

ตารางที่ 3 ผลการทำนายของ protein spot ที่มีการแสดงออกที่แตกต่างกัน โดยการใช้ pl, น้ำหนักโมเลกุลและ peptide mass fingerprint (PMF)

ที่ได้จาก 2D-PAGE และ MALDI-TOF MS. (Searched on 7th November 2007)

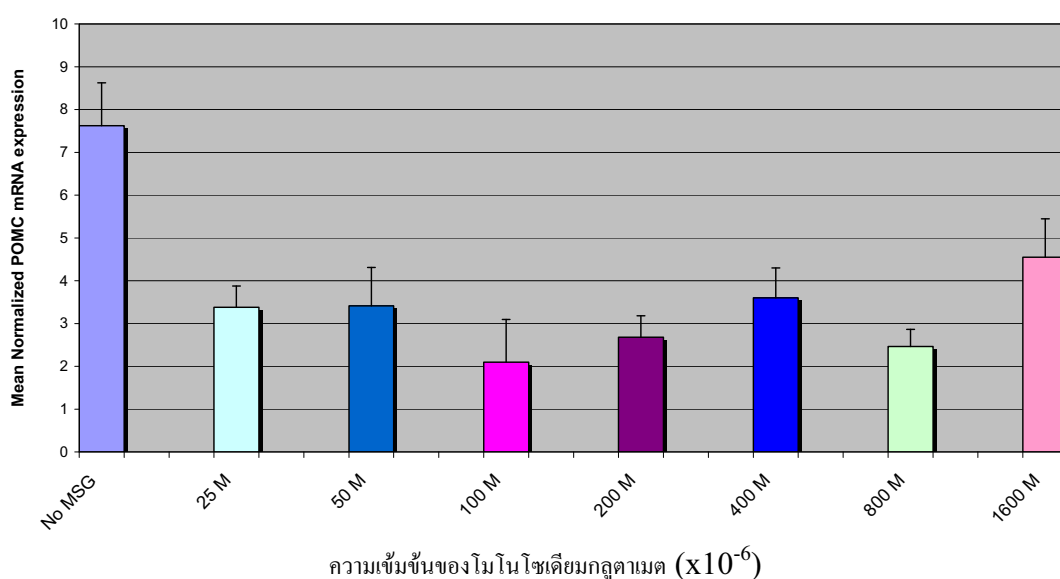
Organs	Assignment Letter	Regulation	Protein Name	Find In	Acc No.	Family of Protein	Number of Peptides	Matched Peptides	Sequence Coverage (%)	Protein Score	Exp. pI/MW (kDa)	Theoretical pI/MW (kDa)	Predicted Function of Protein
Hypothalamus	H1 **	Down	α -N-acetylgalactosaminidase precursor	Swiss Prot	Q66H12	glycosyl hydrolase 27	5	4	9	28	4.95/48	5.55/47.4	Glycoprotein degradation
	H2	Up	unknown	-	-	-	-	-	-	-	8.75/39	-	-
	H3	Up	unknown	-	-	-	-	-	-	-	8.70/35	-	-
	H4	Up	unknown	-	-	-	-	-	-	-	8.39/42	-	-
Pituitary gland	P1	Up	unknown	-	-	-	-	-	-	-	5.76/29	-	-
	P2	Up	unknown	-	-	-	-	-	-	-	7.97/38	-	-
	P3	Up	unknown	-	-	-	-	-	-	-	8.91/39	-	-
Adrenal gland	A1 *	Up	78 kDa Glucose-related protein	Swiss Prot	P06761	heat shock protein 70	9	9	17	61	5.01/75	5.07/72.4	nascent polypeptide folding, anti-apoptosis
	A2 *	Up	Heat shock cognate 71 kDa protein	Swiss Prot	P63018	heat shock protein 70	17	13	26	51	5.58/72	5.37/70.8	nascent polypeptide folding, apoptosis
	A3 *	Up	75 kDa glucose-regulated protein	Swiss Prot	P48721	heat shock protein 70	14	10	19	59	5.78/73	5.97/73.8	cell proliferation, differentiation
	A4	Up	unknown	-	-	-	-	-	-	-	8.18/31	-	-
	A5 *	Down	mitochondrial aldehyde dehydrogenase	Swiss Prot	P11884	Aldehyde dehydrogenase	21	14	36	68	6.82/55	6.63/56.9	Glucose metabolic pathway
	A6 *	Activated	Mitochondrial aldehyde dehydrogenase	Swiss Prot	P11884	Aldehyde dehydrogenase	31	17	39	73	6.30/55	6.63/56.5	Glucose metabolic pathway

*significant protein prediction (p<0.05)

**proteins in these lists are candidate for protein prediction which is non significant predicted.

3.5 ผลการกระตุ้นของโมโนโซเดียมกลูตาเมตต่อเซลล์ pituitary AtT-20 ต่อการแสดงออกของ POMC mRNA และ POMC-derived peptide

Pro-opiomelanocortin (POMC) เป็น propeptide hormone ที่จะถูกตัดแปลงหลังจากกระบวนการแปลรหัส (translation) ได้เป็นเปปไทด์ฮอร์โมนหลายชนิดเช่น ACTH, α -MSH และ β -endorphin การแสดงออกของยีน POMC พบได้ในไฮโปทาลามัส และต่อมใต้สมอง เนื่องจากมีการศึกษาพบว่าโมโนโซเดียมกลูตาเมต สามารถกระตุ้น HPA axis ได้ อย่างไรก็ตามในปัจจุบันยังไม่ทราบกลไกการกระตุ้นดังกล่าวที่แน่ชัด การทดลองนี้ได้นำเซลล์ AtT-20 ซึ่งเป็น mouse anterior pituitary tumor มาทดสอบกับโมโนโซเดียมกลูตาเมตที่ระดับความเข้มข้นต่างๆ แล้วทำการวัดระดับการแสดงออกของยีน POMC ด้วยเทคนิค quantitative real-time RT-PCR พบว่า โมโนโซเดียมกลูตาเมต ที่ความเข้มข้นในช่วง 25-1600 μ M สามารถยับยั้งการแสดงออกของยีน POMC ในเซลล์ AtT-20 ได้มากกว่า 50% (ดังแสดงในกราฟที่ 2)

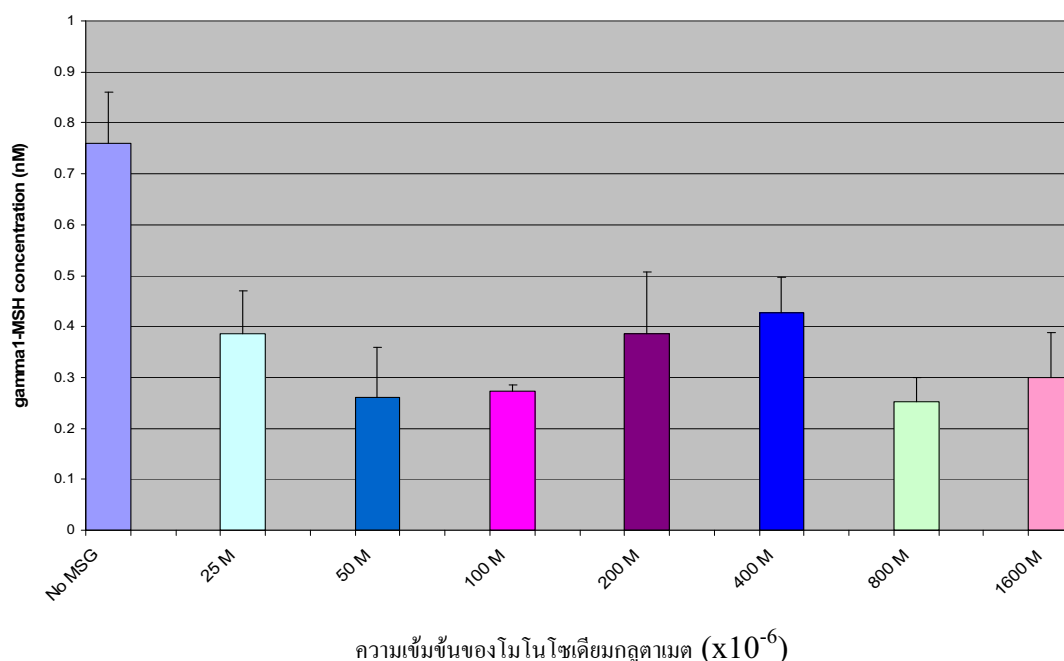


กราฟที่ 2 กราฟแสดงผลของโมโนโซเดียมกลูตาเมตต่อการแสดงออกของ POMC mRNA ในเซลล์ AtT-20

ปริมาณของ POMC mRNA ถูก normalized กับปริมาณของ 18S ribosomal RNA จากการทดลองก็พบว่าระดับของ POMC mRNA ในเซลล์ที่ไม่ได้ถูก treated ด้วยโมโนโซเดียมกลูตาเมตจะมีการแสดงออกของ POMC mRNA ที่สูงกว่า (* $P < 0.005$) ($n=3$) เซลล์ที่ถูก treated ด้วยโมโนโซเดียมกลูตาเมตจะมีที่ความเข้มข้นที่ระดับต่างๆ (50 mM-1600mM)

จากการทดลองด้วยการใช้เซลล์ AtT-20 นั้นก็พบว่าโมโนโซเดียมกลูตาเมตมีผลยับยั้งการแสดงออกของยีน POMC จะถูกยับยั้งได้ด้วยในทุกๆ ระดับความเข้มข้น (25-1600 μM) ซึ่งการยับยั้งการแสดงออกของ POMC mRNA ของโมโนโซเดียมกลูตาเมตมีมากกว่า 50% เมื่อเปรียบเทียบกับกลุ่มควบคุม

