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Altered ubiquitin-proteasome system leads to neuronal cell death in a spontaneous obese rat model

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ABSTRACT

Background: Obesity is associated with various progressive age-related diseases, including neurological disorders. However, underlying molecular basis for increased risk of neurodegeneration in obesity is unknown. A suitable animal model would immensely help in understanding the obesity-linked neurological problems.
Methods: A spontaneously developed obese rat (WNIN/Ob) which is highly vulnerable for a variety of degenerative diseases was isolated from the existing WNIN stock rats. Ultrastructure of neurons in the cerebral cortex of 12-month old obese rats was evaluated by transmission electron microscopy. qRT-PCR and immunoblotting of ubiquitin C-terminal hydrolases (UCHs), ubiquitin, proteasomal sub-units, markers of ER stress and apoptosis were performed in the cerebral cortex. Proteasome activity was assayed by fluorometric method. Immunohistochemistry was performed for mediators of apoptosis, which was further confirmed by TUNEL assay. These investigations were also carried in high-fat diet-induced obese rat model.
Results: Neurons in the cerebral cortex of 12-month obese rats showed swollen mitochondria, disrupted ER and degenerating axons, nucleus and finally neurons. Results showed altered UPS, existence of ER stress, up-regulation of apoptotic markers and apoptosis in the cerebral cortex of obese rats. It appears that UCHL-1 mediated apoptosis through stabilizing p53 might play a role in neuronal cell death in obese rat. Similar changes were observed in the brain of diet-induced obese WNIN rats.
Conclusion: Altered UPS could be one of the underlying mechanisms for the neuronal cell death in obese conditions.
General significance: This is the first report to highlight the role of altered UPS in neurodegeneration due to obesity.

1. Introduction

Obesity incidence is increasing worldwide, affecting both developed and developing countries. The World Health Organization has reported that obesity has been growing at an alarming rate, accounting for approximately 35% of the population [1]. Apart from being a great economic burden upon society, obesity also imposes increased morbidity and mortality. There is accumulated scientific evidence indicating that obesity is strongly related to a vast number of diseases, including hypertension, hypercholesterolemia, type 2 diabetes, respiratory conditions, arthritis and certain types of cancer.

Obesity could have an effect on brain is a recently emerged concept. Associations between obesity and various neurological disorders have been reported including sleep apnea, anxiety, manic depressive disorders, increased risk of developing cerebrovascular accident and other neurological disorders [2,3]. Additional consideration has been raised that obesity may be linked to various progressive and age-related neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease (AD), and autoimmune nervous system diseases like multiple sclerosis. A more recent association between obesity and neurological function was based upon correlations with biological processes of oxidative stress and inflammation [4]. While the causal nature of these processes to neurodegeneration has not been definitively established, it is widely accepted that neuroinflammation and oxidative stress responses occur with clinical manifestation of the disease.

Impaired function of the ubiquitin-proteasome system (UPS) has long been implicated as a contributing factor in various neurodegenerative diseases [5]. The function of the UPS can be impaired by many factors including the aging process, leading to the formation of ubiquitin protein aggregates resulting ultimately in proteinaceous inclusions detected in non-pathologic aging as well as in many neurodegenerative disorders [5,6]. In fact, the presence of various ubiquitin-decorated protein aggregates is pathonumonic for many such diseases. Recent research has shown that UPS plays a role in various neurodegenerative

Abbreviations: DUB, deubiquitinase; ER stress, endoplasmic reticulum stress; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UCH, ubiquitin C-terminal hydrolase; UPS, ubiquitin proteasome system; UPR, unfolded protein response

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diseases, such as Alzheimer's disease and Huntington's disease [7,8]. However, the role of UPS in age related and/or obesity induced neurodegeneration is still to be elucidated. In this context, an animal model that develops neurodegeneration as a consequence of obesity could be ideal to delineate the molecular basis of neurodegeneration due to obesity.

