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Nrf-2 Regulation of Prion Protein Expression is independent of Oxidative Stress

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ABSTRACT

Cellular expression of host prion protein (PrP) is essential to infection with prion disease. Understanding the mechanisms that regulate prion protein expression at both the transcriptional and translational levels is therefore an important goal. The cellular prion protein has been associated with resistance to oxidative, and its expression is also increased by oxidative stress. The transcription factor Nrf-2 is associated with cellular responses to oxidative stress and is known to induce upregulation of antioxidant defense mechanisms. We have identified an Nrf-2 binding site in the prion protein promoter *(Prnp)* and shown that Nrf-2 downregulated PrP expression. However, this effect is independent of oxidative stress as oxidative stress can up-regulate PrP expression regardless of the level of Nrf-2 expression. Furthermore, Nrf-2 has no impact on PrP expression when cells are infected with scrapie. These findings highlight that Nrf-2 can regulate PrP expression, but that this regulation becomes uncoupled during cellular stress.

INTRODUCTION

The prion protein (PrP^{c}) is a normal cellular glycoprotein (Prusiner, 1998) expressed highly in neurons and other cells (Brown, 2004; Brown et al., 1998; Kretzschmar et al., 1986). It is a cell surface protein and binds copper (Brown et al., 1997a), which it may utilize for a function in cell protection (Brown et al., 1999; Jouvin-Marche et al., 2006; Stanczak and Kozlowski, 2007; Treiber et al., 2007; Zocche Soprana et al., 2011). However, the prion protein is better known for its association with a family of neurodegenerative diseases that include Creutzfeldt-Jakob disease, scrapie and bovine spongiform encephalopathy (Collinge, 2001). In disease the protein is converted to an abnormal isoform (PrP^{Sc}), which is associated both with disease transmission and toxicity (Legname et al., 2004; Muller et al., 1993). In this form it is more associated with cell damage than with cell protection. Alterations in the expression of PrP^{c} causes changes in expression of other proteins associated with cellular protection from oxidative stress, supporting the theory that the main cellular role of PrP^{c} defense against oxidative stress (Brown et al., 1997c; Klamt et al., 2001; Kralovicova et al., 2009; Rachidi et al., 2003; White et al., 1999).

Prion protein knockout mice are protected from both infection with the prion agent and the neurodegenerative effects of PrP^{Sc} (Brandner et al., 1996; Bueler et al., 1993). Increased expression of PrP^{c} results in increased susceptibility to prion infection, a shorter disease incubation time and decreased cellular resistance to the toxicity of PrP^{Sc} (Brown, 1998; Fischer et al., 1996). Studies of

conditional knockout mice have shown that during prion disease, inactivation of PrP^c expression causes halting of disease progression (Mallucci et al., 2003). These findings universally demonstrate that understanding the regulation of PrP^c expression is important to determining the mechanism behind neurodegeneration in prion disease. While the promoter of the *Prnp* gene have been studied there is evidence that intron-1 plays a strong role in the regulation of PrP^c expression and even includes its own TATA box (Baybutt and Manson, 1997; Haigh et al., 2007; Wright et al., 2009). Various studies have shown different transcription factors can alter PrP^c expression. Metal transcription factor-1 (MTF-1), Sp1, Sp3, p53 and Atox-1 have all been shown to increase expression (Bellingham et al., 2009; Vincent et al., 2009; Wright et al., 2009). Two repressors of *Prnp* have been identified and these are Yin Yang-1 (YY1) and Hes-1 (Burgess et al., 2009; Wright et al., 2009). A minor effect has also been reported for repression of PrP by Forkhead Box O3a (Foxo3a)(Liu et al., 2013). However, understanding of what regulates PrP expression remains incomplete.

Nuclear Factor–Erythroid2-related factor-2 (Nrf-2) is a transcription factor associated with rapid cellular response to oxidative stress (Johnson et al., 2008). Nrf-2 is associated with the activation of proteins whose genes contain an ARE (antioxidant response element). Studies of Nrf-2 both *in vivo* and *in vitro* have confirmed that increase in its expression diminished many neurotoxic changes from lipid peroxidation (Ansari et al., 2011), excitotoxicity(Li et al., 2007), calcium metabolism (Lee et al., 2003) and mitochondrial changes (Ludtmann et al.,

2014). In particular there has been a lot of interest in the role of Nrf-2 in the protective mechanisms of astrocytes and how this can be modulated to combat neurodegeneration in various animal models(Joshi and Johnson, 2012; van Muiswinkel and Kuiperij, 2005). Modulation of Nrf-2 has been suggested to be of benefit for many diseases including Alzheimer's and Parkinson's disease as well as Amyotrophic Lateral Sclerosis (Gan et al., 2012; Kanninen et al., 2009).

