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Communications
Manuscript Draft

Manuscript Number:

Title: The Role of TonEBP in Regulation of AAD Expression and Dopamine Production in Renal Proximal Tubule Cells upon Hypertonic Challenge

Article Type: Regular Article

Keywords: Keywords: TonEBP; hypertonic stress; AAD; transcriptional regulation

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Cover Letter

Dear Dr. Ng,

We have made the modification in our manuscript according to the reviewer's comments. The modification included citation of references and description of future works in the Discussion (the sentences added in the Discussion were underlined). There are 4,263 words and 4 figures in the present manuscript. The comments and our answers were stated in the following paragraph.

Reviewer comments: A significant fraction of the data in the study was already published in a seminal paper for the field (Miyakawa et al. 1999. PNAS. 96(5):2538-42. Oddly, the authors did not even cite this important paper. The portion on AAD has potential and would be of interest to the readers of BBRC should it be further developed. The authors should review their manuscript carefully and remove data that is already published by others.

Ans:

The report (Miyakawa et al. 1999. PNAS. 96(5):2538-42.) has been cited in the text (Ref. 21).

Although some data of this study are similar to the results of previous papers (e.g., TonEBP protein abundance and immunofluorescent data), those data are basic evidences to prove the activation of TonEBP in the HK-2 cell line which has not been used in TonEBP related studies. These results will provide a complete and logic story for the readers of BBRC. Meanwhile, previous reports having similar experiments to present the activation of TonEBP in other cells were also cited (Ref. 22-24).

Further works were discussed in the last section of Discussion. We hope to develop the *in vivo* animal model to further investigate the mechanisms illustrated in the present study and focus on physiological regulation.

Sincerely Yours,

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*Highlights

Research highlights

- 1) Hypertonic environment induces activation of TonEBP in HK-2 cells.
- 2) TonEBP is involved in AAD up-regulation under the hypertonic condition.
- 3) Dopamine production is dependent on TonEBP activation.

The Role of TonEBP in Regulation of AAD Expression and Dopamine Production in Renal Proximal Tubule Cells upon Hypertonic Challenge

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Abstract

Renal proximal tubule cells overexpress aromatic L-amino acid decarboxylase (AAD) to produce dopamine, which inhibits salt absorption in the hypertonic environment. We examined the effect of TonEBP on AAD expression in human proximal tubule epithelial cells, HK-2 cell line. Confocal microscopy showed that after 2 h of exposure to the hypertonic medium, TonEBP accumulation in nuclei increased as compared to the isotonic control. The activated TonEBP enhanced the mRNA expression of the representative downstream genes (i.e., *SMIT* and *TauT*). Meanwhile, AAD protein abundance also increased with TonEBP activation. EMSA and luciferase reporter assay showed that TonEBP was involved in transcriptional regulation of AAD upon hypertonic stress. Inactivation of TonEBP by the p38 inhibitor SB203580, or TonEBP shRNA significantly reduced AAD expression, which was rescued by re-expressing Myc-tagged TonEBP. Up-regulation of AAD increased dopamine synthesis, and dopamine inhibited NKA activity in hypertonic condition. These results suggested that TonEBP played an important role in the epithelial cells of renal proximal tubule upon hypertonic stress by enhancing AAD expression, which could promote dopamine secretion to negative regulate NKA activity. The elucidation of a new mechanism described in this study combined with previous findings provides more insights into this issue.

Keywords: TonEBP; hypertonic stress; AAD; transcriptional regulation

1. Introduction

Human kidney is the primary organ for maintaining osmotic homeostasis, and renal cells are normally exposed to fluctuations in salt concentrations and encounter osmotic stress. Hypertonic stress causes reduction in cell volume, DNA breakage, cell cycle arrest, and restrictions of transcription and translation [1]. Responses to hypertonic stress are essential for cell survival under this adverse condition. These responses that improve cell adaptation to the hypertonic stress are accomplished by a variety of processes. Among them, the tonicity-responsive enhancer binding protein (TonEBP), a critical transcription factor, is activated to up-regulate downstream genes, including the sodium-myoinositol transporter (*SMIT*), betaine/GABA transporter 1 (*BGT1*), taurine transporter (*TauT*), and heat shock protein 70 (*Hsp 70*) for cytoprotective functions. The elevated expression levels of these target genes leads to the accumulation of organic osmolytes that protect protein synthesis and folding, allowing the cell to compensate for the extracellular osmotic gradient and adapt to hypertonic stress [2-4]. TonEBP is known to be activated by post-translational modification (e.g., dimerization or phosphorylation) [5;6] followed by binding to TonE (TGGAAA), and enhance transcription of target genes after nucleo-trafficking [7].