นอกจากนี้ระดับของ POMC derived peptides, gamma1-MSH (N-terminal POMC (1-28)) ที่เกิดขึ้นจากการตัดแปลงของ POMC ก็ได้ถูกวิเคราะห์ด้วย ELISA พบว่าระดับของ gamma1-MSH ในเซลล์ AtT-20 cell ที่ได้จากการทดสอบกับโมโนโซเดียมกลูตาเมตที่ความเข้มข้นต่างๆ มีความสอดคล้องกับผลที่ได้จากการวิเคราะห์การแสดงออกของยีน POMC การทดลองนี้ได้แสดงให้เห็นว่าโมโนโซเดียมกลูตาเมต มีการกระตุ้นต่อเซลล์ใน in vivo และ in vitro ที่แตกต่างกัน การได้รับโมโนโซเดียมกลูตาเมตไม่น่าจะมีผลต่อการทำหน้าที่ของต่อมได้สมองแต่อย่างใด แต่น่าจะมีผลต่อเซลล์ในเนื้อเยื่อนั้นคือไฮโปธาลามัส หรือต่อมหมวกไต ซึ่งมีความจำเป็นที่จะต้องมีการศึกษากลไกการกระตุ้นดังกล่าวต่อไป

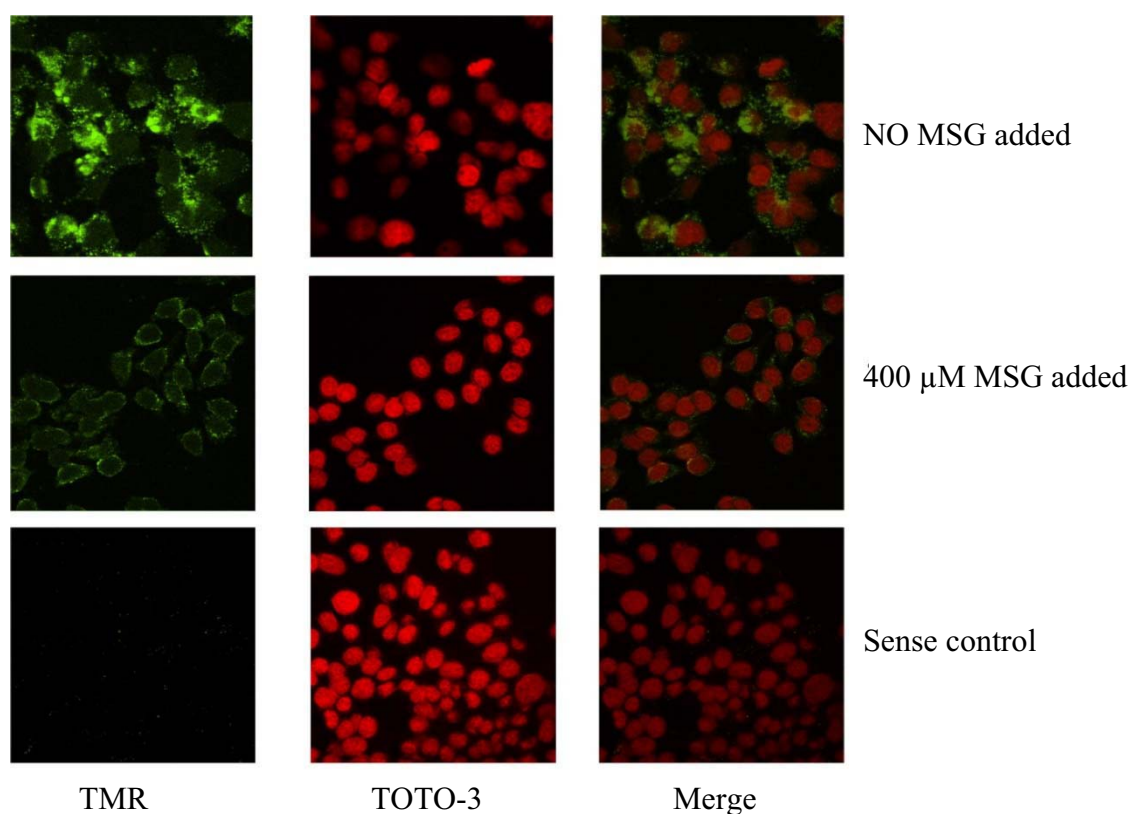


กราฟที่ 3 กราฟแสดงผลของโมโนโซเดียมกลูตาเมตต่อระดับของ POMC derived peptide, gamma 1-MSH ในเซลล์ AtT-20 gamma 1-MSH ถูกวัดโดย ELISA ระดับของ gamma 1-MSH ในเซลล์ที่ไม่ถูก treated ด้วยโมโนโซเดียมกลูตาเมตนั้นมีระดับที่สูงกว่า (* $P < 0.005$) ($n=3$) เซลล์ที่ถูก treated ด้วย โมโนโซเดียมกลูตาเมตที่ระดับความเข้มข้นต่างๆ กัน (50 mM-1600 mM)

3.6 การตรวจวัดการแสดงออกของยีน POMC ในเซลล์ AtT-20 ด้วยวิธี *In Situ*

Hybridization

การทดลองนี้เพื่อที่จะยืนยันผลการยับยั้งของโมโนโซเดียมกลูตาเมตต่อการแสดงออกของยีน POMC ในเซลล์ AtT-20 ดังที่ได้แสดงด้วยการทำ real time RT-PCR ในการทดลองเซลล์ AtT-20 ถูก treated ด้วยโมโนโซเดียมกลูตาเมตที่ความเข้มข้น 400 μ M แล้ว hybridized ด้วย dig-labeled riboprobes เพื่อตรวจระดับการแสดงออกของยีน antisense riboprobe ที่จับกับ POMC mRNA ถูกตรวจวัดด้วย Tetramethylrhodamine tyramide (TMR) ซึ่งจะให้สัญญาณ fluorescent TMR เป็นสีแดง ซึ่งก็จะถูกเปลี่ยนเป็น pseudo green fluorescent ในขณะที่ nuclei จะย้อมด้วย TOTO-3 ให้สีแดงดังแสดงในรูปที่ 17 จากการทดลองนี้จะเห็นได้ว่าโมโนโซเดียมกลูตาเมตมีผลยับยั้งการแสดงออกของยีน POMC ในเซลล์ pituitary AtT-20 ซึ่งสอดคล้องกับผลการทดลองที่ได้จากการตรวจสอบการแสดงออกของยีนด้วย real time RT-PCR



รูปที่ 17 ภาพ Confocal ของเซลล์ AtT-20 ที่ทำการตรวจวัดการแสดงออกของยีน POMC ด้วยวิธี *in situ* hybridization ซึ่ง POMC mRNA จะถูกตรวจพบได้ในส่วน cytosolic part ของเซลล์แสดงด้วย pseudo green fluorescence (TMR) ขณะที่ nuclei ย้อมด้วยสีแดงของ TOTO-3

4. สรุปและวิจารณ์ผลการทดลอง

มีข้อมูลการศึกษาในอดีตที่รายงานว่า การได้รับกลูตาเมตในหนูแรกเกิดในความเข้มข้น 4 มิลลิกรัม/กรัม น้ำหนักตัว/วัน ทำให้หนูเกิด metabolic และ neurodegenerative disorder เช่น hyperlipidemia hyperglycemia โรคอ้วนและโรคอื่นๆ ขึ้นได้ (Ahluwalia, Malik, 1989, Hirata et al., 2003) ซึ่งอาการดังกล่าวมีสาเหตุมาจากการเกิด lesions ในไฮโปทาลามัสของหนูแรกที่ถูกกระตุ้นด้วยโมโนโซเดียมกลูตาเมต (Lemke-Johnson et al., 1974; Magarinos et al., 1988; Oldney, 1971)

การศึกษาผลของโมโนโซเดียมกลูตาเมตต่อการแสดงออกของยีนใน HPA axis เช่น ยีน POMC, NPY, AGRP, Melanocortin 3/4 receptor และ StAR มีความสำคัญและน่าจะมีความเกี่ยวข้องกับความผิดปกติที่พบได้ในหนูที่ได้รับโมโนโซเดียมกลูตาเมตในปริมาณที่สูงอย่างต่อเนื่องและเป็นเวลานาน การทราบข้อมูลการแสดงออกของ metabolic gene เหล่านี้ทำให้เราสามารถเชื่อมโยงความผิดปกติที่เกิดขึ้น กับหน้าที่ของยีนเหล่านี้ที่มีการศึกษามาก่อนหน้า

การวิจัยครั้งนี้ได้แสดงให้เห็นว่าโมโนโซเดียมกลูตาเมตมีผลต่อการแสดงออกของยีนใน HPA axis อย่างไรก็ตามการเพิ่มหรือลดระดับของยีนบางตัวเช่น ยีน POMC ก็ไม่ได้หมายความว่าส่งผลกระทบโดยตรงให้มีการเปลี่ยนแปลงของระดับฮอร์โมนใน HPA axis เสมอไป เช่น กรณีของ corticotrophic hormones เนื่องจากการควบคุมการทำงานของ HPA axis นั้นมีความซับซ้อนและมีการควบคุมในหลายระดับ (Ahima et al., 2002) หรือกรณีการศึกษาการ obese ในหนูที่ได้รับโมโนโซเดียมกลูตาเมตเป็นประจำ นั้นจะพบว่าการลดลงของ α -MSH ซึ่ง α -MSH เป็น agonist ของ MC3/4R และมีการเพิ่มขึ้นของ AgRP ที่จะเข้าไปขัดขวาง hypothalamic-melanocortin signalling system ทำให้หนูกินอาหารมากขึ้นและเป็นโรคอ้วนในที่สุด (Broberger et al., 1998; Tschop et al., 2000) นอกจากนั้นการเปลี่ยนแปลงระดับของ corticotrophic hormones หลังการได้รับโมโนโซเดียมกลูตาเมตอาจจะเป็นผลมาจาก tissue-specific cleavage ของ POMC (post-translational process) ด้วยเอนไซม์ที่จำเพาะที่เรียกว่า prohormone converting enzyme I,II (PC1,II) ที่แตกต่างกันก็เป็นได้ ทำให้ได้ผลิตภัณฑ์เปปไทด์ฮอร์โมนที่เฉพาะ เพื่อทำหน้าที่ควบคุม homeostasis ให้คงที่

