

Full Length Research Paper

Protein profiles of adrenal gland of neonatal rat treated with monosodium glutamate

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Exposure to excessive monosodium glutamate (MSG) during neonatal life has been correlated with loss of function in stress response that might be involved in changes of adrenal activity. This study was designed to investigate the protein pattern and examine the mRNA level of proteins in the adrenal glands following MSG stimulation. Neonatal rats were subcutaneously administered with MSG at a concentration of 4 mg/g body wt for 15 days and adrenal glands were collected. Analysis of 2D-PAGE of adrenal extracts demonstrated that MSG induces an increase expression of HSC70, GRP75 and GRP78. In addition, MSG treatment affected to the pI of ALDH2 to more acidic. Semi-quantitative RT-PCR showed an up-regulation of HSC70 and GRP78 transcripts in adrenal gland of MSG-treated rats, while there were no changes in GRP75 and ALDH2 mRNA levels. This study showed that administration of MSG affects on adrenal gland at both level of protein expression and gene expression differentially suggesting a complex responding process of adrenal gland to MSG stimulation.

Key words. 2D-PAGE, adrenal glands, HPA axis, monosodium glutamate, proteomics.

INTRODUCTION

Monosodium L-glutamate (MSG) is a widely used as a food additive. It has long been known that glutamate is the main excitatory neurotransmitter in the central nervous system (CNS) and is responsible for most fast synaptic neurotransmission (Fonnum, 1984; Robinson and Coyle, 1987; Michaelis, 1998; Ali et al., 2000). The administration of large doses of MSG to neonatal rodents is known to induce anatomico-physiological disturbances which can be directly related to several neuroendocrine, metabolic and behavioral abnormalities (Dolnikoff et al., 1988; Wong et al., 1997; Stricker-Krongrad et al., 1998; Sukhanov et al., 1999). A dose of 4 mg/g body wt of glutamate as MSG to young rats induces neurotoxicity (Olney, 1980). Furthermore, dysfunction of the glutamate receptor in the CNS is correlated with neuronal degeneration in disorders such as Alzheimer's disease, Huntington's disease and stroke (Doble, 1999; Elgh et al., 2005).

In addition, glutamate not only functions as a neurotransmitter in the CNS but it also functions in non-neuronal tissues. There is compelling evidence for the ex-

pression and function of glutamate as a signaling molecule in several sites in the body such as bone, skin, heart, taste buds, stomach, pancreas and intestine (Skerry, 2001). Although there is expression of the glutamate receptors in adrenal glands (Watanabe et al., 1994) their function in the adrenal is unknown. The adrenal glands are an essential part of the hypothalamic-pituitary-adrenal (HPA) axis which plays an important role in normal homeostasis (Herman et al., 1997; Tilbrook and Clarke, 2006). Many abnormalities such as schizophrenia, depression, neurological impairment, cognitive dysfunction, brain atrophy, sclerosis, diabetes, and impaired reproductive function have been linked to dysfunction of the HPA axis then Bergh et al., 2001; Heesen et al., 2002; Schumann et al., 2002; Barber et al., 2003; Claes et al., 2003; Gerra et al., 2003; Ryan et al., 2004; Gold et al., 2005; Turner et al., 2005). Therefore, the effects of MSG on the HPA axis are particularly interesting. MSG might directly activate the glutamate receptors in the adrenal glands or glutamate might indirectly affect adrenal gland function by affecting through the upper gland level i.e. the hypothalamus and/or pituitary.

This study was aimed to (1) investigate the changes in protein patterns in the adrenal gland by proteomic approach using 2D-PAGE and (2) examine an alteration

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of transcripts of differently expressed proteins by semi-quantitative RT-PCR following MSG treatment.

MATERIAL AND METHODS

All experimental protocols were designed to minimize animal suffering and were designed in accordance with the Khon Kean University animal scientific committee.

