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Identification of Differentially Expressed Genes in Murine Hippocampus by Modulation of Nitric Oxide in Kainic Acid-induced Neurotoxic Animal Model

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Kainic acid (KA) causes neurodegeneration, but no consensus has been reached concerning its mechanism. Nitric oxide may be a regulator of the mechanism. We identified differentially expressed genes in the hippocampus of mice treated with kainic acid, together with or without L-NAME, a nonselective nitric oxide synthase inhibitor, using a new differential display PCR method based on annealing control primers. Eight genes were identified, including *clathrin light polypeptide, TATA element modulatory factor 1, neurexin III, ND4, ATPase, H^+ transporting, V1 subunit E isoform 1,* and *N-myc downstream regulated gene 2.* Although the functions of these genes and their products remain to be determined, their identification provides insight into the molecular mechanism(s) involved in KA-induced neuronal cell death in the hippocampal CA3 area.

Key Words: Excitotoxicity, Kainic acid, L-NAME, Differential display PCR, Hippocampus

INTRODUCTION

Kainic acid (KA), a neurotoxic substance that causes neurodegeneration, has been used to produce selective and localized lesions in the central nervous system (CNS). It causes a typical pattern of neurodegeneration in the hippocampus, where the CA3 pyramidal cells and interneurons in the hilus of the dentate gyrus are most vulnerable, followed by the CA1 pyramidal cells (Ben-Ari, 1985; Sperk et al, 1985; Tauck & Nadler, 1985; Phelps et al, 1991). However, pyramidal cells in the CA2 region and granule cells of the dentate gyrus are largely resistant to KA treatment. This reproducible pattern of neurodegeneration can be induced by systemic, intrahippocampal, intra-amygodaloid, or intracerebroventricular (i.c.v.) administration of kainic acid (Tremblay et al, 1983; Ben-Ari, 1985; Tanaka et al, 1992). Kainic acid-induced neurodegeneration occurs through both necrotic and apoptotic mechanisms (Ferrer et al, 1995; Simonian et al, 1996). Neuronal damage produced by systemic administration of kainic acid is believed to be initiated by the activation of kainic acid receptors in the CA3 region of the hippocampus, followed by release of the endogenous excitatory amino acids, glutamate and aspartate, which further activate all types of glutamate receptors.

Nitric oxide (NO) is a diffusible, multifunctional, transcellular messenger that has been implicated in numerous physiological and pathological conditions. Endogenous NO is synthesized from L-arginine by a family of NO synthase (NOS) isoenzymes, such as endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). According to the rate of NO production and the interaction with biological molecules, NO can promote apoptosis or anti-apoptosis (Chung et al, 2001). High concentrations of NO induce apoptotic cell death in several cell types, such as macrophages, thymocytes, pancreatic islets, neurons, and tumor cells (Chung et al, 2001). The factors affecting cell-specific sensitivity to NO-mediated apoptosis can be associated with the redox state, transition metal complexes within cells, and expression of p53. Although NO promotes apoptosis in some cells, it can protect some cells from apoptosis, induced by TNF alpha, oxidative stress, and serum-glucose deprivation. One of the main protective effects of NO has been attributed to the S-nitrosylation of the thiol groups of cysteine residues. In the caspases, cysteine residues are found at the enzymes' catalytic site; S-nitrosylation inhibits caspases 1, 3, and 9 (Kim et al, 1998; Mannick et al, 1999). NO has also other anti-apoptotic roles, including inhibition of the mitochondrial permeability transition pore, cytochrome *c* release, and induction of HSP70 expression (Hao et al, 1999; Brookes et al, 2000).

In the present study, we first examined whether inhibition of nitric oxide signaling increases kainic acid-induced neurotoxicity or not. Then, we identified differentially expressed genes in the hippocampus of kainic acid-treated mice, together with or without L-NAME, a nonselective NOS inhibitor, using a new differential display PCR method based on annealing control primers (ACPs) (Hwang et al, 2003; Kim et al, 2004).

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ABBREVIATIONS: NO, nitric oxide; NOS, NO synthase; ACP, annealing control primer; DEG, differentially expressed gene.