การศึกษานี้เราได้พบว่าการกระตุ้นด้วยโมโนโซเดียมกลูตาเมตทำให้มีการเปลี่ยนแปลงรูปแบบการแสดงออกของโปรตีนและยีนใน HPA axis เมื่อเปรียบเทียบกับหนูกลุ่มควบคุมที่ไม่ได้รับการกระตุ้น การวิเคราะห์ด้วยวิธี 2D-PAGE และ DDRT-PCR ทำให้ทราบการเปลี่ยนแปลงระดับการแสดงออกของโปรตีนและยีนบางชนิดเช่น โปรตีน RafGDS และ α -NAGA ในไฮโปทาลามัส Appbp2 EphA7 H-rev107 Cadherin2 และยีน POMC ในต่อมใต้สมอง และ Paf-ah1b2 cadherin2 Rpl27a และยีน AgRP NPY และ StAR ในต่อมหมวกไต

ในขณะที่เดียวกันการศึกษานี้ได้พบว่าโมโนโซเดียมกลูตาเมตก็มีผลยับยั้งการแสดงออกของยีนและโปรตีนบางชนิดเช่นกันใน HPA axis อย่างเช่น ADAMTS17 Glur4 และยีน POMC ในไฮโปทาลามัส PafAh1b2 ในต่อมใต้สมอง และ Slco3a1 และ Rpl29 ในต่อมหมวกไต การเปลี่ยนแปลงการแสดงออกของยีนและโปรตีนเหล่านี้น่าจะมีส่วนเกี่ยวข้องกับความผิดปกติในพฤติกรรมและสรีระของหนูที่เกิดขึ้น

การเพิ่มการแสดงออกของโปรตีนและยีนบางตัวเช่น GRP78 GRP75 H-rev107 Paf-ah และ EphA7 เนื่องจากการได้รับโมโนโซเดียมกลูตาเมตในปริมาณมากและต่อเนื่อง น่าจะเกี่ยวข้องกับการปรับตัวเพื่อให้อยู่รอดและรักษาหน้าที่ปกติของเนื้อเยื่อใน HPA axis อย่างไรก็ตามกลไกที่แน่ชัดของการกระตุ้นของโมโนโซเดียมกลูตาเมตต่อการเปลี่ยนแปลงการแสดงออกของยีนและโปรตีนเหล่านี้จะต้องมีการศึกษาต่อไป

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อุปกรณ์และสารเคมี

1. 0.5 M EDTA

EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$	9.3	g
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Dissolve and adjust volume to 50 ml with double distilled water.

2. 200 mM TE, pH 8.0

(200 mM Tris-HCl, pH 8.0 containing 50 mM EDTA)

Tris	0.33	g
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0.5 M EDTA	20	ml
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Dissolve in 130 ml double distilled water, adjust pH to 8.0 with HCl and then adjust volume to 200 ml with double distilled water.

3. Protein Precipitation Solution

(10% w/v TCA and 0.07% v/v 2-mercaptoethanol in cold acetone)

TCA	5	g
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2-mercaptoethanol	35	μl
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Dissolve and adjust volume to 50 ml with acetone.

4. 0.07% v/v 2-Mercaptoethanol

2-mercaptoethanol	35	μl
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Dissolve and adjust volume to 50 ml with acetone.

5. Rehydration Buffer

(8 M urea, 2% CHAPS, 0.002% bromophenol blue)

Urea	12	g
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CHAPS	0.5	g
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1% Bromophenol blue stock solution	50	μl
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Dissolve and adjust to a final volume of 25 ml with double-distilled water and stored at 4°C.

6. 10% Ammonium Persulfate (APS)

Ammonium persulphate	0.02	g
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Dissolve in 200 μl double-distilled water. (freshly prepare before used)

7. Rehydration Buffer with IPG

Rehydration buffer	400	μl
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IPG buffer, pH 3-10 L	1	μl
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Mix well and freshly dissolved 1 mg of DTT before used.

8. 1% Bromophenol Blue Stock Solution

Bromophenol blue	100	mg
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Tris-base	60	mg
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Dissolve and adjust to a final volume of 10 ml with double-distilled water.

9. 1.5 M Tris-HCl Buffer, pH 8.8

Tris base	181.7	g
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Dissolve in 750 ml double-distilled water and adjust pH to 8.8 with HCl, then adjust the volume to 1,000 ml with double-distilled water. Store at 4°C.

10. SDS Equilibration Buffer

(75 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 0.002% bromophenol blue)

1.5 M Tris-HCl pH 8.8	10	ml
Urea	72.07	g
87% Glycerol	69	ml
SDS	4.0	g
1% Bromophenol blue stock solution	400	µl

Dissolve and adjust to a final volume of 200 ml with double-distilled water. Store at 4°C. Just prior to use, aliquot the solution for 10 ml and add 100 mg DTT or 250 mg iodoacetamide (for first or second equilibration, respectively).

11. 10% Sodium Dodecyl Sulphate (SDS)

Sodium Dodecyl Sulphate	10	g
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Dissolve in 80 ml double-distilled water and adjust the volume to 100 ml.

12. Agarose Sealing Solution

SDS electrophoresis buffer	100	ml
Agarose	0.5	g
1% Bromophenol blue stock solution	200	µl

Add all ingredients into a 500 ml Erlenmeyer flask. Swirl to disperse. Heat in a microwave oven on low or on a heating stirrer until the agarose is completely dissolved.

13. Bradford Dye

Coomassie brilliant blue G250	100	mg
95% Ethanol	50	ml
85% Phosphoric acid	100	ml

Coomassie brilliant blue G250 is dissolved in 95% ethanol and mixed with 85% Phosphoric acid. The mixture is adjusted the volume to 1,000 ml with distilled water and filtered through Whatman No. 1 filter paper.

14. 30% T, 2.6% C Acrylamide/Bis

Acrylamide	292.2	g
<i>N, N'</i> -methylenebisacrylamide	7.8	g

Dissolve in 800 ml double-distilled water. The mixture is adjusted the volume to 1,000 ml with double-distilled water. When the substances are completely dissolved, filter solution through a 0.45 μm filter. Store at 4°C in the dark.

15. 2X Solubilizing Buffer (SB) with β -Mercaptoethanol

0.5 M Tris-HCl, pH 6.8	2.5	ml
β -Mercaptoethanol	1.0	ml
Glycerol	2.0	ml
0.2% Bromophenol blue-methanol	0.1	ml
10% SDS	2.0	ml

Adjust the volume to 10 ml with double-distilled water.

16. 0.2% Bromophenol Blue-Methanol Solutions

Bromophenol blue	0.02	g
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Dissolve in 10 ml methanol.

17. SDS Electrophoresis Buffer

(25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% SDS)

Tris-base	30.3	g
Glycine	144	g
SDS	10	g

Dissolve and adjust volume to 10 l with double distilled water.

18. 12.5% Separating Gel SDS-PAGE (1 gel)

Acrylamide / Bis (30%T, 2.6%C)	1,667	μl
1.5 M Tris-HCl, pH 8.8	1,000	μl
Double-distilled water	1,290	μl
10% SDS	40	μl
10% APS	40	μl
TEMED	4	μl

The chemicals are mixed consecutively and poured into the gel chamber beware of air bubbles.

Double-distilled water is carefully overlaid above the gel. The mixture is left for three hours for complete polymerization.

19. Tris Buffer Saline (TBS), pH 8.0

(10 mM Tris-base and 150 mM NaCl)

Tris-base	1.21	g
NaCl	8.77	g

The chemicals are dissolved in 800 ml double-distilled water. Adjust the pH to 8.0 with HCl. Make the final volume of 1,000 ml with double-distilled water.

20. Tris Buffer Saline Containing 0.05% Tween 20 (TBST)

Add 500 μ l of Tween 20 to 1,000 ml of tris buffer saline.

21. Transfer Buffer (Towbin Buffer)

(48 mM Tris, 39 mM glycine and 20% methanol)

Glycine	0.586	g
Tris-base	1.162	g
Methanol	40	ml

Glycine and Tris-base are dissolved in 100 ml double-distilled water and added methanol. The mixture is adjusted the volume to 200 ml with double-distilled water.

22. 5% Skim Milk in TBST Buffer (Blocking Solution)

Skim milk	5.0	g
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Dissolve in 100 ml of TBST buffer.

23. Substrate Buffer, pH 9.5

(100 mM Tris-base, 100 mM NaCl and 50 mM MgCl_2)

Tris-base	3.0	g
NaCl	1.5	g
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	2.5	g

Dissolve 3.0 g Tris-base, 1.5 g NaCl and 2.5 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ in 200 ml double-distilled water.

Adjust to pH 9.5 with HCl. Dilute to 250 ml with double-distilled water. Store at 4°C.

24. 1 mg/ml *p*-Nitrophenyl Phosphate (ELISA Substrate Solution)

<i>p</i> -Nitrophenyl phosphate	2.0	mg
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Dissolve in 2 ml substrate buffer, pH 9.5.

25. Substrate Solution for Western Immunoblotting

Alkaline phosphatase Conjugate Substrate Kit (Bio-Rad)

1) AP color reagent A containing nitroblue tetrazolium (NBT)

2) AP color reagent B containing 5-bromo-4-chloro-3-indolyl phosphate (BCIP)

Dissolve 50 μ l of NBT reagent A and 50 μ l of BCIP reagent B in 5 ml of substrate buffer pH 9.5.

Prepare freshly and keep in the dark.

26. DEPC-Treated Water (Nuclease Free Water)

Dissolve 1 ml of DEPC in 1,000 ml of water and place for overnight at room temperature. Water was autoclaved for 15 min to discard DEPC.

27. 0.1% DEPC

DEPC	1	ml
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Dissolve in 1,000 ml of double distilled water.

28. 0.5% SDS

SDS	0.5	g
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Dissolve in 100 ml of nuclease free water.

29. 0.1 M NaCl

NaCl	1.17	g
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Dissolve in 100 ml of nuclease free water.

