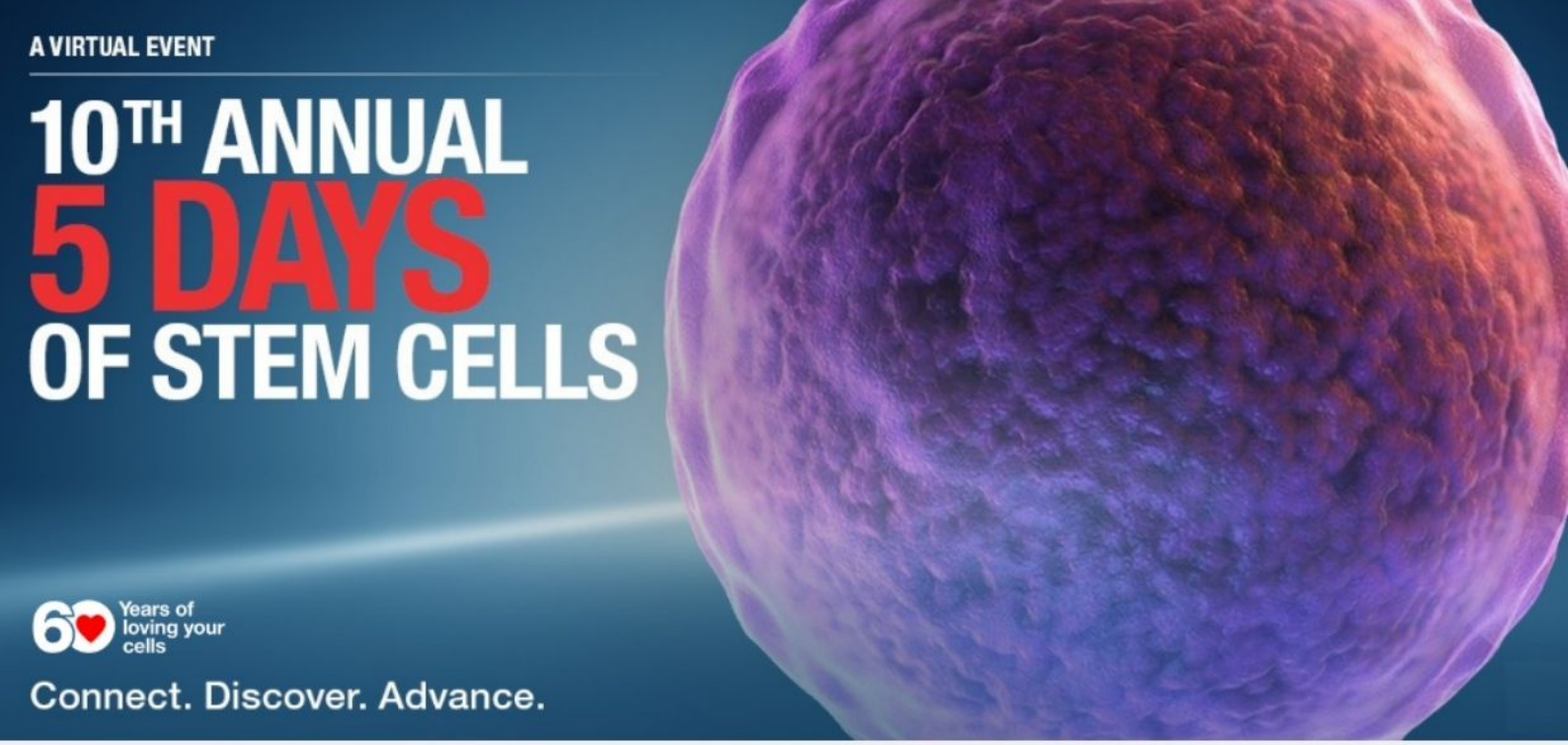

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Identification of tyrosine-nitrated proteins in HT22 hippocampal cells during glutamate-induced oxidative stress

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Abstract

Objectives: Nitration of tyrosine residues in protein is a post-translational modification, which occurs under oxidative stress, and is associated with several neurodegenerative diseases. To understand the role of nitrated proteins in oxidative stress-induced cell death, we identified nitrated proteins and checked correlation of their nitration in glutamate-induced HT22 cell death.

Materials and methods: Nitrated proteins were detected by western blotting using an anti-nitrotyrosine antibody, extracted from matching reference 2-dimensional electrophoresis gels, and identified with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Results: Glutamate treatment induced apoptosis in HT22 cells, while reactive oxygen species (ROS) inhibitor or neuronal nitric oxide synthase (nNOS) inhibitor blocked glutamate-induced HT22 cell death. Nitration levels of 13 proteins were increased after glutamate stimulation; six of them were involved in regulation of energy production and two were related to apoptosis. The other nitrated proteins were associated with calcium signal modulation, ER dysfunction, or were of unknown function.

Conclusions: The 13 tyrosine-nitrated proteins were detected in these glutamate-treated HT22 cells. Results demonstrated that cell death, ROS accumulation and nNOS expression were related to nitration of protein tyrosine in the glutamate-stimulated cells.