A spontaneously developed obese rat was isolated from the existing WNIN (Wistar at National Institute of Nutrition) stock of rats and a colony of WNIN-Obese (WNIN/Ob) rats was established by selective breeding [9]. The phenotype and associated biochemical, histological, and pathophysiological characteristics of WNIN/Ob rats have been reported in detail elsewhere [10–15]. Reddy et al., [11] reported that their body weights increased progressively until the age of 12 months when they weigh as much as 1.2 kg, in contrast to their lean littermates that weigh 500 to 600 g. Obese animals have 47% of fat in the body and are hyperphagic, euglycemic, hyperinsulinaemic, hypertriglyceridaemic and leptin resistant [9,14,15]. Harishankar et al., reported that WNIN/Ob rats have significantly lower brain weights and there is up to 50% of reduction in their organ-to-body weight ratio when compared to their lean littermates [9,10,15]. WNIN/Ob rat is a monogenic mutant and preliminary studies show no molecular defects in leptin and leptin receptor. Positional cloning studies established the presence of mutation on 5th chromosome and QTL (quantitative trait loci) mapping studies have localized the mutation upstream to the leptin receptor [16] and efforts are on to nail the mutation. The average life span of this obese mutant rat strain was found to be 18–20 months as against 30–36 months of their parent WNIN strain. In addition to the obesity trait, these animals were shown to be more prone to the age-related abnormalities such as vision impairment (retinal degeneration and cataract) [11–13], impaired immunity [14] and development of tumors[9,10]. Interestingly, preliminary findings do indicate neurodegeneration in WNIN/Ob rat model.

Therefore this study was designed to investigate neurodegeneration in WNIN/Ob rat model which has two crucial causative factors: obesity and accelerated aging. Further, we examined the possible role of UPS in neurodegeneration linked to obesity using WNIN/Ob rat.

2. Experimental procedures

2.1. Materials

Tri-reagent, acrylamide, bis-acrylamide, ammonium persulphate, β-mercaptoethanol, SDS, TEMED, PMSF, aprotinin, leupeptin, pepstatin, antibodies for UCHL-1 (ubiquitin C-terminal hydrolase-1; U5258), UCHL-3 (U7133) and UCHL-5 (SAB2105178),anti-actin antibody (A5060), horse radish peroxidase (HRP) conjugated anti-rabbit (A6154), anti-mouse (A9044) secondary antibodies were purchased from Sigma Chemicals (St. Louis, MO, USA). Anti-GRP78 (368673) and PVDF membrane were obtained from Merck Millipore (Billerica, MA 01821, US). Anti-XBP1 (sc-7160), anti-GADD 153/CHOP (sc-575), anti-ATF-6α (sc-22799), anti-BAX (sc-6236), anti-p53 (sc-6243), anti-Ub (ubiquitin; sc-9133) antibodies were purchased from Santa Cruz Biotechnology, Inc., (Dallas, Texas 75220, U.S.A.). Anti- BCL2 antibody (AM43) was procured from Oncogene. Alexa Fluor-555 conjugated anti-Mouse IgG (A-21427) and Alexa Fluor-488 conjugated anti-rabbit IgG (A-11008) obtained from Molecular Probes, Inc. Eugene. High capacity cDNA reverse transcription kit and Power SYBR Green Master Mix were obtained from Applied Biosystems (Warrington, UK). All primers were procured from Integrated DNA Technologies (Coralville, IA, USA).

2.2. Animals

The WNIN/Ob rats of different ages along with their respective lean littermates were obtained from National Center for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Hyderabad and were maintained in a temperature and light controlled animal facility, given normal pellet diet and water ad-libitum.

Two-month old WNIN rats were obtained from NCLAS. A group of animals were maintained on AIN-93 diet alone (control) or AIN-93 diet supplemented with 24% fat for a period of six months. Food intake and body weights were monitored periodically. Glucose intolerance and insulin resistance (IR) was assessed in these rats by performing oral glucose tolerance test (OGTT) and HOMA (homeostasis model of assessment)-IR, respectively as described earlier [17]. These animals have developed IR but not hyperglycemia

After the completion of the respective time period, rats were sacrificed by cervical dislocation. The animal was dissected, cerebral cortex of brain tissue was collected and immediately frozen in liquid nitrogen and later kept at −80 °C until further analysis. Few brain tissues were collected in 4% w/v paraformaldehyde fixative solution for immunohistochemistry. Animal care and protocols were in accordance with and approved by Institutional Animal Ethics Committee.

2.3. Transmission electron microscopy (TEM)

Prefrontal cerebral cortex of rats was fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C, and washed with PBS for 4 times each for 45 min, then fixed in 1% aqueous tetroxide for 2 h, later washed with deionized distilled water for 6 times each for 45 min, dehydrated in series of graded alcohol, infiltrated and embedded in araldite 6005 resin followed by incubation at 80 °C for 72 h for complete polymerization. Ultrathin (50–70 nm) sections were made with a glass knife on ultra-microtome (Leica Ultra cut UCT-GA-D/E-1/00), mounted on copper grids and stained with saturated aqueous uranyl acetate and counter stained with Reynolds lead acetate. Finally viewed under TEM (Model: Hitachi, H-7500 from Japan) at required magnifications.