30. 1 mM EDTA

EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$	0.5	mg
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Dissolve in 500 ml of nuclease free water.

29. Sample Loading Buffer

EDTA- $\text{Na}_2 \cdot 2\text{H}_2\text{O}$	3.7	mg
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Bromophenol blue	25	mg
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Xylene cyanol FF	25	mg
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Dissolve completely in 4.25 ml of water and added 5.75 ml of 87% glycerol. Mix well.

30. 10X MOPS Electrophoresis Buffer

MOPS	41.8	g
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Dissolve in 700 ml of nuclease free water and adjust pH to 7.0 using RNase free 2 N NaCl.

Added 20 ml of RNase free 1 M sodium acetate. Adjust volume to 1,000 ml with nuclease free water and filtrated through 0.45 μm Millipore filter. Stored at room temperature and protected from light.

Prior used, the solution was diluted with nuclease free water for 10 times to 1X solution.

31. 50X TAE Electrophoresis Buffer

Tris	48.4	g
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Glacial acetic acid	11.42	ml
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0.5 M EDTA solution	20	ml
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Dissolve and adjust volume to 200 ml of double distilled water and then autoclave for 15 min.

Prior used, the solution was diluted with autoclaved double distilled water for 50 times to 1x solution.

32. 1.5% Agarose Gel

Agarose	0.45	g
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Melt in 30 ml of 1X TAE electrophoresis and pour into a tray and allow it cool down to completely polymerized.

33. Ethidium Bromide Solution

Ethidium bromide	10	mg
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Dissolve in 50 ml of nuclease free water.

Primer sequences

1. Random Primers for DD-RT-PCR

Primer Name	Sequences
P2	5'- ATTACCCCTCACTAAATGCTGGAGG -3'
P3	5'- ATTAACCCTCACTAAATGCTGGTGG -3'
P4	5'- ATTAACCCTCACTAAATGCTGGTAG -3'
P6	5'- ATTAACCCTCACTAAATGCTGGGTG -3'
T1	5'- CATTATGCTGAGTGATATCTTTTTTTTGC -3'
T2	5'- CATTATGCTGAGTGATATCTTTTTTTTAC -3'
T3	5'- CATTATGCTGAGTGATATCTTTTTTTTATAG -3'
T4	5'- CATTATGCTGAGTGATATCTTTTTTTTCA -3'

2. Primer for Real-Time PCR and Riboprobe Synthesis

Gene Name	Primers	Sequences	Application
POMC	Forward	5'-GGTGAAGGTGTACCCCAACG-3'	Real time PCR
	Reverse	5'-GCCGCTCGCCTTCCA-3'	
POMC	Forward	5'-GAAGGTGTACCCCAATGTCG-3'	Riboprobe synthesis
	Reverse	5'-CTTCTCGGAGGTCATGAAGC-3'	

3. Specific Primers for Semi-Quantitative RT-PCR

Gene Name	Primers	Sequences	Product size (bp)
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AgRP	Forward	5'- GAAGGGCATCAGAAGGTC-3'	226
	Reverse	5'- CAGGTCGCAGCAAGGT-3'	
ALDH2	Forward	5'- ATGTGGACAAAGTGGCCTTC-3'	517
	Reverse	5'-TTGAGGATCTGCATCACTGG -3'	
β -actin	Forward	5'- CAACTGGGACGATATGGAGAA-3'	570
	Reverse	5'- AGGAAGGAAGGCTGGAAGAG-3'	
GRP75	Forward	5'-CAGAGCCCCAAGTAAAGCTG-3'	422
	Reverse	5'- TCTGTTGCTCACGTCCTGTC-3'	
GRP78	Forward	5'-TTCCGCTCTACCATGAAACC-3'	422
	Reverse	5'- TCTTTTGT CAGGGGTCGTTC-3'	
HSC70	Forward	5'- CAGAATCCCCAAGATCCAGA-3'	534
	Reverse	5'-ACCATGCGCTCAATATCCTC-3'	
MC4-R	Forward	5'- CGCGCTCCAGTACCATAAC-3'	466
	Reverse	5'- TTCTTGACTCCGCAGGGCA -3'	
NPY	Forward	5'- CTGCGACACTACATCAATCT-3'	283
	Reverse	5'- ATACAACGACAACAAGGGA-3'	
POMC	Forward	5'- CCAGGCAACGGAGATGAAC-3'	453
	Reverse	5'- TCTCGGAGGTCATGAAGCC-3'	
StAR	Forward	5'-CAGGAGAATGGAGATGAAGTGC-3'	437
	Reverse	5'-AGGTCAATACTGAGCAGCCACG-3'	

DNA Sequences of Differently DNA fragments

Band h5

ACTCCTGCCTTCTGCCCAATCGTATGCTTGACACACCATGGCCGTTATTGTAGGGGA
GGAAGCGCGTGGAGACGAGTAGACACGTAGATAATCTATGCTAAAGCGGNGCGCTC
ATGAGATCCACAGCCAGGCGCCGATCATGCGCCCTGGCGCTGTTTGGGGAAATACC
ACTGTAGCGGCCCCGCCCTTTAGCCATAGCATTATCTAGCGTCTGTCACTTCCTCCCA
CGGGCTCCTCCTCTCAAATAGCCTAGG

Band h8

AGCCACTTAGGGGATAGATTTGGCTAATTACCCCTCTTTCAGGAATTCTCTGCAGGTC
CACCCNG TAGGC

Band h11

AGTTTCCTGCACTCGCGTNGACGTTGCAACTCGCGCTTCCCCAAGGTTGATGTGTAT
GTGCCTGTACGCGCGAACGAGCCACCAGTTGGCGACGTGTCCAGACAAGGGGATGC
TAGTGATCTCGAGGGGCCTGCGTGAGCCTACCGAGCCCCTNAGCAATACTAGCCATC
CCTNTATGTGACACGCTGCCAACGTGTGCCTGTCTCGCGGTCAGACGCATTCAACAAC
ACTACCTGTGGAGACGCGAAGTGTACACGCCTGACCCCNAGTCAANAACTCTTA
GGA

Band p2

CGGCCTCTAAAAGCACTTGGCGGCTCTTTATGAGGTCACATATGACCAGGCAATTCTA
CTATGAGGAATCCATACGAGGGGAATAAGAGCATAACGAATCCCCCAAACCTGTATATG
AATATTTATTCTTTTTGGTTCTTTTTTTTCGGAGCTGGGGACCGAACCCAGGGCCTTGC
GCTTCCTAGTAAGCGCTCTACCACTGAAGCTAAATCCCCAGCCCCAATATATGAAGTA
TTTATAATAGCATTACTCATACCAGGTAAAAAAAAGATATCAC

Band p3

TCTACAAGACAGGTGATGAGCAATGCGTAGCACTGGACCTTGTGTGCAAGTACCTAC
ATAATTGGTCTCACATCCACGCTCGAAGAATCGAGCGCTATGTTCCATGCCGATGGG

GCAACTGGTGCCAATGTCTCGAGGCTCGTCTTGTTTCGCGCTCGCCCTTTGCTACAA
 GAACCTCACATTATAGGACCTNTNCTGTGCATGTAGAAAAGAGACTATCTCGAGTACG
 TCGCGATTTCATCTACGGCTACCCGTGTGAAATGCGCTCGCGATCGAAATAGCGTCTC
 GATCACGATCGCGAACCGATTCTCGCACC

Band p4

TTGCCTTTCTGCCAATCGTATGCTTGACACACCATGCCGTTATTAGGGGAGGGAGC
 GCGTGGAGACGGAGTCAGACACGTAGATATCTATGCTAAGCGGGCGCGCTCAAGTG
 GAGATTCCAACACGCCAGGGCGCCGATCATGCGCCTGCGCTTTGGGGATCCACTGA
 GCGGCCCCGCTTTAGCCATAGCATTATCTATGCGTNTGTCACTCCTTNCACGGCTCTC
 TCTCAAAANTAGCCTAGGTGGTGACCA

Band p5

CCTAACCTTCTAAAGAACCCAAGGCCTATTACCTAACCTTCCGTGGGGCATTATAAT
 CCCAAAAGGAATGGTCTCCCACTCTACTAACCCAACCAAAGGATGCCATCTTTTGGTC
 TCCAACTCTGTNGTTTCAACTTAGTTAAGTCGTTACTATCTCAATAACATTAATAACAC
 TAAGAACACACGTAGAGAATAAGCAAACACTCGAGATGTCACTCATTCTACCCTTCGA
 AACAAATGCACTAGGACATAAAAAAGAATAATCATCATTGAGCCTGAAACTGTAGAGT
 G

Band p6

CGAGAGCCACCAGGTTGGCGACTGTCCAAGACAAGGGGATGGCTAGTGATCTCGAG
 GGGCTCGCGTGAGCTCACCGAGCCCCTNAGCNCCTAGCCATCCCTGTCTTGACAC
 GCTGCCAACCGTGTGCCTGTCTCCGGTCAACGCATCCAACCTACCCTGTGGAAGCGCG
 AAGTTTGAACCGCTAGNCCC GGGTNAAAGAAAACCAT CTATTGGAAGA

Band a1

ATCACTTTGACAATATCATCCAGGTTACTTCGGCAAAGTGGCATGAGGCATTACCACT
 TGAAGAGGAACCAGAGCTTCTGCCCAACTGTCAACCTGGATAAATTATGGACGTTGGT
 CAGCGAGCAGACACGGGTCAATGCAGCAAAAAAAGA TATCACTCAG CATAAT

Band a2

CAGTGAATGAGCATGCGTAGCACTGGGACCTTGTGTGCAAGTACCTACATATTGGTTC
 CCTCCACGCTCGAAAATCGAGCGTATGTTCCATGCCGATGGCACATGGTGCCAATGG
 TCCTCGAGGCTCGTCTTTCCGCGCTCGCCCTTTGGCTACAGACTCACTTATAGACCTC
 TTCTGTCATTAGAAAAGAGCTATTCAGTCGTCGCGATACATCTACGGCTACCCGTGTGA
 AATGCGCTCGCGATCGAATAGCGTCTCGATACACGATCGCGAACCGATTCTCGCACG
 GTATNTACTGACATTGGACCCCTGTGCCCACCGGTTTAA