Currently, there is no evidence linking Nrf-2 to either the expression of PrP^{c} or a protective role in prion disease. Our initial studies suggested that the Prnp gene has a binding site for Nrf-2 in intron-1, but that increased expression of Nrf-2 did not alter luciferase reported activity. However, in the current study we discovered a different Nrf-2 binding site on the Prnp promoter. We found that Nrf-2 decreases PrP expression through this site. Curiously, in cells where PrP expression has been repressed by Nrf-2 oxidative stress causes a much greater increase in PrP expression. These finding suggest that Nrf-2 acts as a regulator of PrP expression under healthy conditions, but oxidative stress and prion infection overrides this regulation.

MATERIALS AND METHODS

Reagents were purchased from Sigma-Aldrich unless otherwise stated.

Cell Culture

All cell lines were maintained at 37°C and 5% CO_2 in a humidified incubator. Cell lines used for these studies were predominantly mouse N2A cells (Neuro-2A, ATCC Number: CCL-131). Other cell lines used were C8-D1A (mouse type 1 astrocyte) cells and scrapie mouse brain cells (SMB)(Birkett et al., 2001) and the pentosan sulphate cured control cells (SMB-PS). Cell lines were cultured in DMEM with 10% fetal bovine serum (Lonza) and 1% penicillin–streptomycin. Preparation of protein extracts from cells and proteinase K (PK) treatment of extracts was as previously described (Davies and Brown, 2009).

Promoter Analysis

The Prnp promoter construct was as previously described (Wright et al., 2009). Basically, 6 kB of bovine Prnp up tp the translation initiation site was clone in pGL3Basic, a firefly luciferase reporter construct. A mutation of the promoter (PromoM) was created by introducing an EcoR1 site at -3.0 kb and excising the 5’ -6.0 to -3.0 kb fragment and religating the plasmid. Both constructs were then transfected into N2A cells with Fugene (Promega) transiently, along with a control plasmid contain the thymidine kinase (TK) promoter and a *Renilla* based luciferase reporter. Activity was anlysed after 48 hours. The N2A cells were either stably transfected to overexpress Nrf-2 or were transfected with the empty

vector (pCDNA3.1) as previously described (Wright et al., 2013). Media was removed from the cells and washed with PBS 100 µL of passive lysis buffer was used to lyse the transfected cells. Analysis of dual luciferase activity was carried out with the dual liferase assay kit (Promega) according to the manufacturer's instructions. Measurement of both firefly and Renilla luciferase activity was with a FLUOstar Omega (BMG Labtech) plate reader. Activity of the *Prnp* reporter constructs was then normalized to the TK activity and presented as relative luciferase units (RLU).

EMSA

Electrophoretic mobility shift assays (EMSAs) were carried out to assess Nrf-2 binding to its recognition site. N2A cells were stably transfected with empty expression vector (pcDNA3.1) or pcDNA3.1+Nrf-2. Cells were washed in ice cold PBS and the protein was extracted using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, UK). Protein was quantified using the Bradford assay (Bio-Rad Laboratories Ltd, Hercules, CA). Synthetic biotin and non-biotin labelled probes (MWG, Germany) of a fragment of the bovine Prnp containing the Nrf-2 recognition sequence (-3057 to -3015) 5'TGGTCACTGTCTGTATGG**CTTCCTGT**TATCCTCTTTGAGGT3' were obtained for EMSA analysis. Binding reactions containing equal amounts of protein (15 mg) and probes were performed using the LightShiftH Chemiluminescent EMSA kit (Thermo Scientific, UK) as per the manufacturer's instructions. Protein-DNA complexes were electrophoresed on 6% non-

denaturing polyacrylamide gels, and transferred to Hybond-N+ (GE Healthcare, UK); signals were detected using LightShiftH Chemiluminescent EMSA kit (Thermo Scientific). In order to validate specific binding reactions were also run with the addition non-labelled probe at 1 x and 5 x and 10 x excess of the labelled probe.