Chemicals

Monosodium glutamate and trichloroacetic acid (TCA) were purchased from Fluka (Buchs, Switzerland). Tris was from Amresco (USA). Hydrochloric acid (HCl), glycerol, and acetone were from Carlo Erba (Italy). Ethylenediamine tetraacetic acid (EDTA) was from BDH (UK). Phenylmethanesulphonyl fluoride (PMSF), aminobenzimidazole (ABZM), bromophenol blue and 2-mercaptoethanol were from Sigma (USA). Urea, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS) and dithiothreitol (DTT) were from USB (Canada). IPG drystrip, IPGphor (pH 3-10L), illustra RNAspin Mini, illustra Hot Start Master Mix 2-D Clean-up™ kit, 2-D Quant™ kit, IPGphor cover fluid, iodoacetamide, ammonium persulphate, tetramethylethylenediamine (TEMED) and PlusOne Silver Staining™ Kit were from GE healthcare Biosciences (UK). Glycine was from Fischer Scientific (UK). Sodium dodecyl sulphate (SDS), acrylamide, N, N'-Methylene-bis-acrylamide and AP-conjugate substrate kit were from Bio-Rad (USA). Rabbit monoclonal to HSC70 antibody was from Abcam (UK). RevertAid™ M-MuLV RT was from Fermentas (Canada). Ribonuclease inhibitor was from Promega (USA).

Animals and tissue material

Time mated Wistar rats (*Rattus norvegicus*) were housed in a temperature ($25 \pm 1^\circ\text{C}$) and light-controlled (lights on from 07.00 am to 07.00 pm) room with free access to food and water. Twenty newborn rats were subcutaneously injected with MSG dissolved in saline at a dose of 4 mg/g of body wt at a volume of 200 μl daily, for the first 15 days of life. The same number of control rats received normal saline on the same days. On the 30th day of life, rats were anesthetized with sodium pentobarbital (Mebumal i.p.) and perfuse via the ascending aorta with formalin-picric acid, and adrenal glands were collected.

Sample preparation and protein extraction

The dissected adrenal glands were homogenized in 500 μl of 20 mM Tris-HCl buffer, pH 8.0 containing 5 mM EDTA and 10 mM PMSF/ABZM. The proteins were precipitated by the addition of 10% w/v TCA and 0.07% v/v 2-mercaptoethanol in cold acetone. The sample was left for 2 hr at -20°C and then centrifuged at 14,000 g at 4°C . The precipitated material was washed three times in 1 ml 0.07% v/v 2-mercaptoethanol in cold acetone. After washing, the precipitate was air-dried for 30 min and subsequently dissolved in 50 μl rehydration buffer containing 8 M urea, 2% w/v CHAPS and 0.002% w/v bromophenol blue, and then sonified three times for 5 sec on ice-cooled water. The dissolved protein was centrifuged at 12,000 g at 4°C for 15 min and the supernatant was collected. The protein preparation was further purified using the 2-D Clean-up™ kit and proteins concentration was determined using 2-D Quant™ kit.

Two-dimensional electrophoresis

2-D electrophoretic analysis was carried out on immobilized pH gradient (IPG) system with ready-made gradient gels developed by

GE healthcare Biosciences followed by SDS-PAGE. The procedure was based on the procedure described by Görg et al. (1995). In the first dimensional electrophoresis, a premade IPG drystrip (13 cm) was placed in the electrophoresis holder containing 50 μl of sample (containing 50 μg proteins) and 200 μl rehydration buffer (8 M urea, 2% w/v CHAPS, 0.5% v/v IPGphor (linear gradient pH 3-10), 0.28% w/v DTT and 0.002% w/v bromophenol blue). The holder was then filled with 1 ml IPGphor cover fluid and put under an electric field of 50 μA per strip at 20°C . Rehydration was took place for 12 h. Isoelectric separation was performed in three steps going from 500 V for 1 h (500 Vhr), 1,000 V for 1 h (1,000 Vhr) and 8,000 V for 2 h (16,000 Vhr). Before each strip was subjected to second dimensional electrophoresis, strip was incubated in equilibration buffer (6 M urea, 30% v/v glycerol, 2% w/v SDS and 0.002% w/v bromophenol blue dissolved in 0.05 M Tris-HCl buffer, pH 8.8), containing 2% w/v DTT for 15 min, and then incubated in equilibration buffer containing 4% w/v iodoacetamide for 15 min. The strip was washed quickly in SDS electrophoresis buffer before placed on the SDS-PAGE gel (12.5% w/v acrylamide, 1 mm thickness). Electrophoresis was carried out using a Hoefer SE 600 system in two steps under the following condition: 15 min at 10 mA per strip and 5 h at 20 mA per strip. Following electrophoresis, proteins were visualized by silver staining of the SDS-PAGE gels using the PlusOne Silver Staining™ Kit.