METHODS

Animal care and treatment

All animal experiments were conducted in accordance with the animal care guidelines of the National Institutes of Health (NIH) and the Korean Academy of Medical Sciences (KAMS).

ICR male mice, weighing 23~25 g, were purchased from Daehan Experimental Animal Center (Seoul, Korea). Five mice were housed per cage in a room maintained at 22±2°C, with an alternating 12/12 h light/dark cycle. Food and water were available *ad libitum*.

Three groups of animals were prepared. For the KA treatment group, KA (0.1 μg/5 μL; Sigma, St. Louis, MO, USA) was injected i.c.v., according to the procedure of Laursen and Belknap (1986). Briefly, KA was injected at the bregma with a 25-μL Hamilton microsyringe fitted with a 26-gauge needle that was inserted to a depth of 2.4 mm. For L-NAME (n=5) and KA treatment group (n=5), mice received intraperitoneal (i.p.) injections of L-NAME (50 mg/kg) 1 h prior to KA injection. A vehicle-treated control group (n=5) was also prepared. After 24 h, animals were anesthetized with Zoletil 50 (0.5 mL/kg, i.p.; Virbac Labs, Carros, France) and killed.

Nissl (cresyl violet) staining

Sections (40 μm thickness) were mounted on gelatin-coated microscope slides (Fisher, Fair Lawn, NJ, USA) and allowed to air-dry overnight. The slides were then rehydrated in distilled water and submerged in a 0.5% cresyl violet solution (Sigma) for 10 to 15 min, until the desired degree of staining was achieved. The slides were then cover-slipped using Permount (Fisher), following dehydration through graded ethanols and clearing in xylene.

RNA extraction and ACP-RT PCR analysis

After KA treatment, the hippocampus was isolated. Total RNA was extracted using a Mini RNA isolation II kit (ZYMO Research, Orange, CA, USA). Total RNA was immediately examined by gel electrophoresis and stored at −70°C until use.

Extracted total RNAs were subjected to annealing control primer (ACP)-RT PCR analysis (GeneFishing DEG kits; Seegene, Seoul, Korea). In brief, an anchor ACP (Oligo dT-ACP; Seegene) was used to synthesize the first-strand cDNA by annealing and extending the 3'-end core portion of ACP with the poly(A) tails of mRNAs. The arbitrary ACP (Seegene) is able to bind to the first-strand cDNA with a 10-bp match. The arbitrary ACP consists of 20 sets of primers (Table 1). DNase-treated RNA (3 μg) was isolated, and anchor ACP-T was annealed at 80°C for 3 min. Complementary DNA, serving as a template for further amplification, was synthesized by addition of dATP, dCTP, dGTP, dTTP, and 200 units of M-MLV reverse transcriptase, using the anchor ACP to synthesize the first-strand cDNA at 42°C for 90 min. The second-strand cDNA was then amplified with 40 cycles of PCR, priming with the ACP-T anchor and

Table 1. List of the sequence of arbitrary ACPs

ACP1	5'-GTCTACCAGGCATTCGCTTCATXXXXXGCCATCGACC-3'
ACP2	5'-GTCTACCAGGCATTCGCTTCATXXXXXAGGCGATGCC-3'
ACP3	5'-GTCTACCAGGCATTCGCTTCATXXXXXCCGGAGGATG-3'
ACP4	5'-GTCTACCAGGCATTCGCTTCATXXXXXGCTGCTCGCG-3'
ACP5	5'-GTCTACCAGGCATTCGCTTCATXXXXXAGTGCGCTCG-3'
ACP6	5'-GTCTACCAGGCATTCGCTTCATXXXXXGGCCACATCG-3'
ACP7	5'-GTCTACCAGGCATTCGCTTCATXXXXXCTGCGGATCG-3'
ACP8	5'-GTCTACCAGGCATTCGCTTCATXXXXXGGTCACGGAG-3'
ACP9	5'-GTCTACCAGGCATTCGCTTCATXXXXXGATGCCGCTG-3'
ACP10	5'-GTCTACCAGGCATTCGCTTCATXXXXXTGGTCGTGCC-3'
ACP11	5'-GTCTACCAGGCATTCGCTTCATXXXXXCTGCAGGACC-3'
ACP12	5'-GTCTACCAGGCATTCGCTTCATXXXXXACCGTGGACG-3'
ACP13	5'-GTCTACCAGGCATTCGCTTCATXXXXXGCTTCACCGC-3'
ACP14	5'-GTCTACCAGGCATTCGCTTCATXXXXXGCAAGTCGGC-3'
ACP15	5'-GTCTACCAGGCATTCGCTTCATXXXXXCCACCGTGTG-3'
ACP16	5'-GTCTACCAGGCATTCGCTTCATXXXXXGTCGACGGTG-3'
ACP17	5'-GTCTACCAGGCATTCGCTTCATXXXXXCAAGCCCACG-3'
ACP18	5'-GTCTACCAGGCATTCGCTTCATXXXXXCGGAGCATCC-3'
ACP19	5'-GTCTACCAGGCATTCGCTTCATXXXXXCTCTGCGAGC-3'
ACP20	5'-GTCTACCAGGCATTCGCTTCATXXXXXGACGTTGGCG-3'