In renal proximal tubule cells, dopamine is produced to regulate sodium excretion. Dopamine synthesis relies on aromatic L-amino acid decarboxylase (AAD), to convert the precursor, L-3, 4-dihydroxyphenylalanine (L-DOPA) into dopamine. The secreted dopamine in the proximal tubule bind to dopamine D₁-like receptors (D₁Rs) to inhibit Na⁺/K⁺-ATPase (NKA) activity through (1) protein kinase C (PKC) phosphorylates the catalytic α -subunit of NKA [8] or (2) endocytosis of NKA from the cell membrane, which leads to a decrease in sodium absorption [9].

Previous studies demonstrated that hypertonicity enhanced AAD expression to increase synthesis of dopamine [10-12]. As described above, TonEBP directly stimulates transcription

of several genes in the hypertonic condition. However, the relationship between AAD expression and TonEBP activation is unclear. Therefore, the aim of the present study was to determine whether TonEBP was involved in transcriptional regulation of AAD upon hypertonic stress.

2. Materials and Methods

2.1 Cell cultures and transfection

HK-2 cells (CRL-2190, ATCC) were grown in DMEM/F12 medium (GIBCO) containing 10% FBS, 100 µg/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 5% CO₂ atmosphere incubator. For the hypertonic condition, the cultured medium was supplemented with NaCl to reach 600 mOsm/kg osmolality and examined by an osmometer (Wescor). The TonEBP shRNAs (purchased from Academia Sinica) as well as pCMV-TonEBP (gifts from Prof. Kwon) were transfected to HK-2 cells with jetPEITM (Polyplus-transfection). The stable transfectional TonEBP shRNA cells were maintained in a medium containing puromycin (10 µg/ml).

2.2 Real-time PCR

Total RNA was isolated with the RNA-BeeTM reagent (Tel-Test). The first-strand cDNA was synthesized using a SuperScriptTM reverse transcription kit (Invitrogen). The quantitative real-time PCR for SMIT (sense 5'-ACCCAGTGGCATCCTTAGGT-3', anti-sense 5'-CCCATTGGAGTAAGCGTCA-3') and BGT1 (sense 5'-CCCTGGGGATACTCCA TTG-3', anti-sense 5'-CGAAGAGTGGGACACAGACC-3') was performed with the Mini Opticon real-time system (Bio-Rad) using SYBR Green reagent (Bio-Rad). Then, the data were analyzed by the Bio-Rad CFX manager software.

2.3 Primary antibodies and Immunoblotting

The following primary antibodies were used in this study: (1) rabbit polyclonal anti-TonEBP antibody (Santa Cruz), (2) mouse monoclonal anti-Myc antibody (Santa Cruz), (3) mouse monoclonal anti-AAD antibody (Abcam), (5) mouse monoclonal anti-NKA α1 antiserum (Developmental Studies Hybridoma Bank), (6) rabbit polyclonal anti-p38 antibody

(Cell Signaling), (7) rabbit polyclonal anti-phospho-p38 antibody (Cell Signaling), (8) rabbit polyclonal anti-phospho-AFT2 (Thr71) (Cell signaling). Immunoblotting was performed according to protocol of Kwon et al. [13]. The immunoblotting luminescent signal was detected with Gel Doc XR+ image system (Bio-Rad), and images were exported with Image lab software (Bio-Rad).

2.4 Confocal microscopy

Cells seeded on cover slips were fixed in 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100. Subsequently, the cells were incubated with the polyclonal anti-TonEBP antibody (200× dilution; Santa Cruz) for 1 h and washed with 0.01% Triton X-100. The goat anti-rabbit IgG conjugated Alexa Fluor 488 (Invitrogen) was used to detect the primary TonEBP antibody. The cover slips were mounted onto microscopy glass slide with mounting solution containing DAPI (Invitrogen) and observed using a confocal laser scanning microscope (FV2000, Olympus, Tokyo, Japan) with a 488 nm argon-ion laser.