Mass spectrometry and protein prediction

Differently expressed protein spots were quantified by ImageMaster 2D Platinum v6.0. Protein spots shown differently expressed greater than five folds were cut from the gel and individually stored in microcentrifuge tubes at 4°C . The gel pieces were prepared for tryptic digestion using an Ettan Spot Handling Workstation (Amersham BioScience, UK) using sequencing grade modified trypsin according to the manufacturer's specifications. Following digestion and extraction, the peptides were spotted onto MALDI targets. The mass spectra were recorded on a reflector Bruker reflex V delayed extraction MALDI-TOF mass spectrometer equipped with a 2 GHz LeCroy digitizer and 337 nm N_2 laser. Instrumental parameter were positive polarity, acceleration voltage 20 kV; IS/2 17 kV; focusing lens voltage 8.90 kV; extraction delay 400 ns. The detector was gate. Typically 100 shots were accumulated from three to five different positions within a sample spot. The Mascot (<http://www.matrixscience.com>) search engine was used to preliminary databases screen for PMF against the SwissProt non-redundant protein sequence databases based on *Rattus* genus. The search parameters were fixed as modification carbamidomethylation of cysteine, variable modification methionine oxidation and were considered for the accuracy of the experimental to theoretical pI and molecular weight. Protein scores are considered to be significant when p value is smaller than 0.05 (p value is the probability that the observed match is random event).

Western blotting

Crude proteins (100 μg) were separated by 2D-PAGE as described above and transferred to a nitrocellulose membrane using 160 mA/gel current. The membrane was then blocked with 5% w/v skimmed milk dissolved in TBST (10 mM Tris, 150 mM NaCl, 0.05% v/v Tween-20, pH 7.5) for 1 h or overnight and then incubated for 2 hr at room temperature with rabbit monoclonal antibody to HSC70. The membrane was washed three times with TBST, and then incubated with anti-rabbit IgG conjugated alkaline phosphatase for 1 h at room temperature. The membrane was washed three times with TBST and then with TBS (10 mM Tris, 150 mM NaCl, pH 7.5). Immunoblotting visualization was achieved by using AP-conjugate substrate kit.