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Introduction

Neuronal disorders such as Huntington's disease, Alzheimer's disease and stroke are typically characterized by neuronal cell death, which is thought to result from extensive oxidative stress (1,2). Oxidative stress begins to accumulate when cells lose the ability to regulate production of reactive oxygen species (ROS) such as singlet oxygen (·O), superoxide ($\cdot O_2^-$), hydroxyl free radicals ($\cdot OH^-$) and hydrogen peroxide (H_2O_2) (2,3). These ROS affect protein modification of tyrosine residues (4), and this process induces pulmonary inflammation (5) or neurodegenerative disease (6,7). Thus, ROS are thought be the principal mediator of such neuronal cell death. Despite recent studies that have explored mechanisms of oxidative stress-stimulated toxicity, much remains unknown concerning how ROS cause neuronal cell death.

Nitration of tyrosine residues [conversion of tyrosine to 3-nitrotyrosine (3-NT)] is a well-established post-translational modification that compromises functional and/or structural integrity of target proteins (3,8). 3-NT has been associated with pathological conditions, which result in gain or loss of functional modifications of proteins, and is a well-established disease promoter (6,9,10). Previous studies suggest that nitrated proteins are directly involved in pathogenesis and that 3-NT residues are potentially useful as markers of disease (2,3,10). Recently, proteomic methodologies have been used to identify protein targets of tyrosine nitration (10–13). Targets of nitration are then detected using anti-nitrotyrosine antibody, and mass spectrometry can be performed to identify gel spots such as succinyl-CoA: 3-oxoacid CoA transferase (SCOT) and glutamate dehydrogenase. These studies have identified that several targets of nitrated proteins were involved in intermediary metabolism, oxidative stress, apoptosis, and ATP production (1,10,11,14).

Mouse hippocampal cell line HT22 has proven to be useful in studies of glutamate-induced oxidative toxicity (15,16). In this system, glutamate toxicity inhibits cysteine

transport and depletes cellular glutathione (GSH), thus excluding excitocytoxicity as cause of glutamate-triggered cell death (17,18). Exposure of HT22 cells to glutamate results in rapid depletion of GSH, and increase in ROS. In addition, previous reports have suggested that ROS gradually increase during the first 6 h of glutamate treatment, and explosive increase in rate of production thereafter (18,19). Subsequent accumulation of ROS triggers a cell signalling pathway that contributes to neuronal cell death. Thus, oxidative glutamate toxicity represents a useful model for studying response of neuronal cells to oxidative stress.

Based on these observations, we have treated HT22 cells with glutamate, which induces cell death. We found that HT22 cell death was regulated by increasing glutamate-stimulated ROS accumulation and nNOS expression. We observed several changes in nitrated proteins during glutamate stimulation of HT22 cells. Further, we have used proteomic approaches to analyse nitrated proteins and have established a proteomic database. Our results show that 13 nitrated proteins were specifically induced by glutamate stimulation. Interestingly, we found that nitration level of calmodulin-4, Hsp90, tubulin, glutamate dehydrogenase, acyl-CoA dehydrogenase and ATP synthase alpha chain was reduced in HT22 cells pre-treated with ROS inhibitor and nNOS inhibitor. As neuronal cell death is a common feature in neurological disorders, our illustration of the nitroproteome involved in glutamate stimulation will shed light on mechanisms of apoptosis in neuronal cells and perhaps identify novel therapeutic targets for neuronal diseases.

Materials and methods

Antibodies and reagents

We purchased the anti-nitrotyrosine monoclonal antibody from Upstate Biotechnology (Lake Placid, NY, USA). Anti-mouse and anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-nNOS, anti-iNOS and anti-eNOS antibodies were purchased from BD Bioscience (Franklin Lakes, NJ, USA). *N*-acetyl cysteine (NAC) and 7-nitroindazole (7-NI) were obtained from Sigma (St. Louis, MO, USA). For confirming identity of nitrated proteins, the following primary antibodies were obtained: anti-calmodulin-4 from Abcam (Cambridge, MA, USA); anti-HSP90 from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-tubulin from Sigma; anti-glutamate dehydrogenase from Rockland Immunochemicals Inc. (Gilbertsville, PA, USA); anti-acyl-CoA dehydrogenase from AVIVA Systems Biology (San Diego, CA, USA); and anti-ATP synthase alpha chain from Invitrogen (Carlsbad, CA, USA).

Cell culture and cell viability assay

HT22, mouse hippocampal cell line, was grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (FBS) (Hyclone, Logan, UT, USA). Cell viability was assessed using the MTT assay (Promega, Madison, WI, USA). Briefly, cells were seeded and grown for 24 h in 96-well plates (1×10^4 cells/well). After treatment with 25 μl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide dye) solution, cells were incubated for 1 h. Cell viability was assessed using a 1420 multilabel counter (VICTOR^3; Perkin-Elmer, Waltham, MA, USA) at 490 nm. Results were expressed relative to control values in each experiment and statistical analyses were performed.