Band a4

ACGTATATTGCCTGGAAATCGATTGGTGGT TTCTCACAAAAATTGGAATA
 TTCTATCTCAAGACCCAGCTCTACCAATCCTGGCATTGTTTAACCCAAAAGATGCTA
 C TAATCATCCC ACAAGGACAA TTATGTT

Band a6

CGGGCCCTAAAAGCACTTGGCGGCTCTTTATGAGGTCACATATGACCAGG
 CAATTCTACTATGAGGAATCCATACGAGGGGAATAAGAGCATACGAATCC
 CCCGAAAACGTATATGAATATTTATTCTTTTTGGTTCTTTTTTTTTCGGAGCTGGGGAC
 CGAACCCAGGGCCTTGCGCTTCCTAGGTAAGCGCTCTACCACTGAGCTAAATCCCCA
 GCCCCAATATATGAATATTTATAATAGCATTACTCATACCAGGT AAAAAAAAAG
 ATATCACTCA GCATGAT

Band a12

AACAACTGTGTTACAGCCGTGATTACCCACATACGGATAGCCTTCGCCCCTATCCAT
 GACCACGAAGACCGGTAGCCAACCTTTTGCCTTCACACTATAGATGGTCCATAGTAA
 TGTATGGCTCCGATCTCGATTAATAGTCCACACCTCATAGAGAAACATGCATGCAGGA
 ATAGGTACTCACTGCGAGTTCAACCCAAGTGACCCTCAGCAAGAAAATATGACTTATA
 CAATCAAAG

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารนานาชาติ

1.1 ส่ง Manuscripts ของงานวิจัย เรื่อง **Differential effects of monosodium glutamate treatment on POMC transcripts between rat pituitary and AtT-20 cell line** และกำลังรอผลการพิจารณาการตีพิมพ์ในวารสารนานาชาติ **Acta Biochimica et Biophysica Sinica** (กำลังรอผลพิจารณา)

Authors : 1. Khomsorn Lomthaisong
2. Anuwat Wanthong

1.2 ตีพิมพ์ ใน African Journal of Biochemistry Research เรื่อง **Protein profiles of adrenal gland of neonatal rat treated with monosodium glutamate**

Authors :

1. Anuwat Wanthong
2. Sompong Thammasirak
3. Khomsorn Lomthaisong* (corresponding author)

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2. การนำผลงานวิจัยไปใช้ประโยชน์

- มีการนำผลงานวิจัยในส่วนของวิธีทำการทดลองไปใช้ประกอบการเรียนการสอน ทั้งภาคทฤษฎีและภาคปฏิบัติ

3. ผลงานด้านอื่นๆ

- การนำเสนอผลงานวิจัยในการประชุมวิชาการ

1. **Lomthaisong K**, Khonsunthea W, Wanthong A. Differential display reverse transcription-polymerase chain reaction (DDRT-PCR) of adrenal gene expression. *Congress on Science and Technology of Thailand 2003*, Bangkok, Thailand, October 19-21, 2004.

2. Wanthong A, Thammasirak S, **Lomthaisong K**. Differential expression of hypothalamic proteins after monosodium glutamate treatment. *Congress on*

Science and Technology of Thailand 2004. Bangkok, Thailand, October 19-21, 2004

3. Wanthong A, **Lomthaisong K**, Thammasirirak S. Analysis of protein expression patterns in the adrenal gland following monosodium glutamate treatment in the neonatal rat. *Second Protein Research Network Symposium 2005*. Bangkok, Thailand, September 22-23, 2005.

ภาคผนวก



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**Differential effects of monosodium glutamate treatment on
POMC transcripts between rat pituitary and AtT-20 cell line**

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**Differential effect of monosodium glutamate treatment on POMC transcripts
between rat pituitary and AtT-20 cell line**

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Abstract

Pro-opiomelanocortin (POMC) is a pro-hormone precursor mainly produced in hypothalamus and pituitary gland. POMC processing in pituitary generates peptide hormones especially adrenocorticotrophic hormone (ACTH) that regulates adrenal function. This study provided that high dose administration of MSG (4 mg/g bw/day) during infant period influenced on POMC mRNA content in pituitary gland at 30 days of age. The result showed that POMC transcripts were increased up to 50% in MSG-treated rat compared to control group. On the other hand, directly administration of MSG to pituitary cell line, AtT-20, inhibited POMC expression. POMC transcripts were reduced more than 50% in MSG-treated AtT-20 cells at concentration of MSG ranging from 25 μ M to 1,600 μ M. In addition, gamma1-MSH, a POMC derived peptide, was also decreased more than 50%. It was suggested that MSG had directly inhibited AtT-20 cells activity on POMC expression. However, pituitary function is controlled by releasing hormone of hypothalamus, therefore, increasing of POMC gene expression in pituitary gland of MSG-treated rats should be more indirectly influenced by MSG activation via neurosecretory cell of Hypothalamic-Pituitary-Adrenal axis differentially directly activation to pituitary cell line.

Key words; monosodium glutamate (MSG), Pro-opiomelanocortin (POMC), HPA-axis, homeostasis

Numerous studies showed that glutamate is the main excitatory neurotransmitter in the central nervous system (CNS) and is responsible for most fast synaptic neurotransmission [1]. However, the neonatal administration of large doses of MSG to rodents is recognized to induce neurotoxicity [2,3]. It has been known that the coactions between the hypothalamus and pituitary are integration between the nervous and endocrine system. Neurohormones, also called releasing hormones secreted from hypothalamus influence on activity of hormone-secreting cells in the anterior pituitary [4].

Pro-opiomelanocortin (POMC) is a 267-amino acid precursor protein that is synthesized in the arcuate nucleus (ARC) of hypothalamus as well as the anterior pituitary. The posttranslational processing of POMC results in a number of peptides with very different biological activities [5]. In particular, adrenocorticotrophic hormone (ACTH) which generated by cleaved POMC regulates adrenal function on homeostasis [6].

In this respect, semi-quantitative RT-PCR and real-time PCR were used for evaluation of mRNA levels of POMC in pituitary cell line (AtT-20) and the rat pituitary gland following MSG treatment.

Materials and methods

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

Animal management and tissue material

Wistar rats were used in this experiment. Before birth, their mothers were raised in a temperature (25±1°C) and light-controlled (lights on from 07.00 am to 07.00 pm)

room with free access to food and water. The dams were kept one to a cage. After weaning, the pups used for this study were reserved in the same conditions as their mothers. Rats were daily given subcutaneous injection of MSG (4 mg/g body weight) for treated animals or normal saline for control on first 15 day after birth. All MSG-treated and control animals were killed at 30th days of age. The pituitary gland were gathered and preserved at -70 °C until used.

The cell lines maintenance

AtT-20 cells were cultured under aseptic condition. Prior to treatment, AtT-20 cells were cultured in DMEM containing 10% FCS, 100 U/ml of penicillin, 100 µg/ml of streptavidine and 2 mM L-glutamine at 37 °C and 5% (v/v) CO₂ in humidified incubator until reached confluence stage. The medium was replaced with FCS free medium and the cells were cultured for overnight at 37 °C and 5% (v/v) CO₂ before treatment. The next day the medium was replaced again with FCS free medium containing MSG at various concentration (two folds concentration) ranging from 25 µM-1600 µM and then incubated for 1 hour at 37 °C and 5% (v/v) CO₂. For the control cultured, MSG was left out. After treatment, cells were harvested by adding 0.5 ml of 0.05% trypsin-EDTA solution and centrifuged at 5,000 x g.

RNA extraction

Total RNA from dissected pituitary gland of rats and AtT-20 cells were isolated using illustra RNAspin Mini RNA isolation kit which included column RNase-free DNase treatment. The glands were broken up mechanically as well as lyses in an appropriate aliquot of buffer RA1 containing β-mercaptoethanol and mix immediately

and then filtrated through a RNA spin Filter unit to reduced viscosity and removed the lysate. The homogenized lysate was added with 70% ethanol and mixed by vortexing to adjust RNA binding conditions and then was applied onto the RNAspin Mini column and centrifuge for 30 sec at 8,000 x g. The column was replaced in a new collection tube. Membrane desalting buffer was added onto the column and then centrifuge at 11,000 x g for 1 min to dry the membrane. The flow-through was discarded and returned the column to the collection tube. The column was incubated with DNAs I solution for 15 min to digest DNA and washed with buffer RA2 and RA3 that provided in kit, respectively. Highly pure RNA was eluted in nuclease free water and centrifuged for 1 min at 11,000 x g.

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 containing formaldehyde stained with ethidium bromide.

RNA analysis by semiquantitative RT-PCR

Primers were designed based on the corresponding cDNA sequences from *R. novogicus* as follows: β -actin sense (5'-CAACTGGGACGATATGGAGAA-3') and β -actin antisense (5'-AGGAAGGAAGGCTGGAAGAG-3') (product size 570 bp); POMC sense (5'-CCAGGCAACGGAGATGAAC-3') and POMC antisense (5'-TCTCGGAGGTCATGAAGCC-3') (product size 453 bp).

Total RNA (1 μ g) from AtT-20 cell culture or pituitary gland of animal 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 μ M 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. The cDNA was used as templates for semi-quantitative PCR analysis. A numbers of cycles were optimized 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 30 cycles consisting of 20 sec at 95 °C for denaturation, 20 sec for annealing at 60 °C and 40 sec at 72 °C extension steps. A final extension cycle of 72 °C for 7 min was included. PCR products were further analyzed by agarose gel electrophoresis. Ethidium bromide intensities in each band resulting from PCR amplification were analyzed using image analysis software (Quantity One, Bio-Rad Laboratories, CA). Data are expressed as means±SEM from at least three independent experiments performed on RNA preparations. mRNA levels of POMC were calculated as the ratio to that of β-actin in each sample. Statistical analysis was performed using a paired *T* test. Differences were considered significant at $P < 0.05$.