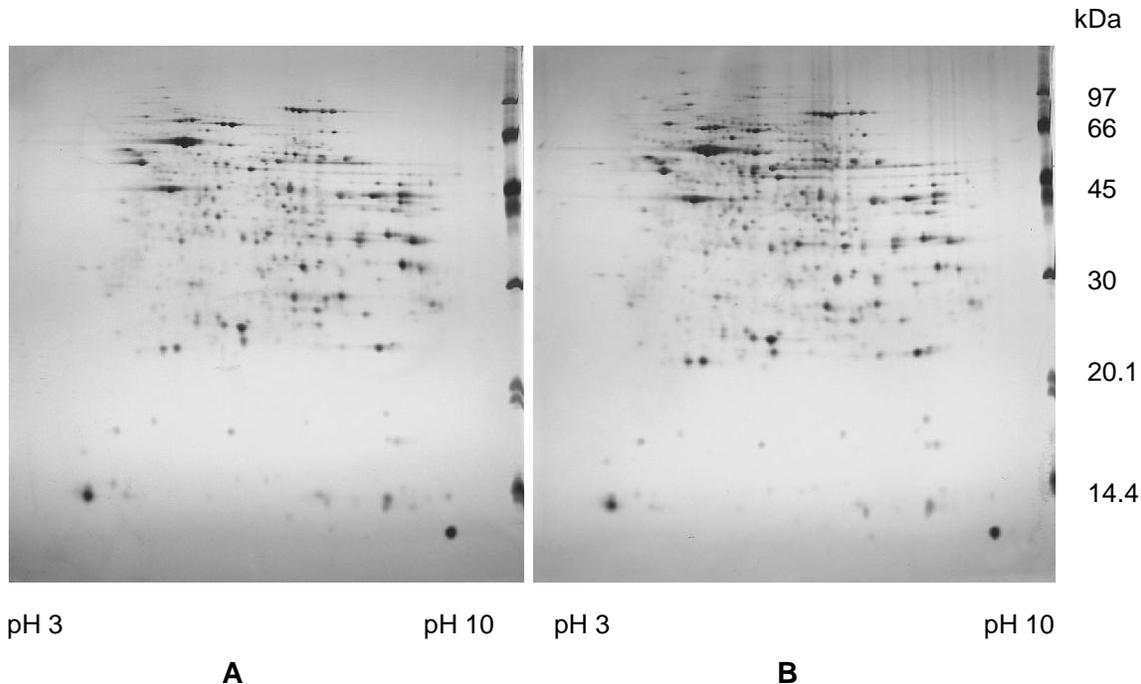


Figure 1. Protein profile of adrenal gland demonstrated by 2D-PAGE. (A) adrenal protein extracted from control group and (B) from MSG-treated group. Isoelectric focusing was performed using an IPG strip between pH 3 and 10 followed by 12.5% SDS-PAGE system.

RNA extraction

Total RNA from both control and MSG-treated adrenal glands of *R. norvegicus* was isolated using illustra RNAspin Mini which included column RNase-free DNase treatment. Concentration and purity of the RNA samples were determined by UV spectroscopy at 260/280 nm, and integrity confirmed by electrophoresis through 1% agarose gels stained with ethidium bromide.

Determination of gene expression by semi-quantitative RT-PCR

Specific primer pairs were designed based on the corresponding cDNA sequences from *R. norvegicus* as follows: - actin sense (5'-CAACTGGGACGATATGGAGAA -3') and -actin antisense (5'-AGGAAGGAAGGCTGGAAGAG -3') (product size 570 bp); GRP78 sense (5'-TTCCGCTCTACCATGAAACC-3') and GRP78 antisense (5'- TCTTTTGTGTCAGGGGTCGTTTC-3') (product size 422 bp); GRP75 sense (5'CAGAGCCCCAAGTAAAGCTG-3') and GRP75 antisense (5'TCTGTTGCTCACGTCCTGTC-3') (product size 422 bp); HSC70 sense (5'CAGAATCCCCAAGATCCAGA-3') and HSC70 antisense (5'ACCATGCGCTCAATATCCTC-3') (product size 534 bp); and ALDH2 sense (5'ATGTGGACAAAGTGGCCTTC-3') and ALDH2 antisense (5'TTGAGGATCTGCATCACTGG -3') (product size 517 bp). Total RNA (1 µg) samples of adrenal gland were heat denatured at 70°C for 5 min and reverse transcribed by incubation the reaction mixture containing 200U RevertAid™ M-MuLV RT, 40 U Ribonuclease inhibitor, 10 µM dNTP mixture and 250 ng of oligo dT primers at 42°C for 60 min. The reactions were terminated by heating at 70°C for 10 min and chilling on ice. For semi-quantitative PCR, different numbers of cycles were optimized for each gene to ensure an amplification of PCR product in the exponential phase. PCR reactions were performed using illustra Hot Start Master Mix and the appropriate primer pairs (1 µM of each

primer). PCR featured of a first activation and denaturing cycle at 95°C for 5 min, followed by the number of cycles determined for the optimal amplification of each gene (see Results) consisting of 45 s at 95°C for denaturation, 45 s for annealing at 60°C and 1.5 min at 72°C extension steps. A final extension cycle of 72°C for 7min was included. PCR products were separated in 1.5% w/v agarose gels and stained with ethidium bromide. Specificity of the PCR procedure was confirmed by exception of the cDNA template in the amplification reaction. Ethidium bromide intensities in each band resulting from PCR amplification were analyzed using Quantity One™ image analysis software (Bio-Rad, USA). Data are expressed as means ± SEM from at least three independent experiments performed on RNA preparations. Statistical analysis was performed using a paired T test. Differences were considered significant at $P < 0.05$.