Western Blot

Cell extracts were electrophoresed on a 10% or 14% polyacrylamide gels. Protein immobilised within the gel was transferred to a PVDF (Millipore) membrane by a semi-dry transfer method. The transfer was run for 120 minutes at 50 mA, 25 V using a Bio-Rad semi-dry blotter. The membrane was then blocked in Tris buffered saline containing 0.1% (v/v) Tween and 5% (w/v) (TBS-T) milk powder for 1 hour, shaking. Membranes were probed with a primary andibody. This was either a monoclonal antibody against PrP (ICMS-18, D-Gen), a monoclonal anti-Nrf-2 antibody (Santa Cruz) or an antitubulin antibody (Sigma). Incubation with the primary antibody was in TBS-T containing 5 % milk powder overnight at 4 ºC overnight. The membrane was then washed again in TBS-T 5 times before being incubated with a secondary HRP-conjugated antibody, in TBS-T containing 5 % milk powder for 45 minutes at room temperature. Following extensive washing in TBS-T, the membrane was developed for 2 minutes with ECL reagent and exposed to film. A Compact X-ray processor was used to develop the film. Membranes were then stripped with 100 mM glycine,

2% SDS pH 3 stripping buffer for half an hour and reprobed with further antibodies as required.

RESULTS

A conserved Nrf-2 binding site

We sought to identify whether the *Prnp* promoter contained a conserved binding site for Nrf-2. We compared 4000bp prior to the translation start site of both the human and bovine (*Bos taurus*) *Prnp* promoter sequences. The sequences were obtained from the USCS genome browser (human: NM_183079, bovine: NM_181015.2). A search was then made to identify conserved transcription factor binding sites using rVista (http://rvista.dcode.org/). Using the Nrf-2 recognition sequence (CTTCCTGT) a single conserved site was found in both bovine (-3.13 kb) and human (-2.5 kb) sequences.

Transcriptional Regulation of PrP Expression by Nrf-2

We have previously used luciferase reporter constructs to study the regulation expression of PrP by its promoter (Wright et al., 2009). Using the promoter reporter construct from that study we made a mutation at -3.0 kb to delete the sequence 5' to this point that included the Nrf-2 recognition sequence described above (PromoM). Using a dual luciferase reporter system we looked at the expression driven by the Prnp promoter in N2A cells transfected to overexpress either Nrf-2 or transfected with the empty vector (pCDNA3.1). Compared to pCDNA3.1, Nrf-2 caused a significant reduction in Prnp promoter reported activity (Figure 1). In comparison the deletion mutant showed no significant difference to the control when Nrf-2 was overexpressed. This data supports the

notion that Nrf-2 decreases Prnp activity through the identified Nrf-2 binding site in the promoter.

Electrophoresis mobility shift assays were also performed to confirm Nrf-2 binding to the predicted sequence in the Prnp promoter. Extracts from Nrf-2 overexpressing and pCDNA3.1 transfected cells were prepared and exposed to the labelled DNA probe. A shift in labelled DNA was observed in the Nrf-2 overexpressing cell extracts and not in the pCNDA3.1 extracts. This interaction could be quenched with unlabelled DNA probe (Figure 2).

Nrf-2 and PrP Expression

We have identified that Nrf-2 repressed Prnp activity at the transcription level. The expression of PrP was also studied using western blot and immunodetection. Protein extracts were prepared from two independently derived cell lines both overexpressing Nrf-2. The level of expression of Nrf-2, PrP and tubulin were detected with specific antibodies and the level of expression in these cell lines compared to the expression in N2A cells transfected with the empty vector (Figure 3). Nrf-2 expression was increased in both transfected cell lines and in comparison, PrP was significantly decreased. These results confirm that Nrf-2 suppresses PrP expression.

