis protease activating factor-1 (Apaf-1), thereby preventing constitution of the apoptosome, the Apaf-1/cytochrome c/caspase-9 activation complex (Beere et al., 2000). Moreover, HSP70 can also inhibit apoptosis by interfering with target proteins other than Apaf-1, one of which is apoptosis-inducing factor (Ravagnan et al., 2001). Thus, an overexpression of HSP70 can be presumed to increase the chance of survival of proliferating cells. In addition, the half-life of HSP70 is 30–48 hr; therefore the overexpression of HSP70 at 4 hr after MAA treatment, as found in the present study, probably reflects its upregulation 24–48 hr after MAA treatment. Meanwhile, histological examinations have clarified that apoptosis predominates in MAA-induced cell death (Surjono and Haryono, 2002). Thus, we hypothesize that the observed overexpression of HSP70 inhibits MAA-induced apoptosis and thereby increases the chance of survival of proliferating cells in the mesoderm of the limb plate. However, additional research is needed to test this hypothesis.

ACKNOWLEDGMENTS

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