Table 1
Sequence of primers used in qRT-PCR.

S.No	Target gene		Sequence (5′-3′)
1	UCHL-1	Forward (F)	TCAGTGCCATCTCCTGCCTG
		Reverse (R)	CTGCGTGGATCAGCCCAATG
2	UCHL-3	F	TGGCAGTTTGTGGACGTGTA
		R	GGCGTTGCTGATGGTTTGTT
3	UCHL-5	F	GGATTACTGCAGTGAGGCCA
		R	GTTCCTCCTCTGCAAGCTGT
4	GRP78	F	AAGGGGAGCGTCTGATTGGCGA
		R	ATGAGGCGCTTGGCGTCGAAGA
5	ATF6	F	TCGGCAAGCGGAGAACCACCTT
		R	ACAGGGATGCACGGCCATCAGA
6	IRE1	F	GCGCACCTGCATTCCCTCAACA
		R	TCCTGCCATGTGCGTTGGGCAT
7	XBP1	F	AGGCAGAGTCCAAGGGGAATGG
		R	AGAGGCGCACGTAGTCTGAGT
8	PERK	F	TACGGCATTTGGCTTGGGGGCA
		R	TCGGCGTCTTCCACGGTCACTT
9	CHOP	F	AAATCGAGCGCCTGACCAGGGA
		R	TTGGCACTGGCGTGATGGTGCT
10	B1	F	ACGCCCTCAGGATCTGCATCGT
		R	AACGTGCCACAGCTCCAAACCC
11	B2	F	TTCACACGCCGAAACCTGGC
		R	TGGCCCCTCATGCTCGTCAT
12	B5	F	TCTGGGAGCGGTTGTTGGCT
		R	GGACAGCCCCATGCCTTTGT
13	Rpn13	F	TGTGAGTTCAAGCGGGTGCC
		R	TGCATCCAGAAGAACAGCCGC
14	p53	F	ACGGGACAGCTTTGAGGTTCGT
		R	AGCTCCGGGCAATGCTCTTCTT
14	β-actin	F	GAGAAGAGCTATGAGCTGCC
		R	CTCAGGAGGAGCAATGATCT

2.4. Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay

To determine apoptosis in cerebral cortex, we performed TUNEL assay using the In Situ Cell Death Detection Kit (Roche Diagnostics GmBH, Mannheim, Germany). The assay was carried out as described by the manufacturer. Briefly, the sections were deparafinized and rehydrated using xylene and ethanol gradings and permeablized using hot 0.1 M citrate buffer pH 6.0 and incubated with the TUNEL reaction mixture containing TdT and fluorescein labeled dUTP for 1 h at 37 ºC. DAPI staining was used as the final step in fluorescent staining procedure to label cell nuclei. The apoptotic cells were analyzed using the fluorescent microscope (Leica laser microscope). For negative control, TdT was not included in the reaction mixture.

2.5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the cerebral cortex of rats using Tri reagent. Isolated RNA was further purified by RNeasy Mini Kit (Qiagen) and quantified by measuring the absorbance at 260 and 280 nm. The quality of RNA preparation was assessed by electrophoresis on a denaturing agarose gel. 4 μg of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription kit. Reverse transcription reaction was carried out with thermocycler (ABI 9700) and reaction conditions were as follows: initial RT for 10 min at 25 °C, followed by 37 °C for 120 min and inactivation of reverse transcriptase at 84 °C for 5 min. Real-time PCR (ABI-7500) was performed in triplicates with 20 ng cDNA templates using SYBR green master mix with gene specific primers (Table 1). The reaction conditions were as follows: 40 cycles of initial denaturation temperature at 95 °C for 30 s followed by annealing at 52 °C for 40 s and extension at 72 °C for 1 min and product specificity was analyzed by melt curve analysis. Relative expression of genes (relative to lean or control) based on the difference in threshold cycles (Cts) between control versus experimental (lean vs WNIN/Ob and control vs high-fat) groups after normalization to internal control (β-actin) in each group. Data were compared between control and experimental samples according to comparative threshold cycle ($2^{-\Delta\Delta ct}$) method and expressed as fold change over control.