RESULTS

2D-PAGE analysis in protein pattern of adrenal gland

The 2D-PAGE gels of the adrenal protein obtained from neonatal MSG administrated and control rats are shown in Figure 1. There were over 100 protein spots observed on each gel using gel image analysis apparatus. In a preliminary screening, 21 protein spots were found that have been differently expressed (data not shown). However, after several repetitions of the same experiment, only 6 protein spots were shown to have consistently difference in their levels of expression. As shown in Figure 2, protein spots no. 1 - 4 showed an increase in level of expression in the MSG-treated rats, while protein spot no. 5 showed higher level of expression in the control rats.

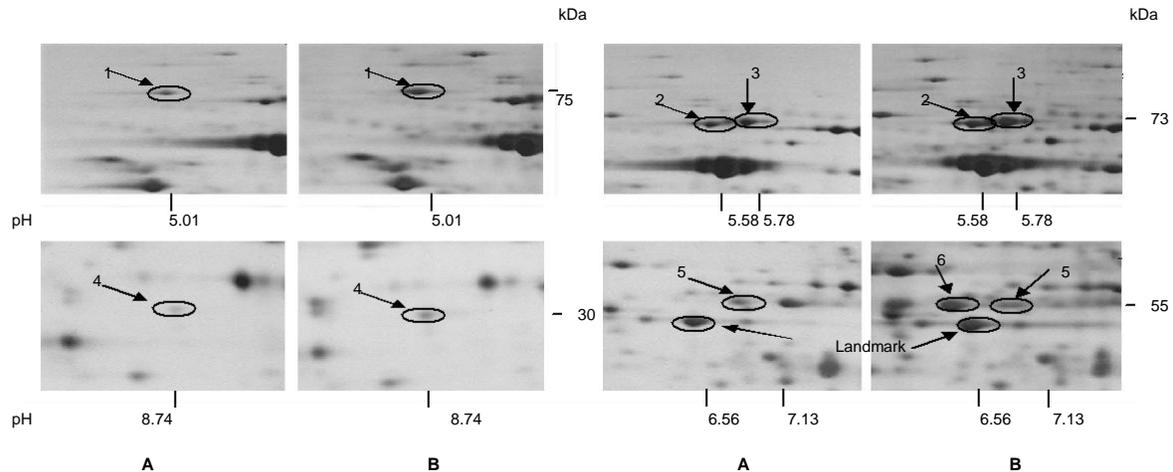


Figure 2. Comparison of protein spot intensity expressed in adrenal gland between (A) control rats and (B) MSG-treated rats. Protein spots no. 1- 4 were up-regulated in the MSG-treated group while spot no. 5 was down-regulated. Protein spot no. 6 was only expressed in the MSG-treated group.

Table 1. Summary of the spots predicted using 2D PAGE and protein mass fingerprint in the MSG treated group compared to the control group.

Spot number	Status (Compared to Control)	Protein name	Accession and identity codes	Experimental pI/Mw (kDa)	Theoretical pI/MW (kDa)
1	Up-regulated	78 kDa glucose-related protein (GRP78) ^a	P06761	5.01/75	5.07/72.4
2	Up-regulated	Heat shock cognate 71 kDa protein (HSC70) ^a	P63018	5.58/72	5.37/70.8
3	Up-regulated	75 kDa glucose-related protein (GRP75) ^a	P48721	5.78/73	5.97/73.8
4	Up-regulated	Unknown	-	8.18/31	-
5	Down-regulated	mitochondrial aldehyde dehydrogenase (ALDH2) ^a	P11884	6.82/55	6.63/56.5
6	Change in pI	mitochondrial aldehyde dehydrogenase (ALDH2) ^a	P11884	6.30/55	6.63/56.5

^a $P < 0.05$

However, interestingly protein spot no. 6 was found to be present only in the MSG-treated group.