POMC transcript analysis in AtT-20 cells using Real-time PCR

Quantification Real-time PCR was performed to quantify POMC mRNA level using an ABI PRISM 7700 system. We used POMC -forward primer 5'-GGTGAAGGTGTACCCCAACG-3' and POMC-reverse primer 5'-GCCGCTCGCCTTCCA-3' together with 5'-FAM-TCGGCGGAGGCCTTTCCCCT-3' as TaqMan probe. 18S ribosomal RNA amplification was used as internal control for experiments. Amplification mixtures (50 μl) contained 1 X TaqMan buffer, 200 μM dNTPs, 5.5 mM MgCl₂, 300 nM each primer, 175 nM TaqMan probe and AmpliTaq Gold (0.025U/μl).

Determination of gamma1-MSH using ELISA

POMC derived peptide, gamma1-MSH level expressed in AtT-20 cells was determined using Enzymatic-Linked Immunosorbent Assay (ELISA). ELISA was carried out to quantify POMC-derived peptide, gamma1-MSH (N-POMC 1-28) using rabbit anti-N-POMC 1-28. Gamma1-MSH was separated from the cells and culture medium. The peptides were coated on microtiter-plate and placed at 4 °C for overnight. Plates were rinsed with TBST to remove weakly bound peptides and then blocked with 100 µl of 5% skimmed milk in TBST and placed at 37 °C for 1 hr. The blocking solution was discarded and plate was washed three times with TBST to make sure that skimmed milk was removed. The washed plate was then incubated with rabbit anti-N-POMC 1-28 at 37 °C for 1 hr. The washing step was repeated and plate was then incubated at 37 °C for 1 hr with the secondary antibody against rabbit antibody linked with alkaline phosphatase. After that, the antibody was discarded and plate was washed three times with TBST and another three times with TBS to removed TWEEN-20. The detection was performed using freshly prepared chromogenic substrate (1 mg/ml of *p*-nitrophenyl phosphate, 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl₂). The absorbance of colorimetric products was determined at 405 nm using an ELISA plate reader.

***In situ* hybridization**

AtT-20 cells were grown up on slice to 40-50% confluence (normally for about 48 hr) and then treated with 400 µM MSG for 1 hr. Cultured cells were washed twice with HBSS solution before being fixed in 4% paraformaldehyde in phosphate buffered saline. In hybridization, cells were washed and dehydrated in

an ethanol series (70%, 95% and 100% ethanol) for 3 min each, delipidated in chloroform, and then rehydrated in an ethanol series (100%, 95% and 70% ethanol) for 3 min each. To block non-specific binding, the cells were prehybridized in prehybridization buffer (50% formamide, 40 ng of salmon sperm DNA, and 5x SSC). The slides were then hybridized in hybridization buffer containing either sense or antisense riboprobes at different probe concentrations ranging from 1 in 250, 1 in 500 and 1 in 1000 dilution (equivalent to 1 µg/ml, 500 ng/ml and 250 ng/ml respectively). Hybridization was carried out at 55 °C for at least 24 hr.

After hybridization, slides were washed in 5x SSC and incubated with RNase A for 30 min in RNase-free buffer. Subsequently, slides were washed with 5x SSC at room temperature for 30 min and at 55 °C for 30 min and then washed with 0.1x SSC at 55 °C for 30 min. Then the slides were blocked with blocking buffer for 2 hr before being incubated with sheep anti-digoxigenin conjugated to horse radish peroxidase (HRP), then washed with buffer I. The signal detection was performed using the tyramind amplification system, TSATM-Directed (Red) kit (NEN[®] Life Science, USA) to detect digoxigenin-11-dUTP in riboprobes. Tetramethylrhodamine tyramide (TMR) was activated by peroxidase, providing the deposition of red fluorescence. The slides were washed in TNT buffer for 15 min three times, and incubated in TMR solution for 10 min at room temperature. Slides were then washed again in TNT buffer for 3x15 min and in TE buffer for 15 min prior to counter staining with the nuclear stain TOTO-3 (Molecular probes, USA). The slides were visualized using the confocal microscope.

The cells were then scanned at 570/30 nm band-pass emission filter for the TRITC and at 660 nm long-pass for TOTO-3 using image-acquiring software from

Leica TCN-NT software. Initial scanning was performed at the medium scanning speed with the laser intensity at 50% in order to minimize photobleaching of the sample before image acquisition. The photomultiplier tube (PMT) was optimized for intensity of the signal in order to obtain the best photographic resolution. Images were multiple colored using Adobe Photoshop version 6.0 (Adobe Corporation, USA) photographic software. The red fluorescence obtained from the emission of TMR was normally altered to pseudo-green fluorescence, whilst the red fluorescence obtained from nuclei staining from TOTO-3 remained red, with the original fluorescent intensity.

Figure 1

Results

Newborn rats were received dairy high dose (4 mg/g of body weight/day) of MSG during infant period (first 15 day of life). POMC transcript levels were evaluated using semi-quantitative RT-PCR analysis. The results showed that POMC transcripts were increased into 50% in the pituitary gland of 30 days old MSG-treated rats compared to control group (Figure 1). On the other hand, AtT-20 cells were directly treated with MSG at various concentrations (two folds concentration) ranging from 25

Figure 2

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4 μM -1600 μM . Unlike *in vivo* treatment, MSG showed inhibitory effects on POMC gene
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6 expression in AtT-20 cells (Figure 2).
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POMC gene expression level was reduced more than 50% in AtT-20 cells treated with MSG at every concentration comparing with control. In addition, the level of gamma1-MSH that is a POMC-derived peptide was also measured by ELISA. The result demonstrated that gamma1-MSH level was correlated to the POMC transcripts measured by quantitative real-time PCR (Figure 3). Gamma1-MSH level was decreased more than 50% in AtT-20 cell received MSG.

Figure 3

Like the results of real time PCR, *in situ* hybridization analysis showed that MSG also had inhibitory effect on POMC expression in AtT-20 cell (Figure 4). The expression of POMC was indicated by fluorescence (TMR) resulting from a binding between POMC mRNA and anti-sense riboprobes whereas nuclei were stained red that resulted by a binding of TOTO-3 to genomic DNA in nuclease. This result demonstrated that there was inhibitory effect of MSG on POMC mRNA expression in MSG-treated AtT-20 cells. This finding was corresponded to real time PCR and ELISA results.

Figure 4

Discussions

Increasing of POMC transcripts in pituitary gland of MSG-treated rats might be due to an increase in pituitary activity after MSG activation resulting in more ACTH production and secretion. ACTH is secreted from endocrinal pituitary cells and transported to adrenal cortex via blood circulation. ACTH stimulates adrenal cortex

activity to synthesized steroid hormones including glucocorticoid, main cortisol in animal [6,7]. Therefore, a rising of POMC expression in pituitary gland responding to MSG stimulation would also cause over activation of adrenal glands.

Inversely, an inhibitory effect of MSG to POMC gene expression was observed in AtT-20 cells. POMC transcripts were decreased to 50% in MSG-treated AtT-20 cells comparing to untreated-cells. It was possible that directly administration of MSG had an excitotoxic effects and leads to changes in abnormal activity of AtT-20 cells. However, the regulation of POMC expression in pituitary gland of animals were influenced by signal from upstream, hypothalamus, and also negatively feedback regulation of cortisol from downstream, adrenal glands [8]. When the body is exposed to stressful condition, neurosecretory cells in hypothalamus are stimulated by nerve impulse from higher brain center to produces more corticotrophin releasing hormone (CRH) [9]. This releasing hormone drives more secretion of ACTH and cortisol of anterior pituitary and adrenal glands, respectively. Cortisol concentration is provided in higher level until the cause of stress was decreased [10]. Thus, up-regulation of POMC gene expression would result in over production of ACTH and other involving peptide hormones in pituitary gland. Administration of MSG has caused indirectly effects and provided complexity of MSG activation mechanism through neurosecretory cells in the HPA-axis.

In conclusion, receiving high dose of MSG in neonatal period would stimulate changes in pituitary activity on production of POMC at 30 days of age. This result may be due to stress response mechanism of animal body via cortisol regulation. Furthermore, the changes on pituitary activity following MSG treatment were indirectly influenced via hypothalamic stimulation to increase secretion of CRH to initiate stress response mechanism of the body.

Acknowledgement

We thank Prof. Dr. Philip Lowry and Dr. Andrew Bicknell, the School of Biological Sciences, The University of Reading, UK, for the technical assistances in fluorescent detection and Real time PCR quantification.

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Footnote;

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Figure legends

Figure 1 Semi-quantitative RT-PCR analyses of POMC transcripts following neonatal MSG-treatment. Amplification was performed using 2 fold serial dilutions of cDNA templates. Representative images of 1.5% agarose gels stained with ethidium bromide indicating the amplified fragments for POMC. cDNA quantity extracted from both animal groups were normalized to the amount of β -actin cDNA. Relative gene expression level is demonstrated by the ratio of cDNA content of MSG-treated to control rats. Mean_{SEM}; (n = 3). *P < 0.05 vs the corresponding band intensity in MSG-treated animal.

Figure 2 Effect of MSG treatment on POMC mRNA expression in AtT-20 cell line. POMC mRNA level was normalized against 18S ribosomal RNA. The results

demonstrate that POMC mRNA expressed within the cells with no MSG-treatment is significant higher (* $P < 0.005$) ($n = 3$) that those cells treated with MSG at various concentration (ranging from 25 μM -1600 μM). Statistical analysis is performed by the student T-test.

Figure 3 Effect of MSG treatment on POMC derived peptide, gamma1-MSH in AtT-20 cell line. Gamma1-MSH level is measured using ELISA. The result showed that Gamma1-MSH within the cells with no MSG-treatment is significant higher (* $P < 0.005$) ($n = 3$) than those cells treated with MSG at various concentration (ranging from 25 μM -1600 μM). Statistical analysis is performed by the student T-test.