2.5 Luciferase reporter assay

The AAD promoter region (chromosome 7: 50,628,801-50,631,801) was constructed three fragments (AAD1, -1 bp to -1017 bp; AAD2, -1004 bp to -2000 bp; AAD3, -2131 bp to -2751 bp) into pGL-3-promoter plasmid containing a minimal SV-40 promoter with Xho I and Kpn I restriction sites. Cells were cotransfected with pGL-3-AAD and phRL-TK plasmids (Promega) and then exposed to hypertonic treatment. Next, cells were lysed, and luciferase and Renilla activities were measured using the Dual-Luciferase Reporter Assay System (Promega).

2.6 Electrophoresis mobility shift assay (EMSA)

The nucleoprotein preparation was performed according to Nakayama et al. [14]. The

200 pmol of EMSA probes were conjugated biotin (positive control: 5'-CTTGGTTGGAAAATTACCGCTGGT-3'; AAD probe: 5'-GAACCTTGGAAATCTTACCATAT-3'). The reaction was performed following the manual of the EMSA "Gel Shift" Kit (Affymetrix). In competition assay, 10 or 100 fold (2 nmol or 20 nmol) of competitor was used to compete the protein-DNA binding. In supershift experiment, anti-TonEBP antibody was used to confirm the specific binding between TonE and TonEBP. The antibody was preincubated with nucleoprotein for 1 h at 4°C, before adding other reaction mixture. Finally, the DNA-protein complex was detected using chemiluminescence ECL (Millipore), and the signal was exposed onto a medical X-ray film (Fujifilm).

2.7 $Na^+ - K^+$ ATPase (NKA) activity

Determination of NKA activity was performed according to Tang et al. [15]. 10 μ l cell extract, 50 μ l of 5 mM ouabain or deionized water as well as 100 μ l of 10 mM Na_2ATP were added to 340 μ l of reaction medium (100 mM imidazole-HCl buffer, pH 7.6, 125 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$). The reaction mixture was incubated at 37°C for 20 min followed by immediate immersion into an ice bath for 10 min to stop the reaction. The reaction mixtures and color reagent (1% Tween-20 and 1% ammonium molybdate in 0.9 M H_2SO_4) were mixed 1:1 (v/v) and incubated for 3 min at 37°C before the samples were assayed by a microplate reader (VERSAmax) at 405 nm.

2.8 Enzyme-linked immunosorbent assay (ELISA)

Determination of dopamine was performed according to the manual of the dopamine ELISA kit (17-DOPHU-E01, ALPCO). Cells (2×10^5) were exposed to hypertonic stress for 24 h after pretreatment with 100 μ M L-DOPA for 1 h, and subsequently, 600 μ l of culture medium was collected and analyzed by ELISA kit. The dopamine content was assayed by a microplate reader (VERSAmax) at 450 nm, and calculated according to the standard curve.

2.9 Statistical analysis

The results are shown as the means \pm SD (standard deviation). In the time-course real-time PCR experiments, statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's test. In the luciferase reporter assay experiments, statistical significance was determined using a Student's *t*-test compared to pGL-3 values. In the Na⁺-K⁺-ATPase activity assay, different groups at the same time point were compared by one-way ANOVA followed by Tukey's pairwise method. Values ($p < 0.05$) were considered statistically significant.

3. Results

3.1 Hypertonic stress induced *TonEBP* activation

TonEBP protein was markedly up-regulated with 600 mOsm/kg treatment (Fig. 1A). The confocal micrographs (Fig. 1B) demonstrated that cytosolic *TonEBP* translocated into nuclei. Assessment of gene expression with real-time PCR showed that the downstream genes, *TauT* and *SMIT* were up-regulated about 28 and 4.8 fold, respectively (Fig. 1C). These results indicated that *TonEBP* was activated when exposed to the 600 mOsm/kg hypertonic environment.

3.2 Hypertonic stress promoted *AAD* transcription via *TonE* in the *AAD* promoter region

AAD protein abundance was increased after 2 h of hypertonic treatment (Fig. 1D). Therefore, luciferase reporter assays determined that *AAD1* region (-1 bp to -1017 bp) revealed the strongest transcription activity which was 23 fold higher than the pGL-3 control (Fig. 1E). This result indicated that the *AAD1* region contained a hypertonicity-specific promoter. The *AAD1* promoter region contained a *TonE* sequence (TGGAAA, -675 bp to -680 bp), and it might be the binding site for *TonEBP*. The EMSA results demonstrated the increase activity of DNA-protein binding (Fig. 1F, lane 3), and the signal could be antagonized by 100× non-biotinylated probes (Fig. 1F, lane 4). In addition, a positive probe was published previously [14] to confirm our EMSA results (Fig. 1F, lane 6 and 7). The supershift assay demonstrated that DNA-protein complex contained *TonEBP* protein (Fig. 1G).