2.6. SDS-PAGE and immunoblotting

Tissues were homogenized in a buffer containing 20 mM Tris, 100 mM NaCl, 1 mM EDTA (TNE buffer; pH7.5) 1 mM DTT, 1 mM PMSF, 1 μg/ml each of aprotinin, leupeptin, and pepstatin. Homogenization of tissues was performed on ice using a glass homogenizer and the homogenate was centrifuged at 12,000 × g for 20 min. The supernatant was collected and used for immunoblot analysis. Equal amount of protein from lean and obese tissues was subjected to 12% SDS-PAGE and proteins were transferred onto PVDF membrane. Nonspecific binding was blocked with 5% BLOT-Quick Blocker reagent (Calbiochem) in PBST (20 mM phosphate buffer; pH 7.2, 137 mM NaCl, 0.1% Tween 20) and incubated overnight at 4 °C with anti-UCHL-1 (1:10,000),

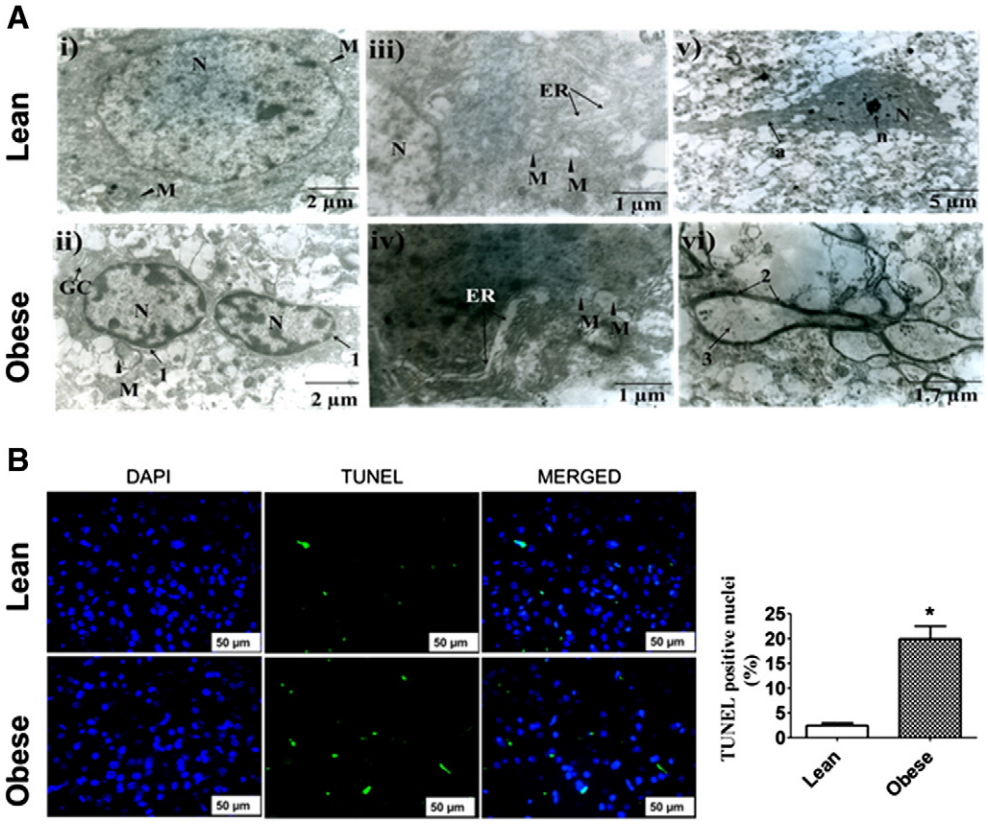

Fig. 1. Ultra structural (TEM) studies and TUNEL assay of neurons in the prefrontal cerebral cortex of 12 month lean and obese rats. Panel A—i) the neurons in lean rats showed intact, normal sized nucleus, mitochondria and Golgi complex. Scale bar 2 μm, original magnification at 9650×; ii) the neurons in obese rats showed dilated nuclear membrane, margination of chromatin, nucleoplasm filled with electron dense material and disappearing ER. The size and shape of the nucleus and mitochondria are altered. Scale 2 μm, original magnification at 93,000×; iii) normal structure of ER is observed in lean rats. Scale 1 μm, original magnification at 19,300×; iv) dilated cisternae of ER and vesicular mitochondria in obese rat. Scale 1 μm, original magnification at 9650×; v) normal neuron showing nucleolus and axon in lean rats. Scale 5 μm, original magnification at 3860×; and vi) degenerating neuron in obese rat with disappeared nucleus, thick neuronal wall, degenerated axon and shrunken cell. Scale 1.7 μm, original magnification at 11,580×. Such neurons were more often found in the cerebral cortex of obese rats. N: nucleus, M: mitochondria, GC: Golgi complex, ER: endoplasmic reticulum, a: axon, n: nucleolus, 1: dilated nuclear membrane, 2: thick neuronal wall, and 3: disappeared nucleus. And panel B—apoptosis was measured by TUNEL assay. Representative fluorescence microscopic images (630× magnification) of the cerebral cortex of lean and obese rats. Bar graph shows % TUNEL-positive cells (data represent mean ± SE from 6 independent experiments; $^{*}p < 0.05$).

anti-UCHL-3 (1:1600), anti-UCHL-5 (1:1000), anti-GRP78 (1:1000), anti-ATF6∝ (1:300), anti-XBP1 (1:300), anti-CHOP (1:200), anti-p53 (1:100), anti-BAX (1:400), anti-BCL2 (1:200), anti-Ub (1:200) and anti-actin (1:500) antibodies diluted in PBS. After washing with PBST, membranes were then incubated with anti-rabbit IgG (1:3500) or anti-mouse IgG (1:3500) secondary antibodies conjugated to HRP. The immunoblots were developed with enhanced chemiluminescence detection reagents (GE Health Care, Buckinghamshire, UK) and digital images were recorded by Image analyzer (G-Box iChemi XR, Syngene, G-box).Images were analyzed and quantitated using image J software (available in the public domain at http://rsbweb.nih.gov/ij/).