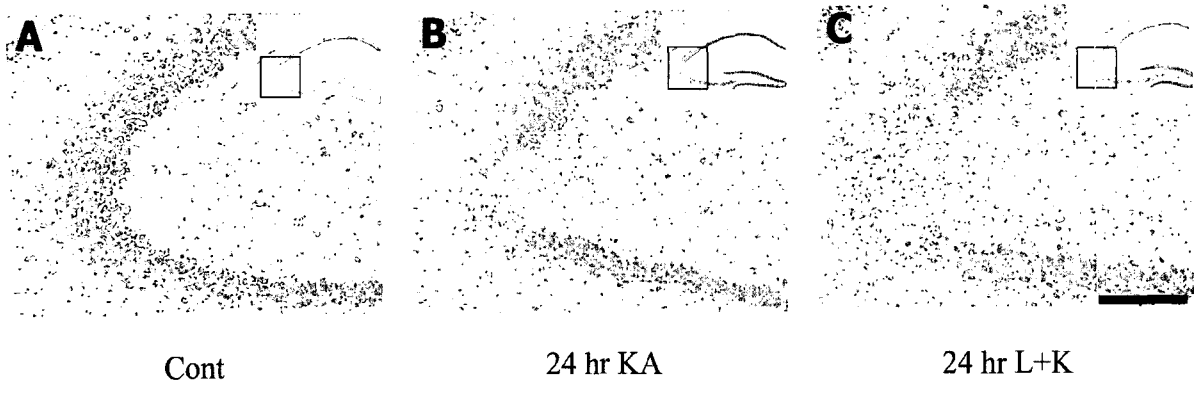

Fig. 1. Effects of L-NAME on kainic acid induced hippocampal neuronal cell death in CA3 region of mouse. Neuronal cell death was examined with cresyl violet staining. Marked losses of pyramidal neurons are shown after KA treatment (B). L-NAME treatment prior to KA (C) increased KA-induced neuronal loss more than KA injection only. Scale bar indicates 100 μm.