Oxidative Stress and PrP Expression

The now classic role of Nrf-2 is as a response factor under conditions of oxidative stress. Data has also been published indicating that there is an increased expression of PrP in response to oxidative stress (Brown et al., 1997b). We therefore looked at the level of PrP expression in cells that were exposed to 1 μM hydrogen peroxide for three and six hours. Cells were either transfected with pCDNA3.1 (empty vector) or the vector containing Nrf-2. Control cells showed an increase in expression of Nrf-2 and PrP (Figure 4). In cells overexpressing Nrf-2 there is also an increase in PrP expression in response to oxidative stress. However, the response is much greater. These results suggest that under conditions of oxidative stress Nrf-2 is not able to suppress PrP expression and changes to PrP expression in oxidative stress conditions is due to other factors and thus independent of Nrf-2.

Nrf-2 and PrP Expression in Astrocytes

Nrf-2 responses in the brain are associated with astrocytes and it is through changes in astrocytes that Nrf-2 is thought to have its protective effects against oxidative stress. In astrocytes, PrP expression is quite low compared to neurons (Brown, 1999). However, PrP expression is increased when astrocytes are exposed to oxidative stress (Miele et al., 2003). It is possible that PrP's low expression level in astrocytes is a response to the higher levels of Nrf-2 expression. SFhERRβ (short form estrogen related receptor beta) has been shown to completely inhibit Nrf-2 when transfected into cells (Zhou et al.,

2007).We tested this by transfecting a C8-DIA astrocytes cell line with the Nrf-2 inhibitor SFhERRβ. Expression of the inhibitor successfully suppressed Nrf-2 expression and caused an increase in PrP expression relative to cells transfected with the empty vector (Figure 5).

Prion Infection and Nrf-2

The SMB cell line is constitutively infected with the Chandler strain of scrapie (Birkett et al., 2001). This cell line continuously produces protease resistant PrP. We looked at the expression of Nrf-2 in this cell line compared to a control cell line cured of the infection by pentosan sulfonate (SMB-PS) (Birkett et al., 2001). Nrf-2 expression levels were clearly elevated in the infected cell line (Figure 6). This is consistent with the previous suggestion that prion infected cell lines exhibit higher levels of oxidative stress(Milhavet et al., 2000). We then transfected the infected SMB cell line with Nrf-2 to increase Nrf-2 expression, and also with SFhERRβ to decrease active Nrf-2. Analysis of the transfected SMB cells demonstrated that Nrf-2 levels had no effect on the level of expression of PrP or the level of protease resistant protein in the cells (Figure 7). This result demonstrates that although Nrf-2 can alter the expression of PrP in uninfected cell lines, in infected cells regulation of PrP by Nrf-2 is uncoupled and changes in Nrf-2 no longer have the same effect.

DISCUSSION

The main reason for advancing understanding of how the expression of PrP is regulated is because expression of PrP is a necessary requirement for the development of prion disease. PrP-knockout mice are resistant to prion infection (Bueler et al., 1993). Indeed, even when a source of PrP^{Sc} generation is implanted into the brain of PrP-knockout mice, this is not sufficient to cause infection(Brandner et al., 1996). Furthermore, induced knockout of PrP during the disease process arrests prion disease development (Mallucci et al., 2003). This absolute requirement for host PrP expression means that an insight into PrP regulation may provide information on how to prevent disease progress in humans.

The normal cellular function of PrP^{c} is an area of great controversy in the field. Despite significant insight into the cellular role of this protein it is still quite common to read in the literature that the function remains unknown. The vast majority of literature on the subject point to a protective role for PrP^{c} at the cellular level (Alfaidy et al., 2013; Barbieri et al., 2011; Brown, 2005; Chiarini et al., 2002; Fleisch et al., 2013; Frigg et al., 2006; Llorens and Del Rio, 2012; Nishimura et al., 2004; Rangel et al., 2007; Sorgato and Bertoli, 2009; Zamponi and Stys, 2009; Zocche Soprana et al., 2010). This role is likely to be related to resistance to oxidative stress (Brown et al., 1997c; Brown et al., 1999; Klamt et al., 2001; Rachidi et al., 2003; Stanczak and Kozlowski, 2007; Treiber et al., 2007; White et al., 1999). However, knockout of PrP expression results in little in

the way of a phenotype in animal models (Bueler et al., 1992). At the cellular level this can be explained by the upregulation of a range of different proteins, many of which are associated with cell survival or resistance to oxidative stress (Kralovicova et al., 2009). Furthermore, alteration in the expression of other antioxidant proteins also results in the increased expression of PrP(Kralovicova et al., 2009). The implication is that PrP is one protein in a network of cell protection proteins that perhaps should not be viewed in isolation.