2.7. Proteasome activity assay

The enzymatic activity of proteasome in the cerebral cortex was assayed using Biovision Proteasome Activity Assay Kit. The kit takes advantage of the chymotrypsin-like activity, utilizing an AMC-tagged peptide substrate which releases highly fluorescent free AMC in the presence of proteolytic activity. The kit also includes a specific proteasome inhibitor MG-132 which suppresses all proteolytic activity due to proteasomes. This permits differentiation of proteasome activity from other protease activity which may be present in samples.

2.8. Immunohistochemistry

The paraformaldehyde-fixed cerebral cortex of rats was embedded in paraffin, and transverse sections (4 μm) were mounted in silane-coated slides. Immunohistochemical study was performed using Vectastain elite ABC kit (Vector Laboratories, CA 94010, USA) that exploits Avidin Biotin Complex (ABC) method. Deparaffinized sections were processed in 10 mM sodium citrate buffer (pH 6.0) and heated for antigen retrieval. After blocking, the primary antibody (p53 and CHOP with 1:50 dilution) was added to the sections in a volume of 100 μl and allowed to incubate overnight at 4 °C. After incubation, the sections were washed three times for 5 min in PBS, and 100 μl of biotinylated secondary antibody solution was added. The sections were incubated for 1 h at room temperature. DAB (3,3-diaminobenzidine tetrahydrochloride) was used as chromogen. Negative controls were run simultaneously with an omission of primary antibody. After staining, the sections were counterstained with hematoxylin. The sections were then dehydrated through ethanol and xylene before coverslips with Permount.

Immunofluorescence staining was also performed to show BAX and cleaved caspase-3 proteins in paraffin sections. The procedure was similar to that of immunohistochemistry with slight modifications. The sections were incubated with primary antibody against BAX (1:50) and cleaved caspase-3 (1:400). Sections were washed 3 times with TBS and the binding of primary antibodies was visualized by Alexa Fluor-555 conjugated anti-Mouse IgG for BAX and Alexa Fluor-488 conjugated anti-rabbit IgG for cleaved caspase-3. After incubation, the sections were then washed three times for 5 min in PBS and mounted with Vectashield mounting medium containing 4′, 6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain (Vector Laboratories, Burlingame, CA). Fluorescently labeled sections were visualized using a Leica fluorescence microscope (Leica Microsystems, Germany) at 400 and 630×.

2.9. Statistical analysis

Student's *t*-test was used for the statistical analysis between two groups. Values of $p < 0.05$ were considered significant. All data are presented as mean ± S.E.