Two-dimensional electrophoresis

Cells were lysed in 1 ml of RIPA (radio-immunoprecipitation assay) buffer followed by precipitation in acetone at −20 °C for 1 h. The cell lysate was then centrifuged at 23 800 ***g*** for 30 min at 4 °C, supernatant was removed and pellets were dried in a SpeedVac Plus SC110A (Savant Instruments, NY, USA) vacuum concentrator for 30 min. Resulting pellets were resuspended in rehydration buffer [7 M urea, 0.5% (v/v) IPG buffer, 2% (w/v) DTT, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate, CHAPS] and protein concentration was measured using Bio-Rad protein assay reagent (Hercules, CA, USA). Isoelectric focusing (IEF) was performed using IPGphor unit (Amersham Biosciences, Uppsala, Sweden) with 18 cm non-linear (NL), pH 3–10, IPG gel strip. Equal amounts (500 μg) of total protein were added to each IPG strip, then rehydrated in 8 M urea, 2% [w/v] CHAPS and 0.001% [v/v] bromophenol blue. Then, IEF was performed at 500 V for 1 h, 1000 V for 1 h and 8000 V for 8 h. The current was limited to 50 mA per gel strip. After IEF separation, gel strips were immediately equilibrated in equilibrium buffer containing 50 mM Tris–HCl, pH 8.8, 8 M urea, 30% (v/v) glycerol and 2% (w/v) SDS. Second-dimension separation was performed on non-stacked 12% SDS–PAGE gel (size = 18 cm). Electrophoresis was performed using Protean II xi 2-D cell (Bio-Rad). Analytical 2-DE gels were stained with colloidal Coomassie blue (Bio-Rad) and separated proteins were subjected to western blot analysis, on nitrocellulose membrane, using anti-nitrotyrosine monoclonal antibody.

Protein identification and database searches

Spots were excised as 1–2 mm^2 slices and destained using freshly prepared solution containing 10% [v/v] ethanol

and 2% [v/v] phosphoric acid. Slices were then washed in solution containing 200 mM ammonium bicarbonate and acetonitrile (ACN), and dried in a SpeedVac Plus SC110A vacuum concentrator (Savant Instruments). Dried gel pieces were rehydrated in 5–15 ml of 10 ng/ml trypsin solution, and were digested at 37 °C for 16 h. Trypsin-digested peptides were extracted from gel slices by incubation in 50 mM ammonium bicarbonate for 1 h followed by incubation in ACN for 1 h. Peptide extracts were dried in a vacuum concentrator and then solubilized using 0.5% [v/v] trifluoroacetic acid (TFA) for analysis using a mass spectrometer (MS). Then, samples were analysed using an AXIMA-CFR mass spectrometer (Shimadzu-Biotech, Manchester, UK), with saturated α-cyano-4-hydroxy-*trans*-cinnamic (CHCA) solution in 0.1% [v/v] TFA/50% [v/v] ACN serving as matrix. The MS was externally calibrated using matrix and trypsin auto-digestion ion peaks at 842.51 and 2211.1064 Da as internal standards, respectively. Mass spectra of peptides were submitted to search the Swiss-Prot database through MS-FIT program (http://prospector.ucsf.edu/) and pI acquired from our image analysis. Molecular mass range of 6–200 kDa and mass tolerance of 50 ppm were used for internal calibration. Protein matches were assessed on the basis of number of peptides matched to target protein. Identification of these peptides expressed as percentage of all submitted peptides was scored (20,21).

DNA fragmentation

Cells were gently lysed for 30 min at 48 °C in buffer containing 5 mM Tris (pH 7.4), 20 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% [v/v] Triton X-100. After centrifugation at 37 700 ***g*** for 15 min, supernatants containing soluble fragmented DNA were collected and treated with 20 μg/ml RNase (Wako Pure Chemicals, Tokyo, Japan), digested with 20 μg/ml proteinase K (New England Biolab, Ipswich, MA, USA) and precipitated in ethanol. DNA fragments were separated on 2% agarose gel and visualized using 0.1% ethidium bromide (22).

Immunoprecipitation and western blot analysis

Total cell lysates were prepared in RIPA cell lysis buffer (20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM ethyleneglycoltetraacetic acid (EGTA), 150 mM sodium chloride, 1% Triton X-100, 2.5 mM sodium pyrophosphate) containing protease inhibitors (1 mM leupeptin, 1 mM pepstatin A and 1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride) and phosphatase inhibitors (1 mM sodium fluoride and 1 mM sodium orthovanadate). Equal amounts (300 μg) of total proteins were precipitated using a target antibody, and then incubated at 4 °C for 1 h on a rocking platform. To precipitate protein–antibody mixture, 20 μl protein A/G-agarose beads (Calbiochem, Madison, WI, USA) were added, and incubated for 12 h on a rocking platform at 4 °C. After incubation, immunoprecipitation complexes were collected by centrifugation at 3000 ***g*** for 5 min at 4 °C, and then these complexes were washed in PBS. After washing three times, immunoprecipitation complex samples were mixed with SDS–PAGE sample buffer [10% (v/v) glycerol, 62.5 mM Tris–HCl, pH 6.8, 2% (v/v) SDS, 0.01 mg/ml bromophenol blue, 5% (v/v) β-mercaptoethanol] and separated on 12% SDS–PAGE gel, then transferred to nitrocellulose membrane (Millipore, Brookfield, MA, USA) (23). Membranes were blocked with 5% skimmed milk in TBS-T buffer (TBS containing 0.05% of Tween-20), and exposed to anti-nitrotyrosine monoclonal antibody (Upstate Biotechnology) diluted (1:3000) in TBS-T buffer containing 5% skimmed milk for 12 h on a rocking platform at 4 °C. Membranes were washed in TBS-T buffer and exposed to diluted HRP-conjugated goat anti-mouse antibody, diluted 1:3000, for 1 h. After incubation, membranes were washed in TBS-T buffer and visualized using chemiluminescent ECL detection kit (Amersham Biosciences).