3.3 *AAD* up-regulation was dependent on *TonEBP* activity upon hypertonic stress

The p38 protein is a common kinase that is activated under hypertonicity [16], and it was reported to contribute to *TonEBP* activation and nucleo-trafficking [6;17]. The p38-specific

inhibitor SB203580 inhibited p38 activation, and resulted in repressing up-regulation of TonEBP downstream genes (Fig. 2A). Thereby, TonEBP transcriptional function was weak after inhibiting p38 activity (P-AFT2). In addition, AAD protein abundance was more significantly decrease when p38 activity was inhibited (Fig. 2B).

We also silenced TonEBP with shRNA to down-regulate TonEBP expression (supplemental data Fig. S1). Similar to the p38 inhibition experiment, the shRNA also inhibited TonEBP activation and expression of downstream genes (Fig. 2A). When Myc-tagged TonEBP was re-expressed in silenced cells, AAD protein abundance increased markedly compared to that of silenced cells (Fig. 2C).

3.4 Dopamine synthesis correlated to AAD expression was regulated by TonEBP

We attempted to analyze dopamine content under hypertonic stress with TonEBP-inhibition. Compared to the hypertonic treatment group, dopamine secretion was increased in the group treated with hypertonic stress and L-DOPA (Fig. 3, column 4). Moreover, when TonEBP activity was inhibited with SB203580 or shRNA, dopamine synthesis was also inhibited (Fig. 3, columns 5 and 7).

3.5 Dopamine secretion affected NKA activity and sodium excretion

In this study, NKA protein expression increased in hypertonic stress both with and without L-DOPA (Fig. 4A and B). However, NKA activity (Fig. 4C) significantly decreased in the L-DOPA treated group, different from the pattern of protein expression (Fig. 4A and B). In the TonEBP silenced group, adding L-DOPA (L-DOPA + shRNA) for 8 h significantly increased NKA activity as compared to the group treated with L-DOPA only. According to our data of dopamine production, the L-DOPA group had the most dopamine production, and the TonEBP shRNA group had less (Fig. 3). Furthermore, NKA protein in the membrane

fraction was less abundant in L-DOPA group (Fig. 4D). The results corresponded to the decrease of NKA activity because dopamine is a hormone inhibiting NKA activity [9;18].

4. Discussion

TonEBP, a major and specific transcription factor, is activated to enhance the expression levels of downstream target genes to regulate osmoprotective mechanisms. It plays a central role in cellular accumulation of compatible osmolytes during hypertonic stress, TauT, SMIT, and BGT1 were demonstrated to be the downstream genes of TonEBP [19;20]. The nucleo-localization and protein up-regulation of TonEBP were identified to indicate the TonEBP-mediated transcriptional activation [21]. Since studies on TonEBP of HK-2 cell line were limited, the immunofluorescent staining and protein expression in nuclear fractions were performed to be the basis of this study. The localization and protein up-regulation of TonEBP in nucleus of HK-2 cells exposed to hypertonic condition (i.e., 600 mOsm/kg) (Fig. A-C) coincided with previous findings of the other cells provided important evidences to define the activation of TonEBP [22-24]. Therefore, our results clearly showed that a hypertonic condition created in this study successfully activated TonEBP in HK-2 cells.

AAD is a key enzyme responsible for dopamine synthesis and highly expressed in the epithelium of proximal tubules [25]. AAD has been illustrated that expression or activity were increased in different cell types upon hypertonic challenge [10;26;27]. In this study, AAD protein abundance increased significantly under hypertonic stress (Fig. 1D). This increasing expression of AAD is through transcriptional regulation. In the luciferase reporter assay, the AAD1 promoter region 1 had the strongest activity in response to hypertonic stress (Fig. 1E). A conserved TonE sequence can be found in AAD1 region (-675 bp to -680 bp). Subsequently, EMSA demonstrated that the directly binding of TonEBP and AAD probe was in response to hypertonic stress (Fig. 1F and G). However, the results revealed two reactive bands in the hypertonic condition as well as the positive control assay (Fig. 1F). This finding is similar to the findings of Ko et al. [28] that two different types of DNA-protein interactions might occur.