3. Results

3.1. Ultra structural studies of neurons

TEM analysis of neurons in the prefrontal cerebral cortex of 12-month old obese rats showed distinct signs of neuronal cell abnormalities. The neurons in obese rats showed vacuolated matrix, shrunken cells, dilated nuclear membrane, margination of chromatin, nucleoplasm filled with electron dense material and disappearing ER (Fig. 1A). Further, dilated cisternae of ER and vesicular mitochondria were observed in obese rat neurons while such abnormalities were not found in lean rat. Fig. 1A(vi) show degenerating neuron in obese rat with disappeared nucleus, thick neuronal wall, degenerated axon and shrunken cell when compared to normal healthy neuron in lean brain. Such disintegrating

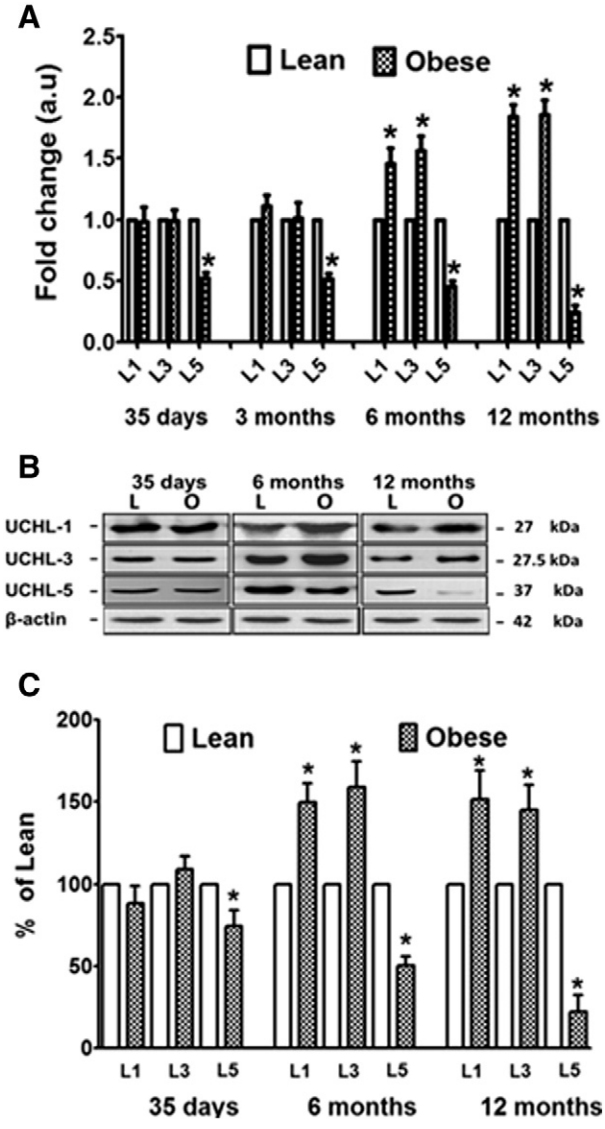

Fig. 2. Differential expression of UCHs in the cerebral cortex of rats at different age points. Panel A—the expression pattern of UCHs in the cerebral cortex of WNIN/Ob and their lean littermates was analyzed by qRT-PCR. Expression values were represented as fold change over control on an arbitrary scale after normalization with actin. Data represent mean ± SEM of three independent experiments. WNIN/Ob rats showed significant up-regulation of UCHL-1 and UCHL-3 genes at 6 and 12 months and down regulation of UCHL-5 at all the four age points when compared with their lean littermates(*$p < 0.05$). Panel B—representative immunoblots of UCHs and panel C—quantification of immunoblots demonstrating the UCH levels in cerebral cortex of WNIN/Ob and their littermates; expression was normalized for actin and are represented as percent of lean littermates. Data represent mean ± SEM of three independent experiments(*$p < 0.05$).

neurons are more often found in the prefrontal cerebral cortex of obese rats.

3.2. TUNEL assay

Consistent with the results of TEM, TUNEL assay indicated only very few TUNEL-positive cells in lean rats (Fig. 1B). In contrast, there was an apparent increase in the number of apoptotic neuronal cells in cerebral cortex of obese rats.

When the ratio of brain weight to body weight was calculated, the obese rats were found to have lowered ratio of brain weight to body weight compared to their lean counterparts (0.1815 ± 0.08 vs 0.317 ± 0.05). There is up to 58% of reduction in their organ-to-body weight ratio in obese rats.