Results

Effects of glutamate stimulation on cell viability

To determine whether oxidative stress was sufficient to trigger neuronal cell death, we assessed viability of glutamate-stimulated HT22 cells. As shown in Fig. 1a, there were no changes in cell viability from 0 to 2 h after glutamate stimulation. However, viability gradually decreased after 4 h and more than 80% of glutamate-stimulated cells had died by 12 h. In addition, glutamate stimulation induced apoptotic morphology, characterized by accumulation of shrunken and rounded cells (Fig. 1b); this was consistent with previously published findings (24). DNA fragmentation is known to be a characteristic phenomenon of apoptosis resulting from endonuclease enzymes having cleaved genomic DN; this can be visualized by agarose gel electrophoresis. Thus, we examined whether DNA fragmentation was induced by glutamate treatment. As shown in Fig. 1c, DNA fragmentation was observed markedly at 12 h in glutamate-stimulated cells. Previous studies have reported that ROS plays a central role in glutamate-induced neuronal cell death and its production in massive quantities was required for cell death (15,25). *N*-acetyl cysteine (NAC) is known to be a potent antioxidant that is shown to decrease ROS production in cultured neuronal cells (26,27). Thus, we checked whether exogenous ROS inhibitor (NAC) would block cell death in glutamate-stimulated HT22 cells. As shown in Fig. 1, cell

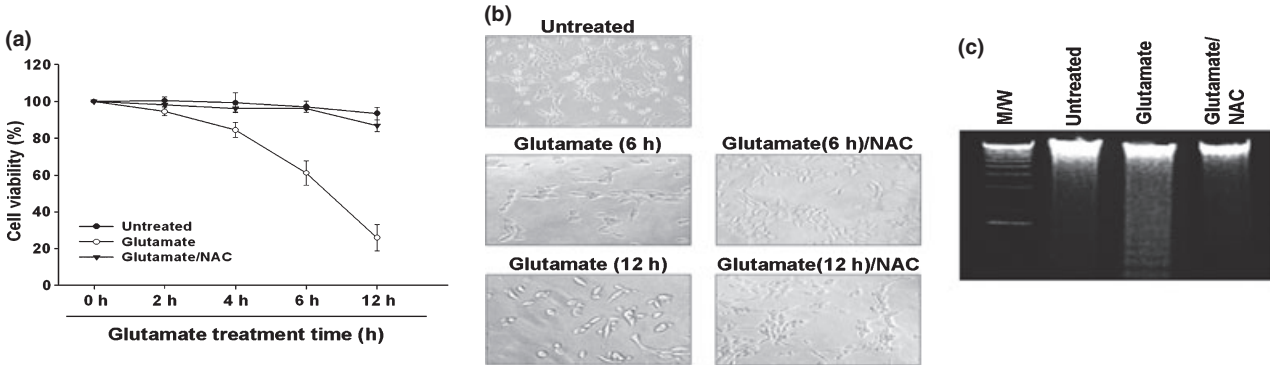

Figure 1. Effects of glutamate treatment on HT22 cell viability. Cells were grown on 96-well plates (1×10^4 cells per well) for 24 h, pre-treated with *N*-acetyl cysteine (NAC, 50 μM) for 30 min and then treated with 5 mM glutamate. Cell viability at each time point was measured using the MTT assay (a). Morphology of glutamate-stimulated cells, as visualized by light microscopy at 200× magnification (b). Cells were pre-treated with *N*-acetyl cysteine (50 μM) for 30 min and then treated with 5 mM glutamate for 12 h. DNA was extracted and DNA fragments were analysed by 2% agarose gel electrophoresis (c).

viability and DNA fragmentation analysis revealed that ROS inhibitor (NAC) pre-treatment significantly blocked glutamate-stimulated cell death. Consistent with previous results, these findings also support the notion that ROS production by glutamate stimulation induces HT22 cell death.

nNOS is related to apoptosis in glutamate-stimulated cells

In the central nervous system, glutamate elicits many of its effects by activating nitric oxide synthase (NOS). In addition, three isoforms of NOS have been characterized in brain cells: nNOS produced from neurons, iNOS synthesized from glial cells and eNOS derived from endothelial cells (28–30). We examined alteration in NOS expression levels induced by glutamate stimulation in HT22 cells. Our results indicated that glutamate stimulation increased nNOS expression but we failed to detect *iNOS* and *eNOS* gene expression (Fig. 2a). As nNOS is known to be the main NO synthase in brain during neuronal disorders such as ischaemia (31), we investigated whether exogenous nNOS inhibitor, 7-nitroindazole (7-NI), influenced cell death by glutamate stimulation. Cells were pre-treated with 7-NI and then glutamate-stimulated cell death was monitored using a cell viability assay. As expected, glutamate stimulation induced cell death, but treatment with nNOS inhibitor blocked glutamate-stimulated cell death (Fig. 2b,c). These results suggest that increasing glutamate-stimulated nNOS expression induces HT22 cell death by glutamate stimulation.