Regulation of PrP expression at the transcriptional level is complex. As well as a traditional promoter, PrP shows considerable regulation from the first intron and also Exon1 (Haigh et al., 2007; Wright et al., 2009). Some of the effects are cell type specific. We have previously shown that intron1 contains its own TATA box and that transcription factors binding to site in intron1 have strong effects on PrP’s expression. In particular we identified Hes1 as a repressor and Atox1 as an activator mediating their effects by binding to intron1. The repressor role on intron1 has been confirmed by others (Xue et al., 2012). In this case roles for Sp1 and RP53 were identified. There have also been reports that other transcription factors play a role in PrP expression. These include the repressor Yin-Yang-1 (YY1) (Burgess et al., 2009) and activators such as p53 (Vincent et al., 2009)and metal-activated transcription factor-1 (MTF-1) (Bellingham et al., 2009). Other reports indicate that indirect effects alter PrP. As *Prnp* has heat shock elements then it can respond to many forms of cellular stress(Shyu et al., 2000). Such stress events that have been shown to up-regulate PrP include

insults from nitric oxide (NO) radicals (Wang et al., 2005) and oxidative stress induced by hyperbaric oxygen (Shyu et al., 2004), hypoglycemia (Shyu et al., 2005) or other sources. Copper-induced oxidative stress also up-regulates PrP indirectly via binding of p53 following phosphorylation by ataxia-telangiectasia mutated (ATM) (Qin et al., 2009). In contrast, All-trans retinoic acid (ATRA) has been observed to suppress PrP expression(Rybner et al., 2002).

Nrf-2 is well known for its role in regulating antioxidant defense proteins. So, it is not unexpected that if PrP plays a role in antioxidant defense that it can be regulated by Nrf-2. In support of this we found that the *Prnp* promoter does contain an Nrf-2 recognition sequence and this sequence was found to bind Nrf-2 in EMSA assays. However, Nrf-2 repressed PrP expression rather than induce it. Nrf-2 has largely been characterized as an activator of genes with an ARE domain (antioxidant response element) (Johnson et al., 2008). However, there are other examples where Nrf-2 has been seen to act as a repressor such as for RON (Recepteur d' origine nantais) tyrosine kinase (Thangasamy et al., 2011). Astrocytes have a higher Nrf-2 level (Williamson et al., 2012) and low PrP expression (Brown, 2004) and we found that inhibiting Nrf-2 activity increased PrP expression in an astrocyte cell line.

Despite this repressing role of Nrf-2 in PrP expression, we found an increased expression of PrP in response to oxidative stress. This response was greatest in Nrf-2 overexpressing cells. This suggests that the pathway that increases PrP

expression in response to oxidative stress is independent of Nrf-2. If these findings hold true *in vivo*, then Nrf-2 might play a role in "priming" PrP's response which could make it of greater benefit to the cells under oxidative stress. There is also considerable evidence that prion infected cells have higher levels of oxidative stress (Drisko, 2002; Haigh et al., 2011; Milhavet et al., 2000). When we looked at the impact of prion infection on the Nrf-2 response we saw that there was no change in expression in response to altered Nrf-2 levels. Therefore, under both oxidative stress conditions and prion infection, the response of PrP was uncoupled from the transcriptional repression of Nrf-2.

These findings clearly do not provide an avenue of research that would benefit treatment of prion disease, other than highlighting the key role of oxidative stress in the process. Identifying the regulation of PrP^{c} remains an important endeavor, but it is quite possible (as in this case) that transcriptional mechanisms identified as being of relevance for healthy cellular metabolism do not operate, or are bypassed under pathological conditions.

FIGURE LEGENDS

Figure 1 Promoter Analysis

A N2A stable cell lines stabley transfected either with pCDNA-Nrf-2 (Nrf-2(1) and Nrf-2(2)) or the empty vector (pCDNA) were transiently tranfected with luciferase based Prnp reporter vectors. The vectors were either pGL3Basic with the full length bovine Prnp promoter (Promo) or 3kb fragment (PromoM). A luciferase based reporter assay was carried out. Values are presented as relative luficerase units (RLU, a ratio of the luciferase values to pTK renilla reporter cotransfected) and are the mean and s.e. of four experiments in triplicate.