Previous findings revealed that p38 was required for TonEBP activation under hypertonic stress [6]. Hence, we used SB203580 and TonEBP shRNA to inhibit TonEBP activation and clarify its impact on AAD expression. Compared to the hypertonic group, the inhibition of TonEBP activation decreased TauT and SMIT mRNA (Fig. 2A). Meanwhile, the protein expression of AAD was also inhibited (Fig. 2B) and the inhibition could be recovered by over-expression of TonEBP (Fig. 2C). Taken together, this study provides the strong evidence that TonEBP is directly involved in up-regulation of AAD upon hypertonic challenge.

Dopamine has been identified to be an important modulator of central and peripheral physiological functions in humans [29]. Within the kidney, the highest abundance of dopamine receptors is in proximal tubule epithelial cells [29]. In addition, dopamine secretion significantly increases in hypertonic stress (Fig. 3) as reported in previous studies [10;30]. AAD is the key enzyme for converting L-DOPA to dopamine, and its expression is regulated by TonEBP. Thus, it made sense that dopamine secretion is dependent on TonEBP activity (Fig. 3). This study connects the regulatory function of TonEBP not only to AAD expression but also to dopamine secretion in proximal epithelial cells upon hypertonic challenge. Furthermore, to reduce reabsorption of salt in the hypertonic condition, NKA activity is inhibited by dopamine [31]. Previous studies illustrate that phosphoinositide 3-kinase (PI3K) binds to NKA $\alpha 1$ and induces the recruitment and activation of endocytosis [32]. The NKA internalization results in reduced Na^+ transport in proximal tubule epithelial cells [8;18]. In this study, NKA protein expression of HK-2 cells was up-regulated when exposed to the hypertonic condition, and it was not affected by addition of L-DOPA (Fig. 4A and B). However, NKA activity dramatically decreased when treated with L-DOPA to elevate dopamine production. When inhibited by TonEBP shRNA, decreased NKA activity was recovered at 8 h after exposure to the hypertonic condition with L-DOPA (Fig. 4C). This

finding may be because dopamine production was triggered by hypertonic challenge with TonEBP activation. The pattern of NKA activity did not parallel its protein abundance. Subsequently, to separate cytoplasmic and membrane enriched fractions, we demonstrated that NKA protein abundance was decreased in membrane enriched fractions with L-DOPA treatment (Fig. 4D). It was presumed that NKA activity was decreased through the endocytotic mechanism. This study used an *in vitro* culture system to illustrate that dopamine-mediated inhibition of NKA activity might provide a mechanism through blockade of salt reabsorption of proximal epithelial cells under hypertonic challenge. Future works will focus on the regulation of dopamine degradation in hypertonic stress because two enzymes (monoamine oxidase and catechol-*O*-methyltransferase) were involved in dopamine degradation and their activity could be inhibited by hypertonic media [31]. The mechanism is important for cellular physiology, however, it is not clear so far. On the other hand, the TonEBP transgenic mice and knock out model should be created and treated with high salt diet or dehydrated without drinking water. This model can be used to explore the physiological regulation and verification of the summary of this study *in vivo*.

Acknowledgments

We thank to Dr. H. Moo Kwon for contributing the Myc-TonEBP plasmids, Dr. Jeremy Jian-Wei Chen for contributing the pLKO.1 plasmid, and Dr. Biing-Hui Liu for contributing the anti-phospho-ATF2 antibody. This study was supported by a grant from the National Chung Hsing University to T.H.L. (CC-98118)

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Figure Legends

Fig. 1. AAD transcription was enhanced by activated TonEBP. (A) TonEBP protein expressed with hypertonic treatments for 4 h. (B) TonEBP was activated in cells exposed to 600 mOsm/kg for 2 h. The activation was reflected by nuclear translocation of TonEBP (Iso: isotonic; Hyper: hypertonic) (scale bar: 20 μ m). (C) Cells were treated with 600 mOsm/kg, and mRNA expression of *TauT* (left panel) and *SMIT* (right panel) was analyzed by real-time PCR ($n = 4$). (D) AAD protein expression was detected in cells exposed to 600 mOsm/kg for 4 h ($n = 3$). (E) Cells were transfected with pGL-3 containing SV-40 promoter and inserted one of three AAD promoter fragments and phRL-TK (renilla luciferase) and then exposed to 600 mOsm/kg for 2 h ($n = 4$). (*, $p < 0.05$; **, $p < 0.01$) (F) TonEBP protein bound to the probe (200 pmol) designed from the AAD promoter region in the hypertonic condition. The interaction between TonEBP and the designed probe was absent when treated with 100 \times competitor (20 nmol). (G) Supershit assay was used anti-TonEBP antibody to form higher molecular weight complex (arrow point).