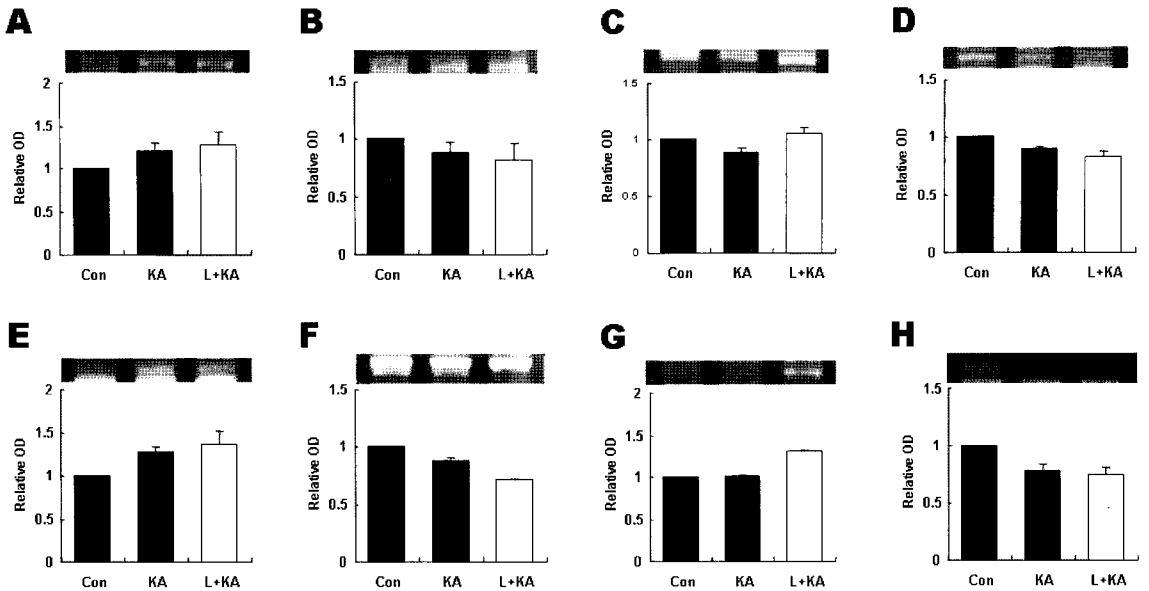

Fig. 2. Relative expression and representative agarose gel profiles of differentially expressed transcripts that were obtained from hippocampus of different groups, Control (Con), KA only treated (KA) and KA with L-NAME treated (L+KA). Using 20 ACPs, 8 DEGs were identified (A~H), and similar banding patterns were altained three times. These DEG bands were excised from the gel for further cloning and sequencing.

Table 2. The DNA sequences of differentially expressed transcripts

DEG1						
1	ACAGGTTGAG	AAGAACAAGA	TCAACAACAG	GATCGCTGAC	AAAGCGTTCT	ACCAGCAGCC
61	AGATGCTGAT	ACCATTGGCT	ATGTGGCATC	GGAAGAGGCT	TTTGTGAAAG	AATCCAAGGA
121	GGAGACCCCG	GGCACAGAGT	GGGAGAAGGT	AGCCCAGCTG	TGTGACTTCA	ATCCCAAGAG
181	CAGCAAGCAG	TGTAAAGATG	TGTCCCGCCT	GCGCTCGGTG	CTCATGTCCC	TGAAGCAAAC
241	ACCACTGTCC	CGCTAGGTGC	CCGTCACGAG	AACGGCCGCG	AAGCACATGG	GCCTTGCTGG
301	GGCAGAGGAG	CAGCTGCTTC	CGCCAGAGTG	GAACTTCCTC	TGGCAGCTGC	CACACACAGC
361	CTGTTCTGTT	CCTCTGAATC	TCTGGGAGCT	AGGAAGTGGG	ACCCTTACCC	CTTTCACCCC
421	ACAACTCCTT	CCTGGTCCCT	GGCCCAGCCC	TCATGACTCC		
DEG2						
1	GGGGTGGACT	CATGCTAGCG	CTGTAGCGGG	CGGAGCAGCA	GCTGCCAGAA	AAGAGGATTA
61	CTTACGCCAT	GAGATCAGTG	AACTTCAGCA	GAGACTCCAA	GAAGCAGAGA	ATCGAAACCA
121	AGAGCTGAGT	TAAAGTGTTT	CATCAACAGC	CAGACCGTTG	CTGCGACAGA	TAGAAAACTT
181	ACAAGCAACT	CTGGGGTCCC	AGACGTCCTC	GKGGGAGACA	CTGGAGAAGA	GTCTTTCTGA
241	TAGGCTTGGT	GAATCACAGA	CCTTGTTGGC	TGCAGCAGTT	GAAAGAGAAC	GTGCAGCTAC
301	AGAAGAACTC	CTGGCCAACA	AAATCCAGAT	GTCTTCAGTG	GAGTCACAGA	ATACGTTATT
361	ACGACAGGAA	AACAGTAGAC	TTCAGGCCCA	GCTAGAGTCA	GAG	
DEG3						
1	CTGACATTGT	TGTTTCCTAA	TGCTTTGGTG	AACCCATGNT	GGGATGACAA	GCCGTTTGAA
61	AAGCAGGTCC	CCAGATCTAA	TTCCAATATT	TGCCATTCAA	ACCTGTAACA	GATGGGTTTG
121	GGGCTGGTGT	TATCGGAGCT	ATTGGTTTCA	CTTAACAAAT	ACTGTCCCTG	TCCATCAACC
181	CAAAGTGTGT	GAACGGGGAA	ATGTAACCCC	CAAGAATTAA	AGCAGAGCCT	TTCTAAGGGA
241	GGAAAAAAG	GGAAAGGGGG	TGGACTGGAT	CTGTGAAATG	CATGC	
DEG4						
1	TTATTGCATC	AATCATAATC	CAAACTCCAT	GAAGCTTCAT	AGGAGCAACA	ATACTAATAA
61	TCGCACATGG	CCTCACATCA	TCACTCCTAT	TCTGCCTAGC	AAACTCCAAC	TACGAACGGA
121	TCCACAGCCG	TACTATAATC	ATGGCCCGAG	GACTTCAAAT	GGTCTTCCCA	CTTATAGCCA
181	CATGATGACT	GATAGCAAGT	CTAGCTAATC	TAGCTCTACC	CCCTTCAATC	AATCTAATAG
241	GAGAATTATT	CATTACCATA	TCATTATTTT	CTTGATCAAA	CTTTACCATT	ATTCTTATAG
301	GAATTAACAT	TATTATTACN	GGTATATACT	CAATATACAT	AATTATTACC	ACCCAACGCG
361	GCAAACTAAC	CAACCATATA	ATTAACCTCC	AACCCTCACA	CACACGAGAA	CTAACACTAA
421	TAGCCCTTCA	CATAATTCCA	CTTATTCTTC	TAACTACCAG	TCCAAAACTA	ATTACAGGCC
481	TGACAATAT					