Prediction of protein spots with peptide mass fingerprint (PMF) data

These 6 protein spots were cut out of the 2D-PAGE gel and digested with trypsin prior to analyze by mass spectrometry using MALDI-TOF MS analysis. The generated peptide mass fingerprint, pI and molecular weight of each protein were used for searching a matched protein in the Swissprot protein databases using the Mascot tool on the Matrix Science biology server (<http://www.matrixscience.com>). By using an appropriate scoring algorithm, five of six differential expressed protein spots were predicted for protein identity (Table 1). Protein spot no. 1 was matched to the 78 kDa glucose-related protein

(GRP78), also called BiP protein, protein spot no. 2 to the heat shock cognate 71 kDa protein (HSC70) and protein spot no. 3 to the 75 kDa glucose-regulated protein (GRP75), respectively. However, protein spot no. 4 could not find any matched protein in databases. Protein spot no.5 was detected in lower level of expression in adrenal gland of MSG-treated and predicted as mitochondrial aldehyde dehydrogenase (ALDH2). Protein spot no. 6 was present only in adrenal sample from MSG-treated rats and predicted as protein similar to spot no. 5, that is, ALDH2 but with different pI value.

Prediction of protein identity was confirmed by western immunoblotting using monoclonal antibody shown antigen-antibody binding position corresponding to protein position shown on the 2D-PAGE (data not shown). Although, confirmation of protein identity by this immunoblotting approach was not give the exact protein identifi-