Tyrosine-nitrated proteins were identified in glutamate-stimulated cells

A proteomic approach was used to identify nitrated proteins in glutamate-stimulated HT22 cells. Cells were

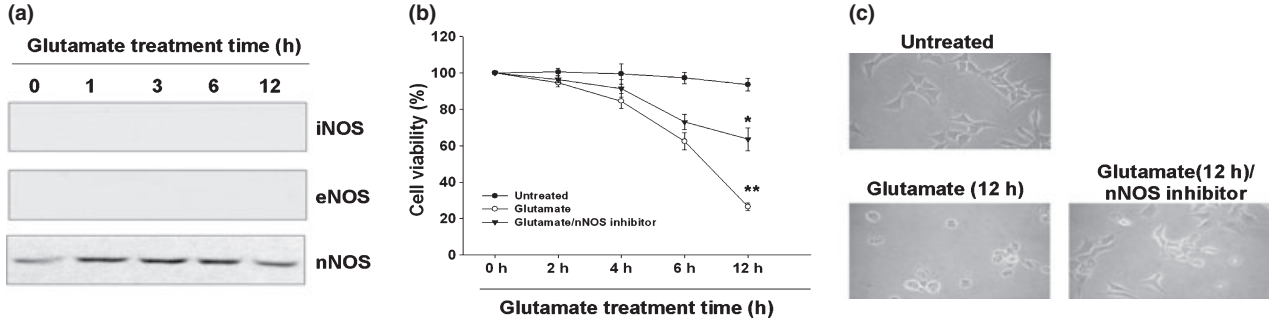

Figure 2. nNOS expression was increased in glutamate-stimulated HT22 cell death. Cells were subjected to time-dependent treatment with 5 mM glutamate. NOS isoforms were determined by western blot analysis using anti-iNOS, anti-eNOS, or anti-nNOS antibody (a). Cells were pre-treated with nNOS inhibitor (7-nitroindazole, 100 μM) for 30 min and then treated with 5 mM glutamate. At each time point, cell viability was measured by MTT assay. * and ** represent statistically significant differences relative to untreated cells (*$P < 0.05$; **$P < 0.01$). Data are mean ± SD of four independent experiments (b). Cells were pre-treated with nNOS inhibitor (100 μM) for 30 min and then treated with 5 mM glutamate for 12 h. Morphology of glutamate-treated cells, as visualized by light microscopy at 200× magnification (c).

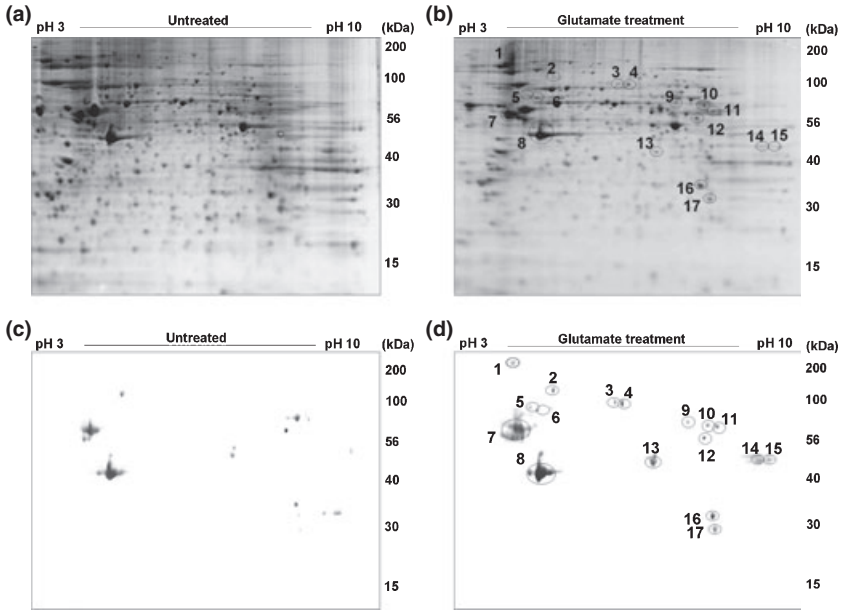

Figure 3. Tyrosine-nitrated proteins were identified by 2-DE gels and MALDI-TOF MS in glutamate-stimulated HT22 cells. Proteomics analyses were performed by treating cells with 5 mM glutamate for 6 h and applying cell lysates (500 μg) to first dimension of pH 3–10 non-linear IPG strips (18 cm) and second dimension of 12% SDS–PAGE gels, which were visualized by Coomassie blue staining. Representative 2-DE protein profiles of normal (a) and 5 mM glutamate-stimulated cells (b). Cell lysates were run on to 2-DE gels, and transferred to nitrocellulose membranes. Tyrosine-nitrated protein levels of normal (c) and 5 mM glutamate-stimulated cells (d) was determined using western blot analysis with anti-nitrotyrosine monoclonal antibody. Circled spots indicate proteins that were more highly expressed in glutamate-stimulated cells than in normal cells.

treated with 5 mM glutamate for 6 h, and cell lysates were analysed on 2-DE gels. Gels were transferred on to nitrocellulose membranes and proteins were detected using anti-nitrotyrosine monoclonal antibody. Nitrated proteins were matched using 2-DE western blotting and Coomassie blue-stained images of the same sample. Expression levels of nitrated proteins showed that 17 proteins were increased in glutamate-stimulated HT22 cells than in control cells (Fig. 3). Thirteen nitrated proteins were selected from the 2-DE gels and analysed by MALDI-TOF mass spectrometry analysis (Table 1) and peptide fingerprinting. Proteins were further identified, based on database searches. Among nitrated proteins identified in this analysis, a cytoskeletal protein (spots 7 and 8), glutamate dehydrogenase (spots 10 and 11), acyl-CoA dehydrogenase (spot 12), ATP synthase alpha chain (spots 14 and 15) and glutathione *S*-transferase Mu 2 (spot 17) were consistent with earlier observations of nitrated proteins (5,6,9,12,13,32).