Fig. 2. AAD expression positively correlated to TonEBP activity. The shRNA (silencing TonEBP) and SB (10 μ M SB203580, the specific inhibitor of p38) were used to inhibit TonEBP activation. (A) *TauT* and *SMIT* mRNA abundances were determined by real-time PCR under 600 mOsm/kg of hypertonic stress for 4 h (Iso: isotonic; Hyper: hypertonic). (B) Immunoblotting analyzed the expression of AAD with SB treatment, and P-ATF2, a target protein phosphorylated by activated p38 was used to indicate p38 activity. (C) AAD expression was determined via immunoblotting in TonEBP shRNA stably silenced cells, and cells were made to re-express myc-tagged TonEBP ($n = 4$). (*, $p < 0.05$)

Fig. 3. Dopamine secretion was effected by TonEBP activation and the presence of L-DOPA. HK-2 cells were pretreated with or without L-DOPA (100 μ M) and then exposed to the hypertonic condition (600 mOsm/kg) for 24 h with or without TonEBP inhibition (shRNA:

TonEBP shRNA; SB: 10 μ M SB203580). The cultured media were collected, and the dopamine contents were determined by ELISA ($n = 3$). The secreted dopamine concentration was compared to a dopamine standard curve. (**, $p < 0.01$)

Fig. 4. NKA activity was inhibited by dopamine secretion in the hypertonic environment. HK-2 cells were pretreated with L-DOPA (100 μ M) and exposed to 600 mOsm/kg. (A) NKA protein abundance was determined by immunoblotting, and (B) the quantification showed no evident difference between the mock- and L-DOPA-treated groups. (C) NKA activity was assayed in cells cultured in hypertonic medium with a concomitant inhibition of TonEBP activity by shRNA. Significant differences among all groups at 4 and 8 h are shown as different letters or symbols ($n = 3$). (D) Protein accumulation of NKA was determined by immunoblotting in different fractions of cells cultured in media with or without hypersaline and L-DOPA treatment. α -tubulin was used as a marker of cytosolic fraction (Cyto: cytosolic fraction; Mem: membrane fraction).