Figure 4 Confocal photographic images demonstrate the expression of POMC mRNA in the AtT-20 cells following MSG treatment. The POMC mRNA that located in cytosolic part of cells is indicated by green fluorescence (TMR) while nuclei are stained red (TOTO-3). The control AtT-20 cells were hybridised with sense riboprobe. *In situ* hybridisation shows lower production of POMC mRNA (green fluorescence) in MSG-treated AtT-20 cells compared to the cells that untreated with MSG.

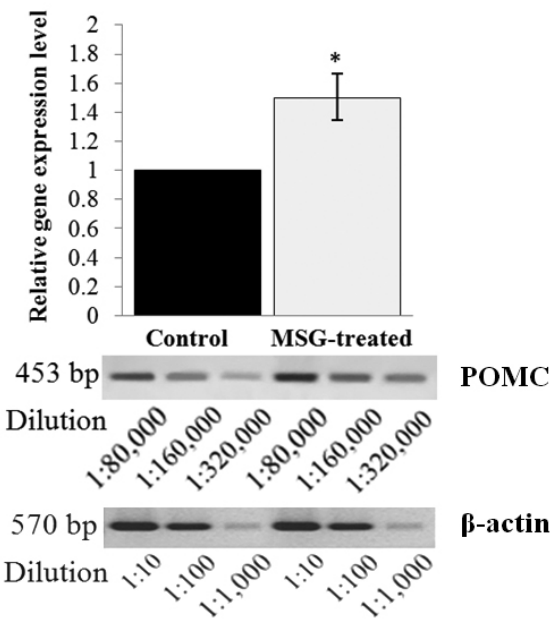


Figure 1

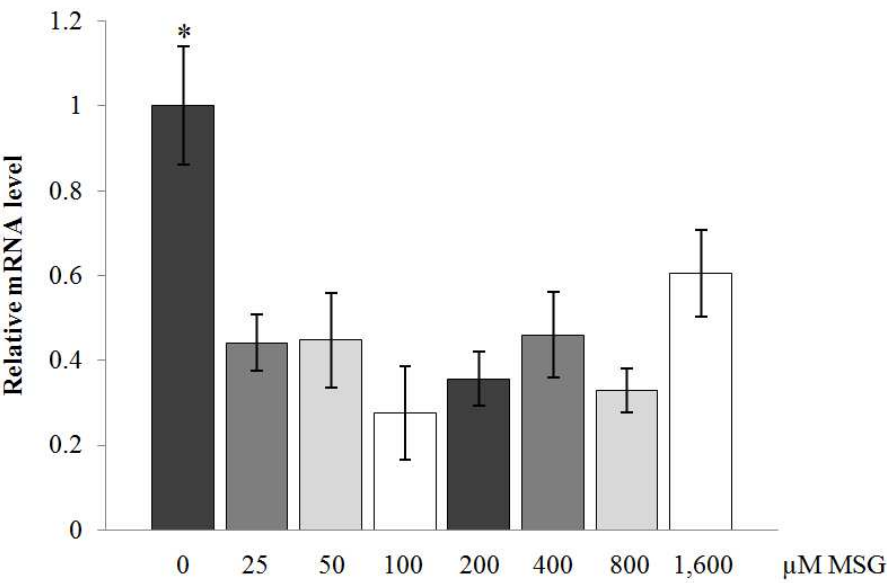
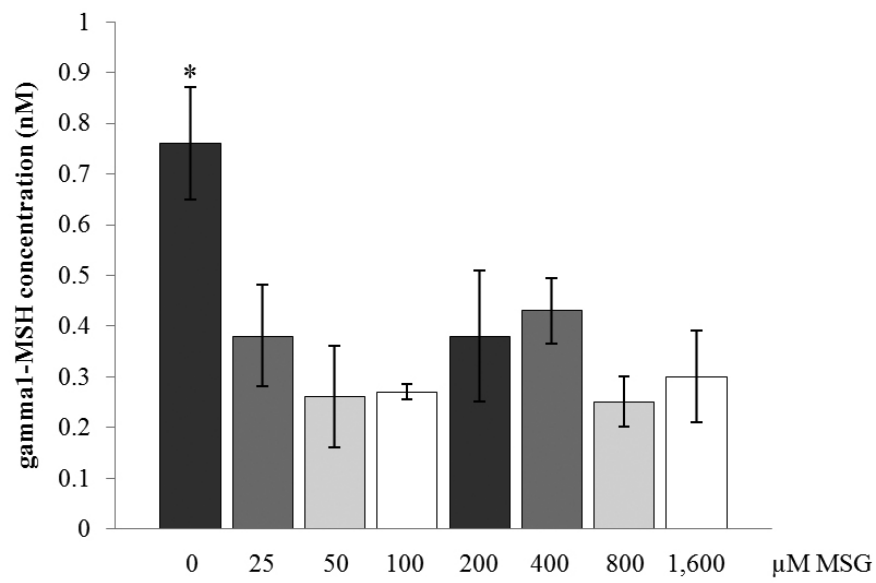
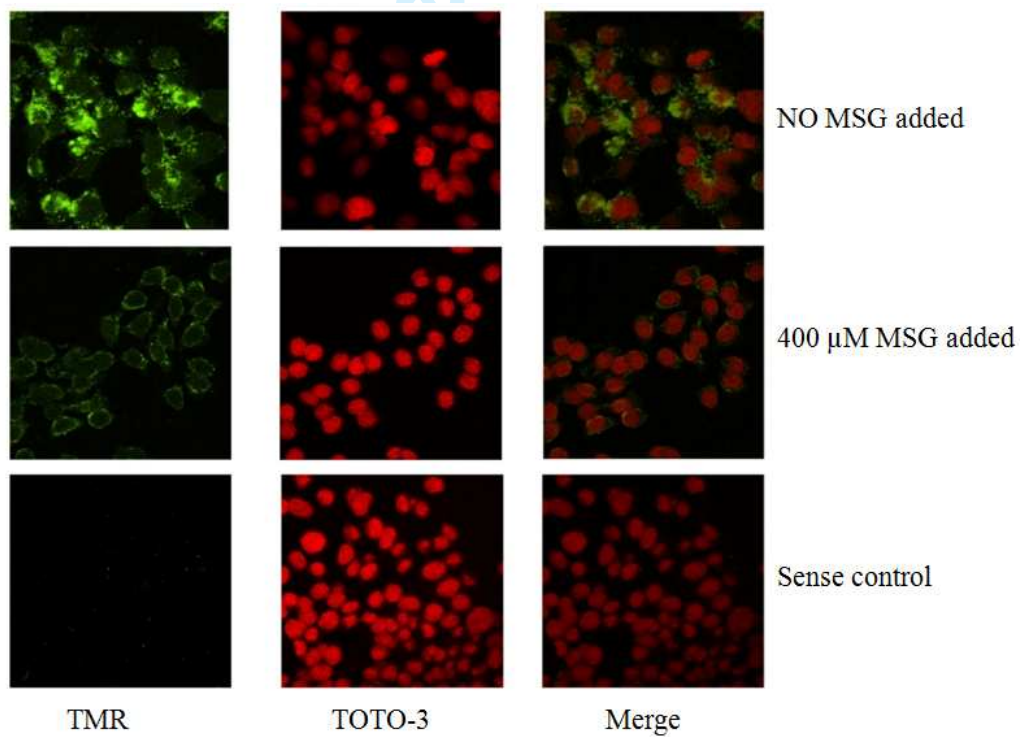


Figure 2

**Figure 3****Figure 4**

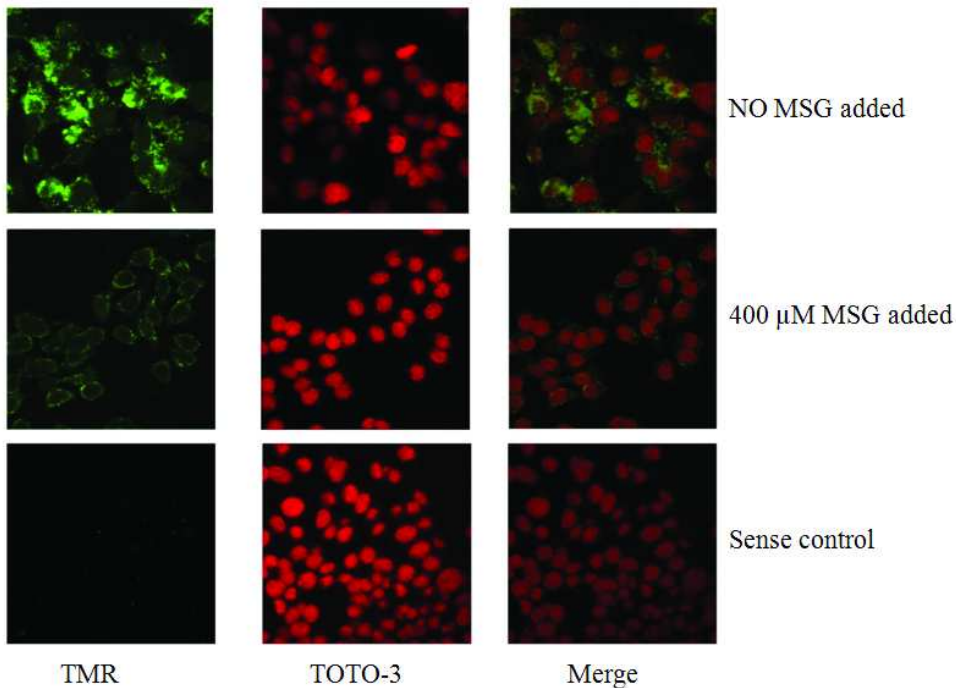


Figure 4 Confocal photographic images demonstrate the expression of POMC mRNA in the AtT-20 cells following MSG treatment. The POMC mRNA that located in cytosolic part of cells is indicated by green fluorescence (TMR) while nuclei are stained red (TOTO-3). The control AtT-20 cells were hybridised with sense riboprobe. In situ hybridisation shows lower production of POMC mRNA (green fluorescence) in MSG-treated AtT-20 cells compared to the cells that untreated with MSG. 67x48mm (300 x 300 DPI)

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 anatomic-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). Phenylmethanesulphonylfluoride (PMSF), aminobenzamidine (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 dodecylsulphate (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 ± 1 °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 N₂ 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.