B Shown are the raw pTK renilla values for the above experiments. The data is provided as evidence of the equivalent transfection efficiency between experiments.

Figure 2 EMSA

A representative EMSA assay for band shift of a labelled Prnp fragment. Nuclear extracts were prepared from N2A cells stabley transfected with pCDNA-Nrf-2 (lane 1) or the empty vector (pCDNA3.1) (lane 5). Increasing amounts of cold Prnp fragment were added to the reaction with the Nrf-2 transfected cell extracts to ensure specificity of the band (lane: 2, 1:1; lane 3,1:5; lane 4 1:10). The shifted band is indicated by the arrow.

Figure 3 PrP Expression

Western blot of protein extracts from N2A cells stabley transfected with pCDNA-Nrf-2 (Nrf-2(1) and Nrf-2(2), two separate cell lines overexpressing Nrf-2) or the empty vector (pCDNA). Immunodetection with specific antibodies to Nrf-2, PrP and tubulin was carried out. While tubulin verified equivalent protein loading of the lanes, Nrf-2 verified overexpression of the protein in the cell lines. In the cell lines PrP expression was decreased relative to pCDNA. This was quantitated for four separate blots and the mean and standard error shown in the graph below. For Nrf-2 the control (pCDNA) value was equated but for PrP the control value was equated to 10. The relative change for the two proteins was then represented relative to these normalisations to allow them to be presented on the one graph. Therefore a significant increase in Nrf-2 expression resulted in a significant decrease in PrP expression for the two Nrf-2 cell lines (Student's t test, $p < 0.05$).

Figure 4 Oxidative Stress

Western blot of protein extracts from N2A cells treated with 1 μM hydrogen peroxide for 0, 3 and 6 hours. The cells were either transfected to overexpress Nrf-2 or were transfected with the empty vector (pCDNA). Immunodetection with specific antibodies was carried out to assess levels of Nrf-2, PrP and tubulin. For pCDNA cells both Nrf-2 and PrP were significantly (Student's t test, $p < 0.05$) elevated as result of treatment with hydrogen peroxide. For Nrf-2 transfected cells only PrP was significantly elevated. All results were compared to the values

at 0 h (which was equated to 1) and are shown in the lower graph. Sown are the mean and standard error for three experiments.

Figure 5 Astrocytes

The astrocyte cell line C8-DIA was transfected to overexpress the Nrf-2 inhibitor SFhERRβ or the empty vector (pCDNA). The level of PrP, Nrf-2 and tubulin expression in the cells was assessed by western blot and immunodetection. In astrocytes PrP is expressed predominantly as the double glycosylated isoform as indicated by the higher band. The expression of the SFhERRβ caused a significant ($p < 0.05$) increase in PrP expression (3.5 ± 0.6 fold increase, n=3).

Figure 6 Infected Cells

Protein extracts were prepared from SMB (scrapie infected) and SMB-PS (non-infected) cells. Extracts were divided in two and half one half treated with proteinase K (PK) at 20 μg/ml for 30 minutes The protein was electrophoresed on a 12% PAGE gel and transferred to a membrane via western blot. Immunodetection of both PrP and Nrf-2 was carried out with specific antibodies. Tubulin was also analyzed to confirm equal loading. PrP expression was typical for the two cell types, with SMB cell showing higher levels of total PrP due to the presence of PK resistant protein. SMB cells showed significantly higher levels of Nrf-2 (4.6 ± 0.7 folding higher, n=4, $p < 0.05$).

Figure 7 Nrf-2 and SMB Cells

SMB cells were transfected to overexpress either Nrf-2 or SFhERRβ or were transfected with the empty vector (pCDNA). Extracts were prepared from the cells and treated with and without PK as in Figure 6. After PAGE gel electrophoresis and western blot, immunodetection of PrP, Nrf-2 and tubulin was carried out. Analysis of the bands densitometrically was carried out and presented in the graph for both total protein (PK -) and PK resistant protein (PK +). The values were compared to those for the pCDNA SMB cells, which were equated to 1. There was no significant difference between any of the different cell types for either total or PK resitant protein ($n=3$, $p > 0.05$). The levels of Nrf-2 and tubulin are also shown (both proteins are degraded by PK).

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