Figure 1

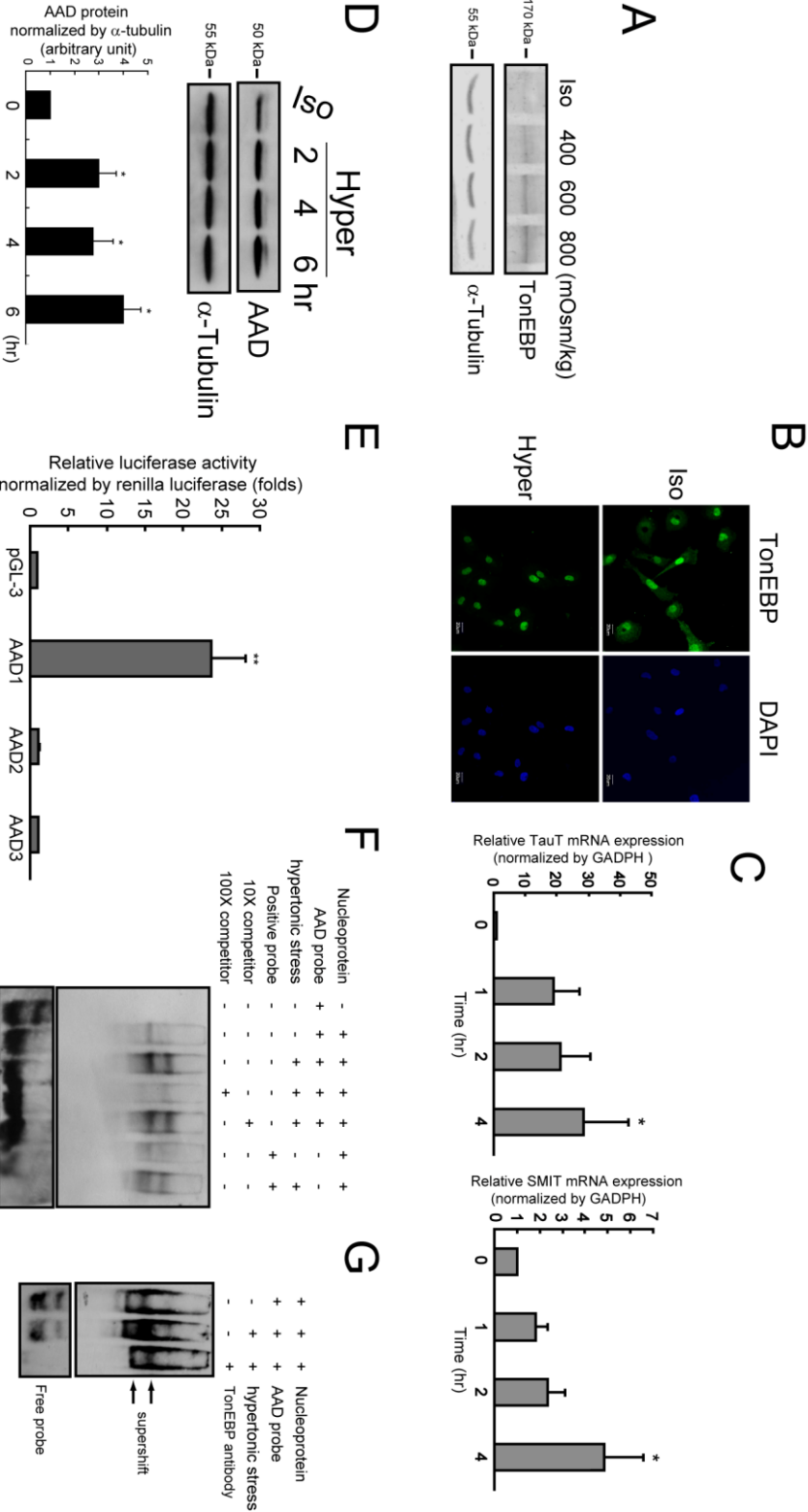
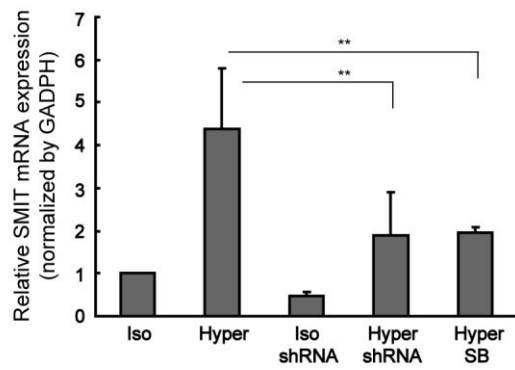
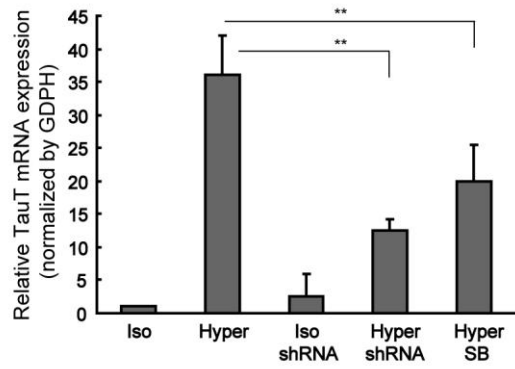
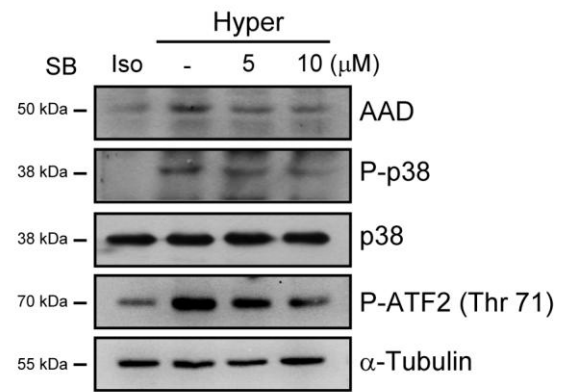