Table 2. Continued

DEG5						
1	AGCAGATTAT	CTGTGTGGCT	TCCTCTTTCT	GTTCTAATAC	TGGTAATCAG	TGGACACCCT
61	CTACAGCTAAT	GCCCTCGGC	TCAGTGTCAA	CAGCAGGGCC	ACTTGGTGTG	GGCAAGAACC
121	CAGCCCCAGC	TCATCTGAGG	CCACAGCTGT	TCACTGGGTT	TGCAGCAGGA	CGGGAGGGTC
181	AGATGTGCTG	CACGGCCTCA	CCCAGGACCC	ATTCTTTCCC	TGTCCTGCTC	TAGTCTAGAG
241	TGAGTTGTGC	ATGCTGTCCC	TGCTCTTTGG	GCATAAAGTG	CATGTGGGAC	TCACATTCCT
301	CCAGGAACAC	TACCTTCGCT	GTTACTGTTT	AGGTCTAGTC	TTCCTTCTCT	GTAAATGCGA
361	TTTAAAATCT	AAGCCACAAA	TATTCTTTAT	TTATTAAAAC	AGAGGGCTTA	T
DEG6						
1	CCTCAGAATC	TGTGCAAATG	TGGTCCGACG	NTAAGTTATG	TGTCTAAGGC	TTGGCAGAGC
61	CGCACAGCTG	GCCAGAGGCT	AGACACTGGG	CAGGCAGGGC	TCGCCCCTTC	TCACCTGAGA
121	CAAAGTGTGT	GTTCTCATGT	GGACGGACAC	AGGAGGCAGA	TCCCAAGAAA	GTGGTCCTCT
181	GCGTGGTCGG	TGGGAGTTTG	CTGGATTTGT	GTATCTCCTG	ATCTTTGTAG	AATAAACACG
241	AGTCACATGG	T				
DEG7						
1	CTGCAGCTCG	CTTTGCTTCC	TGCCAGCTGT	TTTCCTCCTG	TGCCTGGTGA	TGTGAGTGGT
61	GTGAAATGAC	ACCTTTAATG	TTCCATGCTT	GGTGGAGATC	TTTTTATCAA	AAGGCATTTT
121	CCAAAAAATC	ATGTTTTGAT	AGAGGATATC	TGGTGTCCTT	AGGAATGACA	CAAATCATGA
181	CCATTACATT	TGATCTGTAA	CTGCTGCTAG	TTTCATGGAG		
DEG8						
1	ATGCTCTGAA	CCACCCGGAC	ACAGTTGAAG	GTCTTGTTCT	CATCAACATT	GATCCCAATG
61	CCAAGGGCTG	GATGGACTGG	GCAGCCCACA	AGTTAACTGG	CCTTACGTCT	TCCATTCCGG
121	ACATGATTCT	TGGACATCTT	TTCAGCCAGG	AAGAGCTTTC	TGGAAATTCT	GAATTGATAC
181	AAAAGTACAG	AGGTATCATC	CAACACGCAC	CCAACCTGGA	GAACATTGAA	CTCTACTGGA
241	ACAGCTACAA	CAACCGCCGA	GACCTGAACT	TTGAGCGAGG	TGGTGAGACG	ACCCTCAAGT
301	GCCCTGTGAT	GCTGGTGGTT	GGAGACCAAG	CACCTCATGA	AGATGCCGTG	GTGGAATGTA
361	ACTCAAAGCT	GGACCCCACA	CAGACCTCGT	TCCTCAAGAT	GGCCGACTCT	GGAGGTCAGC
421	CACAGCTGAC	TCAGCCCGGC	AAGCTGACAG	AGGCTTTCAA	GTACTTCCTG	CAAGGCATGG
481	GCTACATGGC	CTCTTCCTGC	ATGACTCGAC	TATCTCGGTC	TCGCACAGCA	TCTCTGGCAG
541	TGCAGCATCA	TCGATGGCAG	TCGGTCCC			