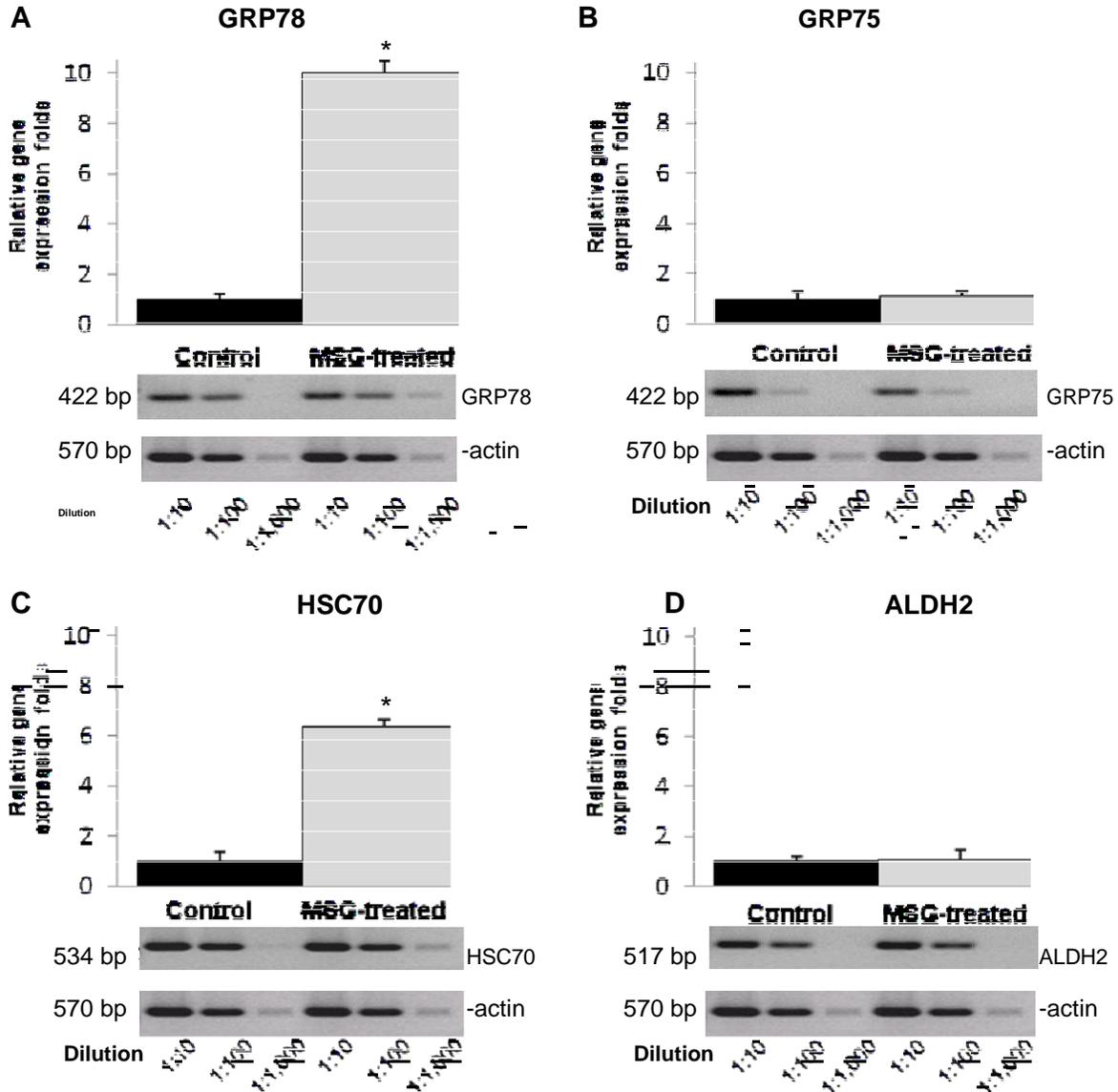


Figure 3. Analysis of mRNA levels in adrenal glands following MSG treatment by semi-quantitative RT-PCR. Amplification was performed using 10 fold serial dilutions at 1:10 to 1:1,000 dilutions of cDNA templates. Representative images of 1.5% agarose gels stained with ethidium bromide indicating the amplified fragments for (A) GRP78, (B) GRP75, (C) HSC70 and (D) ALDH2 in the control and MSG- treated rat adrenal glands. cDNA quantity of adrenal glands extracted from both animal groups were determined and normalized to the amount of - actin cDNA. Relative gene expression fold is demonstrated by relative amount of cDNA to -actin gene. Mean SEM; $n = 3$. * $P < 0.05$ vs the corresponding band intensity in MSG-treated animal.

cation due to possible binding of antibody to other protein homolog, by using monoclonal antibody at least it was in part demonstrated the correction of protein identity by bioinformatically PMF searching method.

Determination of gene expression in adrenal gland

Semi-quantitative RT-PCR revealed up-regulation of GRP78 and HSC70 mRNA approximately ten folds and six folds in adrenal samples of MSG-treated rats respect-

tively (Figure 3A, 3C). This result showed a corresponding to an increase in protein expression of these proteins analyzed by 2D- PAGE. However, ALDH2 mRNA content was not affected by neonatal MSG treatment (Figure 3D). Interestingly, expression of GRP75 mRNA was not up-regulated in the adrenal gland of MSG-treated rats and was not corresponded to level of protein expression shown by 2D-PAGE (Figure 3B). It suggested that neonatal treatment with 4 mg/g body wt/day of MSG affect differentially to gene and protein expression in the adrenal gland.

DISCUSSIONS

Exposure of neonatal rats to MSG has effect to protein expression pattern in the adrenal glands. Using 2D-PAGE and mass spectrometric approach, GRP78 and GRP75 were found to have higher expression in the gland of MSG- treated rats. These proteins are also members of the heat shock proteins 70 (HSP70) families in which their expression is increased when exposed to elevated temperatures or other stress. GRP78 is thought to function in Ca^{2+} sequestration or as a molecular chaperone in the folding and assembly of membrane or secreted proteins in the endoplasmic reticulum (ER) (Little et al., 1994; Nigam et al., 1994; Lievremont et al., 1997). Additionally, it is also thought to function as an anti-apoptotic protein (Reddy et al., 2003). Over expression and antisense approaches in cell systems show that GRP78 can protect cells against cell death caused by disturbances of ER homeostasis (Miyake et al., 2000). GRP78 can suppress elevations of intracellular Ca^{2+} levels following exposure of neurons to glutamate, and this effect of GRP78 apparently results from decreased release of Ca^{2+} from ryanodine-sensitive stores (Yu et al., 1999). In ER-stressed cells, GRP78 mainly localized within the mitochondria and decorated the mitochondrial membrane compartment (Sun et al., 2006). Although mitochondria are central for the integration of signals that induce apoptosis, there is emerging evidence that suggests mitochondria are important components of the ER -stress-induced apoptotic pathway (Breckenridge et al., 2003). Similar to the GRP78, the increasing of GRP75, also called mitochondrial HSP70 or mortalin, is not surprising. GRP75, a member of the HSP70 family of chaperones, has been shown to have different subcellular localizations in normal and immortal cells. It has been assigned to multiple sub-cellular sites and implicated in multiple functions ranging from stress response, intracellular trafficking, antigen processing, and control of cell proliferation, differentiation, and tumorigenesis (Merrick et al., 1997; Wadhwa et al., 1998; Wadhwa et al., 1999; Wadhwa et al., 2000; Rivolta and Holley, 2002; Wadhwa et al., 2002; Jin et al., 2006).