Figure 2

A



B



C

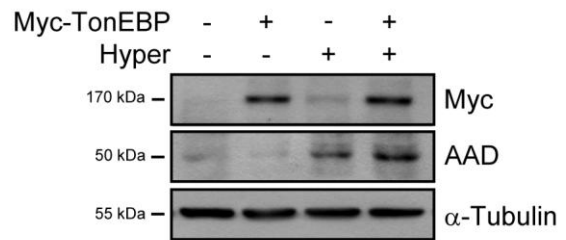


Figure 3

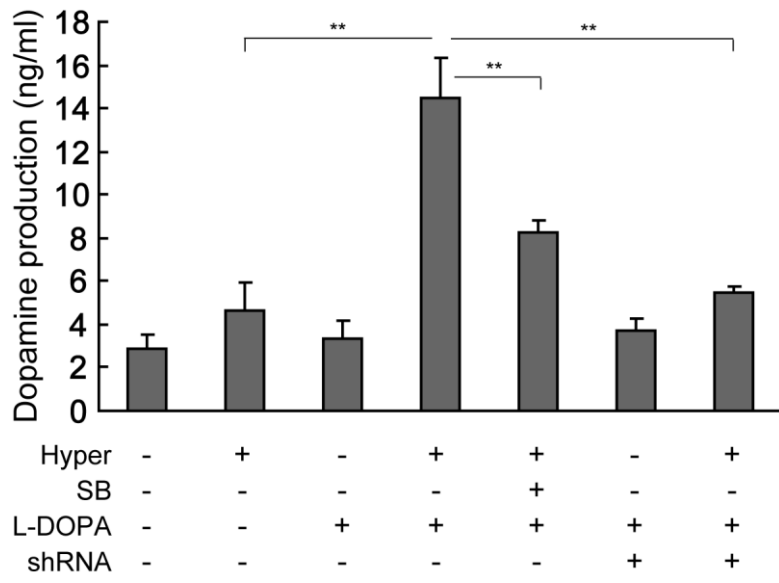
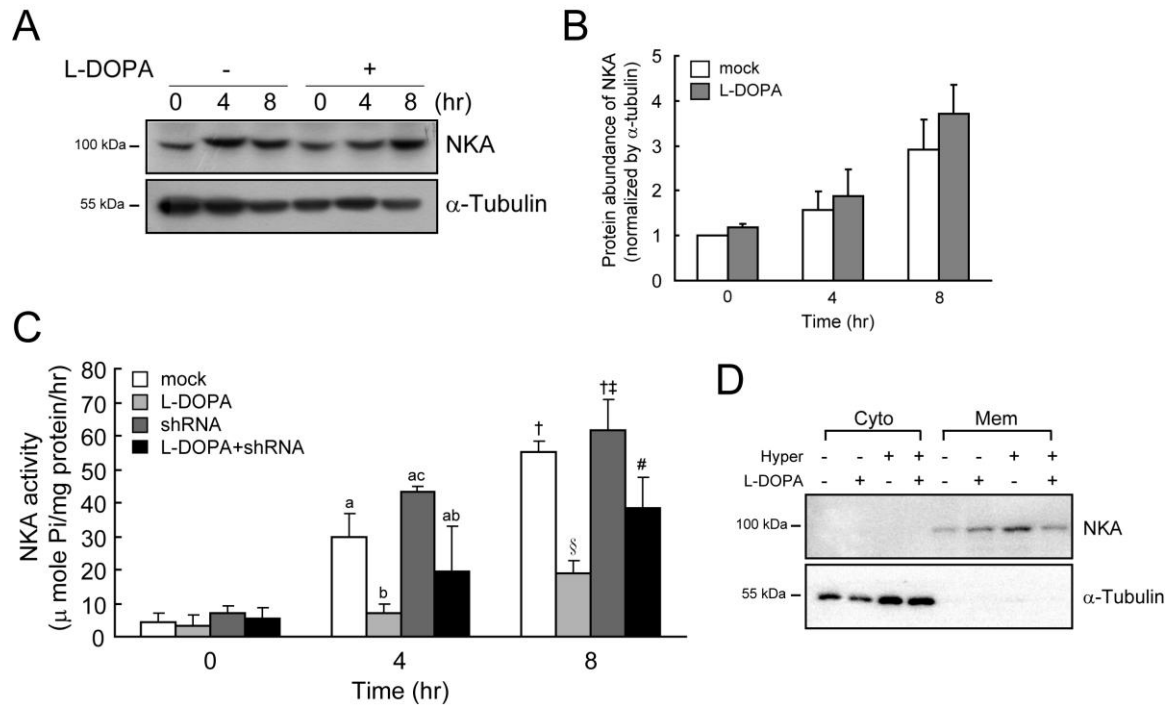


Figure 4



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