Because inhibited HT22 cell death by ROS and nNOS inhibitor treatment was shown to be involved in glutamate-stimulated cell death (Figs 1b and 2b), we investigated expression levels of nitrated proteins to discover whether ROS production or nNOS expression would be related to apoptosis after glutamate stimulation. Expression levels of nitrated proteins were checked by immunoprecipitation and western blot analysis of total extracts from glutamate-stimulated cells. Total cell lysates were immunoprecipitated with anti-calmodulin-4, anti-HSP90, anti-tubulin, anti-glutamate dehydrogenase, anti-acyl-CoA dehydrogenase and anti-ATP synthase alpha chain antibodies. Immune complexes were collected on protein A/G-agarose beads, separated by SDS–PAGE, transferred to nitrocellulose membranes and immunoblotted with anti-nitrotyrosine monoclonal antibody. As shown in Fig. 4, we found that nitration level of calmodulin-4, HSP90, tubulin, glutamate dehydrogenase, acyl-CoA dehydrogenase and ATP synthase alpha chain was significantly increased in glutamate-stimulated cells. However, expression levels of nitrated proteins were reduced in cells pre-treated with ROS inhibitor (NAC) and nNOS inhibitor (7-NI). Taken together, these results suggest that nitration of these proteins may contribute to neuronal cell death by oxidative stress-stimulated ROS accumulation and nNOS expression.

Discussion

Previous studies have suggested that nitrated proteins are highly expressed during pathogenesis of Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (3,7). Oxidative stress-stimulated protein contributes to

Table 1. Nitrated proteins were identified in glutamate-stimulated HT22 cells using 2-DE and MALDI-TOF MS

Spot no.	Protein name	Accession no.	Mass (MW/pI)	Peptide matched	Score	Alteration	Fold*
1	Calmodulin-4	Q9JM83	167.5/4.79	26/65	0.4	↑	3.3
2	Transitional endoplasmic reticulum ATPase	Q01853	89.3/5.1	22/50	0.44	↑	0.4
3	Heat shock protein	P11499	83.3/5.0	27/75	0.36	↑	0.4
4	HSP 90-beta			26/75	0.43	↑	0.3
5	Neurosecretory protein	P20156	68.1/4.7	5/11	0.44	↑	0.2
6	VGF procurer	P20156	68.1/4.7	5/11	0.44	↑	0.2
7	Tubulin (Beta-tubulin)	P04691	49.9/4.8	4/20	0.2	↑	0.3
8	Actin (Beta-actin)	P60710	41.7/5.3	13/35	0.37	↑	0.3
9	Unnamed protein product	P20585	65.2/7.5	5/19	0.26	↑	0.5
10	Glutamate dehydrogenase	P26443	61.3/8.1	20/91	0.22	↑	1.2
11				20/91	0.22	↑	1.3
12	Acyl-CoA dehydrogenase	Q9DBL1	47.8/8.0	14/28	0.5	↑	1.1
13	Unnamed protein product	P27797	45.5/7.0	10/73	0.13	↑	0.5
14	ATP synthase alpha chain	Q03265	59.7/9.2	16/66	0.24	↑	0.8
15		Q03265	*59.7/9.2*	*16 /66*	*0.24*	↑	*0.8*
16	NADP-dependent malic enzyme, cytosolic NADP-dependent	P06801	63.9/7.2	7/27	0.25	↑	1.0
17	Glutathione *S*-transferase Mu 2	P15626	25.7/6.9	18/90	0.2	↑	0.5

↑: increased spot in glutamate-treated HT22 cells.
*The relative value of glutamate-stimulated protein expressed as the fold of that in the presence of untreated cells.

mitochondrial dysfunction, which also plays a major role in these diseases (33–36). Here, in our study, we demonstrate that oxidative stress (glutamate stimulation) in mouse hippocampal HT22 cells induces cell death that regulates both ROS accumulation and nNOS expression. Our findings reveal the extent of tyrosine nitration in the cell line during oxidative stress-stimulated cell death. Seventeen nitrated proteins were identified from the cells during oxidative stress-induced death, using anti-nitrotyrosine monoclonal antibody, after separation on 2-DE gels. By characterizing the nitrated proteins involved in neuronal cell death, we hoped to contribute to greater understanding of neuronal apoptosis.