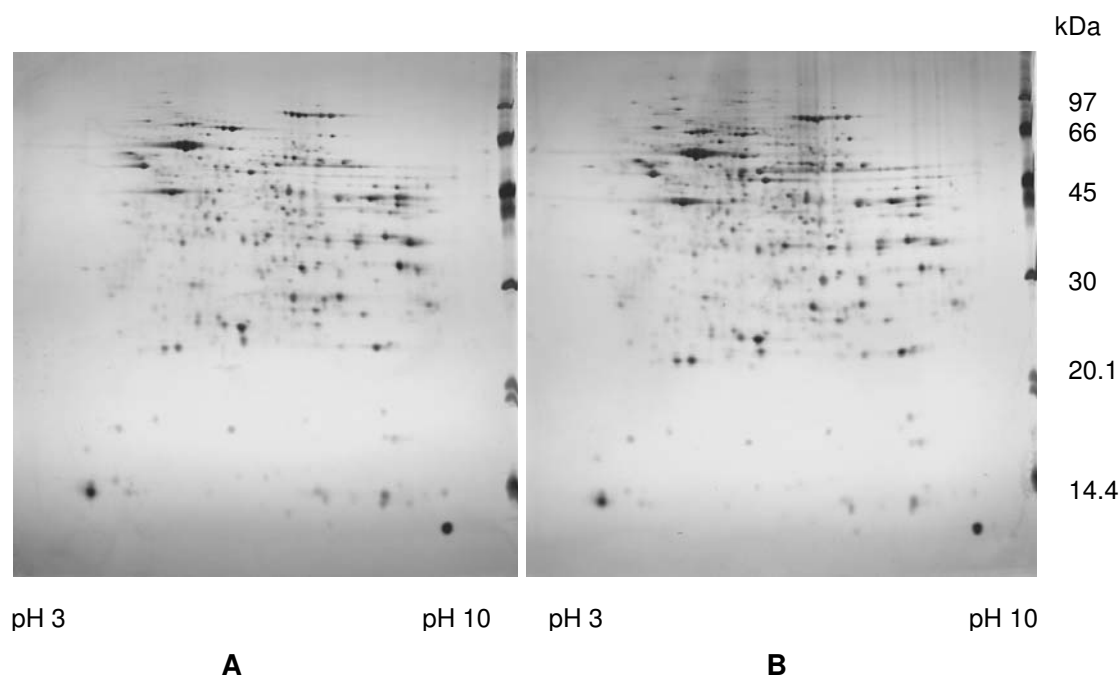


Figure 1. Protein profile of adrenal gland demonstrated by 2D-PAGE. (A) adrenal protein extracted from control group and (B) from MSG-treated group. Isoelectric focusing was performed using an IPG strip between pH 3 and 10 followed by 12.5% SDS-PAGE system.

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: β -actin sense (5'-CAACTGGGACGATATGGAGAA-3') and β -actin antisense (5'-AGGAAGGAAGGCTGGAAGAG-3') (product size 570 bp); GRP78 sense (5'-TTCCGCTCTACCATGAAACC-3') and GRP78 antisense (5'-TCTTTTGTGACGGGTGCTTC-3') (product size 422 bp); GRP75 sense (5'-CAGAGCCCCAAGTAAAGCTG-3') and GRP75 antisense (5'-TCTGTTGCTCAGTCCTGTC-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 μ g) 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 μ M 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 μ M 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 $\pm$ 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.

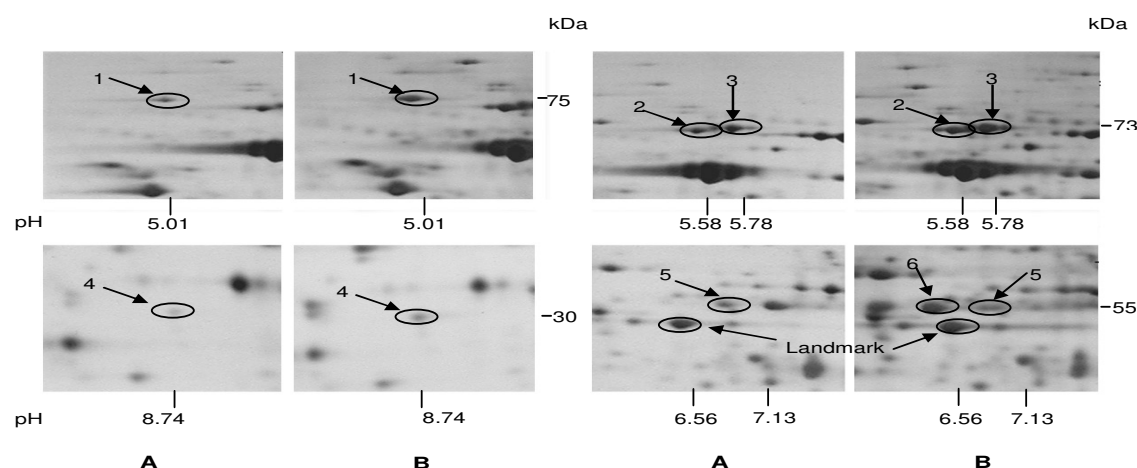


Figure 2. Comparison of protein spot intensity expressed in adrenal gland between (A) control rats and (B) MSG-treated rats. Protein spots no. 1- 4 were up-regulated in the MSG-treated group while spot no. 5 was down-regulated. Protein spot no. 6 was only expressed in the MSG-treated group.

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-

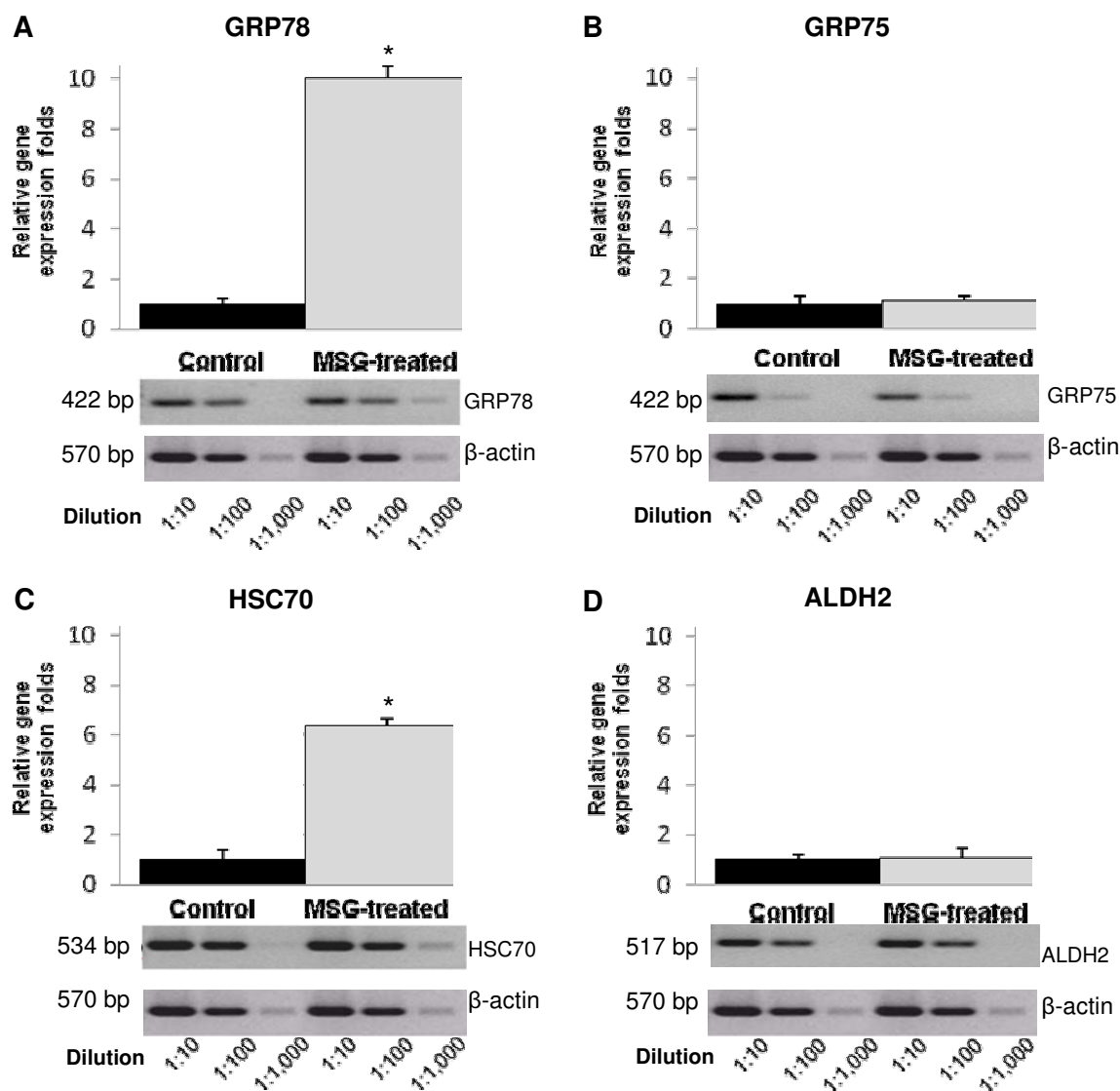


Figure 3. Analysis of mRNA levels in adrenal glands following MSG treatment by semi-quantitative RT-PCR. Amplification was performed using 10 fold serial dilutions at 1:10 to 1:1,000 dilutions of cDNA templates. Representative images of 1.5% agarose gels stained with ethidium bromide indicating the amplified 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 β -actin cDNA. Relative gene expression fold is demonstrated by relative amount of cDNA to β -actin gene. Mean $\pm$ 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; Lievremonet 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 encourage 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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