a set of primers comprising one of 20 arbitrary ACPs at the 3'- and 5'-ends, respectively. Differentially expressed genes were identified with an anchor ACP in combination with 20 arbitrary ACPs (Table 1), and their levels were determined using an Imaging Densitometer (Bio-Rad, Hercules, CA, USA).

DNA sequencing and analysis

PCR products were excised from a 2% agarose gel, cloned into the pGM-T Easy Vector (Promega, Madison, WI, USA), and then sequenced using the ABI PRISM 373 BigDye Terminator Cycle Sequencing kit (V2.0; Applied Biosystems, Foster City, CA, USA). Sequences were compared with the National Center of Biotechnology Information (NCBI) database using the BLAST program.

RESULTS

To determine whether NOS protects against or accelerates neurotoxicity, we studied the effects of L-NAME in a KA-induced neurotoxicity model. Twenty-four hours after kainic acid injection, we observed extensive death of hippocampal neurons in the CA3 region (Fig. 1B). Compared to kainic acid treatment alone, administration of L-NAME 1 h prior to kainic acid injection further increased kainic acid-induced hippocampal neuronal death in the CA3 region (Fig. 1C).

On the basis of differential expression analysis of mRNA fragments observed on agarose gels, eight bands were detected (Fig. 2), which were named DEG1 to DEG8. Expression banding patterns of DEG1 and DEG5 showed that the L-NAME-and-kainic-acid-treated group had the highest-intensity bands (Fig. 2A, 2E). For DEG2, DEG4, DEG6, and DEG 8, the highest intensity bands were observed in the controls (Fig. 2B, 2D, 2F, 2H). The bands were excised from the gels, cloned, and sequenced (Table 2). The sequences obtained were searched using BLASTN or BLASTX at the NCBI Web page. DEG1 showed significant similarity with the *clathrin light polypeptide* gene; DEG2 with the *TATA element modulatory factor 1* gene; DEG3 with the *neurexin III gene*; DEG4 with the *ND4 gene*; DEG5 with *ATPase, H^+ transporting*, and the *V1 subunit E isoform 1* genes; and DEG8 with the *N-myc downstream regulated gene 2* (Table 3).