Recently, Guo *et al.* (37) reported that green tea polyphenols (GTP) prevent cell death and suppress accumulation of ROS and intracellular NO level in an *in vitro* experimental model of Parkinson's disease, by 6-hydroxydopamine (6-OHDA), a commonly used neurotoxin. In addition, GTP decreased levels of nNOS and nitration of protein tyrosine by 6-OHDA stimulation. Thus, this group suggested that protective effects of GTP in the model of Parkinson's disease, occurred by controlling the ROS-NO signal pathway. Glutamate is known to be a major contributor to pathological cell death in the nervous system and appears to be mediated by ROS (24). Excess NO mediates neurotoxicity in pathological situations, and NO toxicity is accentuated in the presence of oxidative radicals such as O_2-, which can also be generated by nNOS (38). Consistent with those findings, our observation was that induction of oxidative stress-stimulated HT22 cell death was due to accumulation of ROS production by glutamate (Fig. 1). We also observed that induction of HT22 cell death by glutamate stimulation was related to nNOS expression (Fig. 3). Glutamate treatment has been reported to induce neuronal cell death by enhancement of ROS production, increase in nNOS expression and activation of caspase cascades (39). In this study, pre-treatment with ROS inhibitor completely prevented glutamate-stimulated cell death, whereas the effect of nNOS inhibitor to prevent glutamate-stimulated cell death was significant ($P < 0.05$) but only moderate. As glutamate-stimulated neuronal cell death seems to depend on several types of intracellular signalling induced by ROS production, inhibition of nNOS-mediated nitration alone may not be enough to provide full protection against apoptosis in glutamate-stimulated cells.

Nitrated proteins were identified in glutamate-stimulated HT22 cells. Peptide mass fingerprinting data generated in this study are consistent with an earlier observation of nitrated proteins, including glutamate dehydrogenase, acyl-CoA dehydrogenase, ATP synthase alpha chain and glutathione *S*-transferase Mu 2 (6). These mitochondrial target proteins are key enzymes in energy production and are involved in the citric acid cycle, electron transport chain and ATP production. The role of cytoskeletal protein modification has been exten-