Unlike canonical heat shock proteins, HSC70 was placed in the heat shock protein family due to homology with other heat shock proteins but is constitutively expressed and performs functions related to normal cellular processes. Thus, increases in the expression of HSC70 after MSG-treatment is more surprising. HSC70 plays an important role in cells by transiently associating with nascent polypeptides to facilitate correct folding. However, previous studies have shown that HSC70 and HSP70 have different and antagonistic effects on the promotion of cell survival (Goldfarb et al., 2006; Matsui et al., 2007). HSC70 has been shown to stabilize Bim mRNA, an apoptotic factor that regulates total blood cell number, and promoted cell death. The finding that MSG up-regulates the expression of both apoptotic and anti

apoptotic proteins in the adrenal gland is interesting. It is possible that MSG might stimulate adrenal gland activity and increase cell degeneration via an activation of HSC70 functions. However, to encouraged cell survival, proteins in HSP70 family are higher expressed to promote cell survival and might have opposite function against the HSC70.

The changes of spot no. 5 and spot no. 6 are of particular interest. Both spots were predicted as the same proteins, ALDH2. This protein belongs to the aldehyde dehydrogenase family of proteins and its main function is to catalyze the conversion of acetaldehyde to acetic acid. Aldehyde dehydrogenase is the second enzyme of the major oxidative pathway of alcohol metabolism. There are two major isoforms of aldehyde dehydrogenase; that is, cytosolic and mitochondrial isoform distinguished by their electrophoretic mobilities, kinetic properties, and subcellular localizations. ALDH2 is the mitochondrial isoform. In addition, ALDH2 activity is involved in many metabolic pathways in addition to alcohol metabolism such as glycolysis and gluconeogenesis, amino acids metabolism, glycerolipids metabolism, urea cycle, and the metabolism of other compounds e.g. ascorbate, 1,2-dichloroethane, propanoate, butanoate, limonene, and pinene (Yoshida et al., 1984; Ikuta et al., 1986; Chen et al., 2005; Li et al., 2006). Consequently, changes in the expression levels of ALDH2 in the adrenal glands following MSG treatment might reflect changes in the metabolic activities of the gland. However, because protein spots no. 5 and no. 6 were predicted as ALDH2, it suggests that there are consistent expressions of ALDH2 in the gland of both animal groups but there is difference in pI of the two protein spots. We found that the pI of the more acidic spot (spot no. 6, pI 6.37) is similar to the mature ALDH2 while the pI of spot no. 5 (pI 6.86) is similar to that of the ALDH2 precursor.

Moreover, the result of semi-quantitative RT-PCR analysis of GRP78 and HSC70 gene expression was agreed with an increase in protein expression of GRP78 and HSC70 in the adrenal gland of MSG-treated rats. Thus, it is possible that MSG influence on these two proteins through regulating factors that act at gene transcriptional level. Although GRP75 showed an increase in protein level after MSG treatment, however, there was no differentiation in GRP75 transcript compared to control animals. MSG, therefore, might have an effect on the regulation of GRP75 protein at translational level. Interestingly, administration of MSG to neonatal rat seemed not to affect on ALDH2 gene transcription but affect in shifting of ALDH2 protein pI value as well as no difference in its protein level. Thus, it is probable that MSG have an effect on the regulation of ALDH2 at the post-translation modification level.

At the present, the mechanisms that MSG causes changes in protein pattern of the adrenal gland are not clear. MSG might have directly effect on the adrenal gland by regulating of particular gene expression or by

acting at proteins that maintain normal functions in the gland. However, MSG might have an indirect effect on adrenal function by affecting at the upper organs, such as hypothalamus or pituitary gland resulting in dysregulation of HPA axis.

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