DISCUSSION

As in other studies (Tremblay et al, 1983; Ben-Ari, 1985; Tanaka et al, 1992), neuronal death in the CA3 region was observed in the KA-treated group. Previous studies showed that excitotoxic death of neurons due to induced release of glutamate and activation of NMDA-type glutamate receptors was the result of inhibition of mitochondrial respiration by NO and its derivatives (Brown & Borutaite, 2002). Neuronal death in the CA3 region was further increased

Table 3. Sequence homology and characterization of differentially expressed transcripts

DEG. No	GeneBank Accession No.	Sequence homology search
DEG1	BC070404	Mus musculus clathrin, light polypeptide (Lcb), mRNA (cDNA clone MGC:92950 IMAGE: 5699653), complete cds
DEG2	XM_132740	PREDICTED: Mus musculus similar to TATA element modulatory factor 1 (LOC232286), mRNA
DEG3	AK034555	Mus musculus 12 days embryo embryonic body between diaphragm region and neck cDNA, RIKEN full-length enriched library, clone: 9430005B13 product: neurexin III, full insert sequence
DEG4	AY39599	Mus musculus clone LA9 mitochondrion, complete genome Gene = "ND4"
DEG5	BC055438	Mus musculus ATPase, H^+ transporting, V1 subunit E isoform 1, mRNA (cDNA clone MGC:65310 IMAGE: 3995744), complete cds
DEG6	BC034216	Mus musculus RIKEN cDNA 1810008A18 gene, mRNA (cDNA clone MGC:37648 IMAGE: 5008732), complete cds
DEG7	AK220564	Mus musculus mRNA for mKIAA4238 protein
DEG8	BC012963	Mus musculus N-myc downstream regulated gene 2, mRNA (cDNA clone MGC: 13746 IMAGE: 4211421), complete cds

in the L-NAME-pretreated group compared with the kainic acid-alone treated group. L-NAME is known to block NO formation by inhibiting both the inducible and constitutive isoforms of NOS (Moncada et al, 1992; Harry et al, 2001). While an elevation of NO can be damaging to neurons, inhibition of NO formation is not uniformly protective. Ciani et al, (2002) demonstrated that inhibition of NO by L-NAME resulted in progressive apoptotic death of cerebellar granule cells. Such cell death was rescued by adding NO donors or a cGMP analog. Furthermore, they demonstrated that two cellular pathways, the Akt/Gsk-3 kinase system and transcription factor CREB, may mediate the survival role of NO. In addition, NO appears to have pro-apoptotic effects on neurons, however anti-apoptotic effects on astrocytes, depending on the glycolytic capacity of the cell (Almeida et al, 2001).

In this study, we applied a new differential display RT-PCR technique to compare gene expression in mouse hippocampus among controls, KA-treated, and L-NAME pretreated-plus-KA-treated animals. Using this method, we detected eight genes that were differentially expressed. With regard to the 8 DEGs *Ndrg2* gene, a member of the *N-myc downstream-regulated* genes, is strongly associated with to nitric oxide and cell survival mechanisms. A recent study showed that *Ndrg2* gene expression was down-regulated by Myc via transcriptional repression (Zhang et al, 2006) and, interestingly, the exposure of cultures to L-NAME resulted in increased expression of *N*-Myc mRNA (Ciani et al, 2004). Furthermore, NDRG2 is physiological substrate for serum- and glucocorticoid-induced kinase 1 (SGK1) and Akt (Murray et al, 2004). Both SGK1 and Akt have pivotal roles in cell survival signaling. NDRG2 is widely expressed throughout many developing tissues and organs, particularly in heart and brain (Hu et al, 2006). Especially, it is expressed in glia and is under positive regulation by glucocorticoids, which are involved in neurogenesis or in cell differentiation, in the neurogenic zones of the dentate gyrus (Deng et al, 2003; Nichols, 2003). *Ndrg2* also inhibits glioblastoma cell proliferation without affecting apoptosis, and its mRNA is down-regulated in gliomas, suggesting a role in tumor suppression (Deng et al, 2003). The above mentioned studies together with our results suggest a link between survival mechanisms promoted by NO and NDRG2.

In conclusion, the ACP-based strategy which we used to identify DEGs is easy and avoids false positives. Using our method, we detected eight genes that were differentially expressed in kainic acid-treated mouse hippocampus. Although the detailed functions of these genes and their products remain to be determined, their identification in this study provides insight into the molecular mechanism involved in neuronal cell death in the hippocampal CA3 area.

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