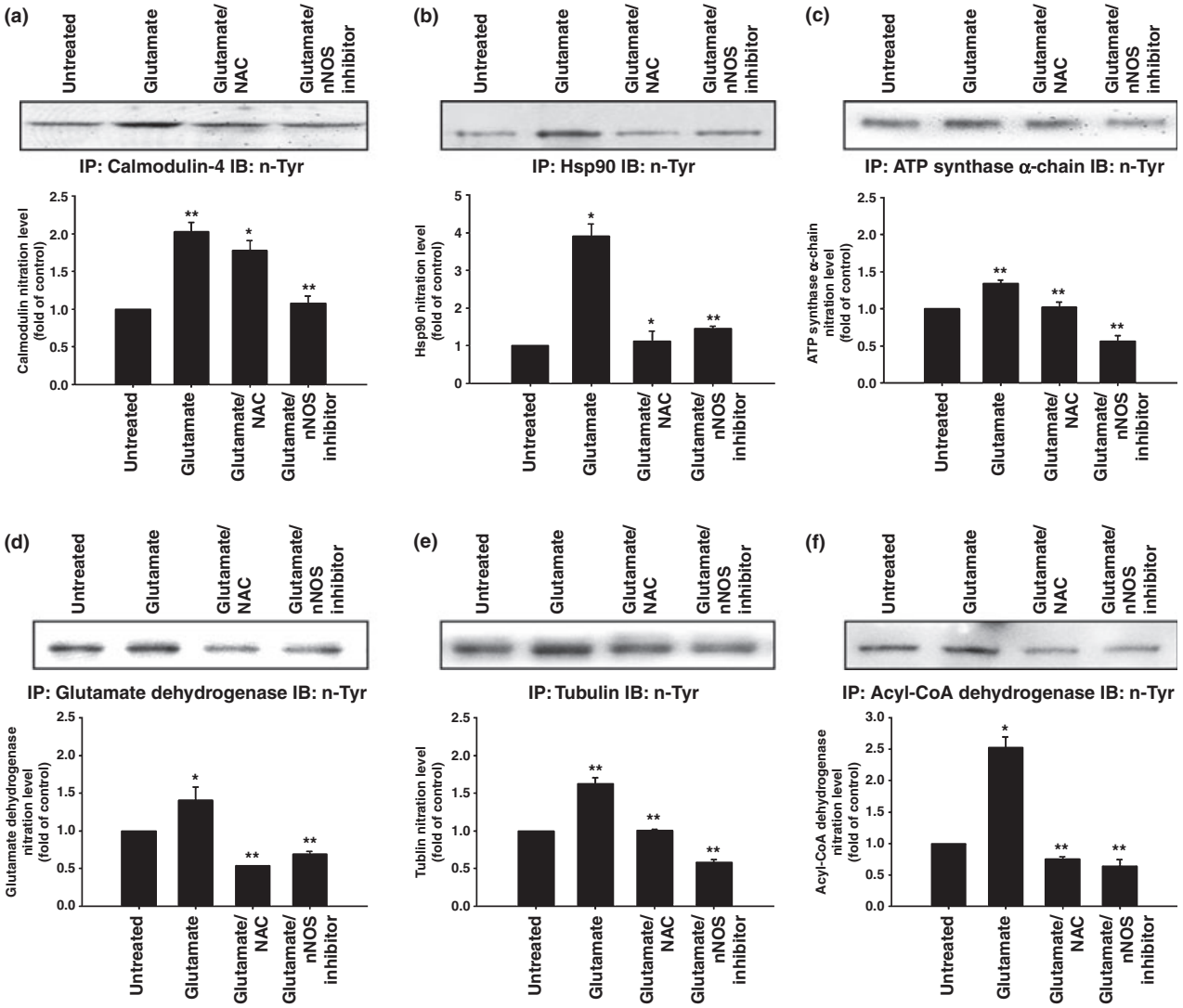

Figure 4. Pre-treatment of inhibitors of ROS (NAC) and nNOS (7-NI) decrease tyrosine nitration levels of proteins identified. Cells were pre-treated with NAC (50 μM) or 7-nitroindazole (100 μM) for 30 min, followed by treatment with 5 mM glutamate for 6 h. Total cell lysates were immunoprecipitated with antibodies against calmodulin-4 (a), HSP90 (b), ATP synthase α-chain (c), glutamate dehydrogenase (d), tubulin (e) and acyl-CoA dehydrogenase (f), then analysed by western blot analysis with anti-nitrotyrosine monoclonal antibody to check levels of protein nitration. Nitrated proteins were quantified by densitometric analysis using image analysis software, ImageQuant v5.2 (Amersham Biosciences). * and ** represent statistically significant differences relative to untreated cells (*$P < 0.05$; **$P < 0.01$). Data are given as mean ± SD of three independent experiments.

sively studied as proteins such as tublin and actin contain many tyrosine residues and are thus potential targets of nitration. Schopfer *et al.* (32) suggested that tyrosine nitration of cytoskeletal proteins activate cell responses such as apoptosis and inflammation. Actin nitration is significantly higher in brain tissues affected by Alzheimer's disease than in unaffected brain tissue (7). From our results, we observed that expression of nitrated proteins was higher in glutamate-stimulated HT22 cells than in control cells. Thus, cytoskeletal protein nitration may be linked to key regulators of oxidative stress-mediated neuronal apoptosis.

Calmodulin is a calcium regulatory protein that functions as the calcium signal to modulate cell metabolism through reversible binding to over 50 different target proteins (40). A report by Smallwood *et al.* (41) showed that tyrosine nitration of calmodulin is induced by oxidative stress stimulation. Thus, this group suggested that calmodulin nitration by oxidative stress stimulation has the potential to dramatically alter intracellular signalling. However, cell function of calmodulin-4, an isoform of calmodulin, remains unclear. Endoplasmic reticulum (ER) stress contributes to accumulation of protein unfolding or misfolding (42). This process is caused by oxidative stress,

which contributes to pathogenesis of neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (14). Transitional ER ATPase interacts with ubiquitin ligase by the ubiquitin–proteasome pathway. This process is the target of Akt signalling of cell survival (43). In our data, nitration of transitional ER ATPase may associate with neuronal cell death by glutamate stimulation. Oxidative stress-induced effects of transitional ER ATPase nitration have not been fully determined. The neurosecretory protein VGF is expressed in both the central and peripheral nervous systems and is thought to have a role in hypothalamic and autonomic outflow pathways that regulate the peripheral energy expenditure (44,45). Cytosolic NADP-dependent malic enzyme is part of a newly discovered family of oxidative decarboxylases that exists in various species and contributes to energy production and other biosynthesis pathways (46). Chaperones help in maintaining proper folding and stability of target proteins. Heat shock protein 90 interacts with signalling molecules and is required by several key regulators of apoptosis (47–49). These proteins are known to be associated with cell signalling and metabolism, and their modified tyrosine residues may be related to apoptotic mechanisms of neuronal cells. However, biological functions of these nitration-modified proteins have not been clearly elucidated.

Recently, Bayir *et al.* reported that tyrosine residue nitration of manganese superoxide dismutase (MnSOD) was increased in brain after traumatic brain injury (TBI). Moreover, MnSOD tyrosine residue nitration is attenuated in the brain of nNOS-deficient mice after TBI (31). This group suggested that nNOS was a key regulator of MnSOD tyrosine residue nitration in injured brain. Immunoprecipitation and western blot analyses were performed on total protein extracts from glutamate-stimulated HT22 cells to assess expression of nitrated calmodulin-4, HSP90, ATP synthase alpha chain, glutamate dehydrogenase, tubulin, and Acyl-CoA dehydrogenase (Fig. 4). Our results revealed that nitration levels of these proteins were lower in HT22 cells that were pre-treated with the 7-NI (a nNOS inhibitor) prior to glutamate stimulation. Recent studies have indicated that post-translational modification of proteins such as phosphorylation and nitration were induced by oxidative stress (50,51). Hara *et al.* (52) suggested that nitration of GAPDH was induced by oxidative stress, which triggered binding of E3 ubiquitin ligase and subsequent induction of apoptosis. As oxidative stress-stimulated cell death is induced after post-translational modification of proteins, significant reduction in glutamate-mediated cell death (12 h) may follow inhibition of nitrated proteins (6 h) by nNOS inhibitors (Figs 3 and 4). However, interaction of ROS accumulation and nNOS expression remains unknown, and additional studies are needed to investigate bioactivity of nNOS during glutamate-mediated cell death.

In conclusion, we have demonstrated that glutamate-stimulated oxidative stress increased both ROS accumulation and nNOS expression, and subsequent induction of HT22 cell death. Furthermore, we used a proteomic approach to identify nitrated proteins in oxidative stress (glutamate)-stimulated cells. Interestingly, some of the nitrated proteins such as calmodulin-4, HSP90, tubulin, glutamate dehydrogenase, acyl-CoA dehydrogenase and anti-ATP synthase alpha chain were related to ROS accumulation and nNOS expression by glutamate stimulation. Taken together, these findings suggest biological functions of nitrated proteins in the HT22 cell under conditions of oxidative stress stimulation, which may provide valuable information concerning neuronal cell death, and deeper understanding of neuronal apoptosis processes.

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