3.3. Differential expression of UCHs

Since the deubiquitinating enzymes (DUBs) play important regulatory role in the UPS, we initially examined differential expression of one class of DUBs; UCHs, in the cerebral cortex of obese rats both at transcript and protein level at different ages (35 days, 3, 6 and 12 months). UCHL-1 and UCHL-3 expression was unaltered at 35 days and 3 months but up-regulated at 6 and 12 months of age in obese rats compared to their respective lean controls (Fig. 2A). However, UCHL-5 expression was down regulated from 35 days onwards in obese rats when compared to their respective lean littermates. Immunodetection data support these observations at protein level (Fig. 2B&C)

3.4. Proteasome activity

Due to down regulation of proteasome associated UCH, UCHL-5; we speculated that the activity of proteasome might be affected and hence we examined chymotrypsin-like activity of proteasome in the cerebral cortex of obese rats at 6 and 12 months of age. The results indicate declined proteasomal activity in the cerebral cortex of obese rats when compared to lean rats at both 6 and 12 months of age (Fig. 3A).

3.5. Proteasomal sub unit expression

The declined proteasomal activity could also be a result of decreased expression of proteasome sub units or other extrinsic factors. To explore this, expression of three genes (B1, B2 and B5) of 20S catalytic subunit and one gene (Rpn13) from 19S regulatory subunit was investigated. B1 subunit is responsible for chymotrypsin-like proteasomal activity, while B2 and B5 are responsible for the trypsin-like, and peptidyl-glutamyl peptide-hydrolyzing activities respectively. qRT-PCR data showed no change in B1, B2, B5 levels whereas there was an up-regulation of Rpn13 in obese rats (Fig. 3B).

3.6. Ubiquitin and its conjugates

Ubiquitin (Ub) as an important component of UPS decides the fate and function of a protein. Immunodetection of Ub in the cerebral cortex of 12 months old obese rats reveals elevated levels of both free Ub and its conjugates in obese rats when compared to respective lean rats (Fig. 3C&D).

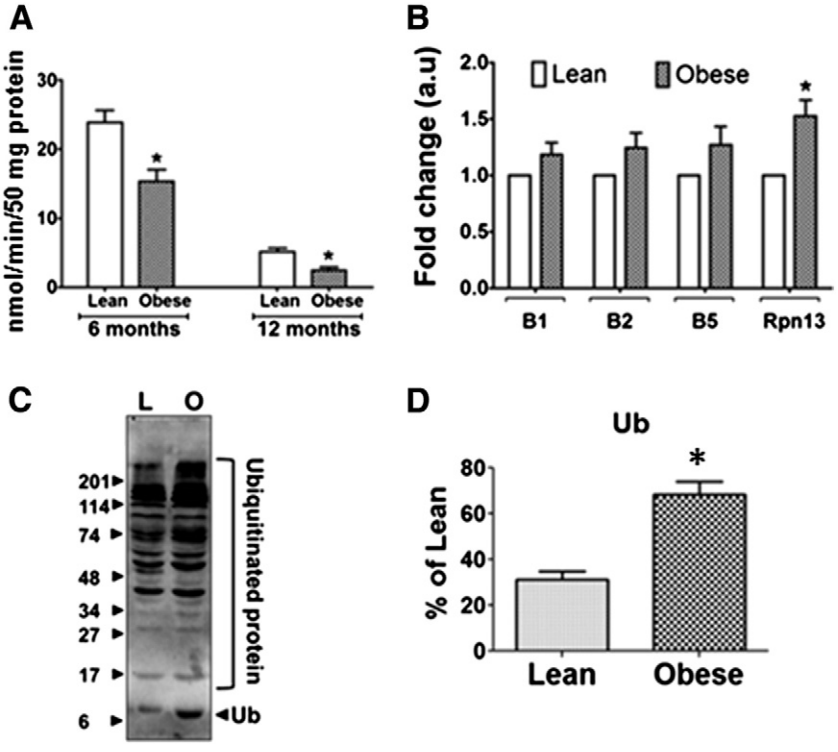

Fig. 3. Panel A—the chymotrypsin-like proteasomal activity in the cerebral cortex of WNIN/Ob and their lean littermates at 6 and 12 months. Data represent mean ± SEM of three independent experiments. The WNIN/Ob rats showed significantly decreased chymotrypsin-like proteasomal activity when compared with their lean littermates ($p < 0.05$). Panel B—the expression pattern of catalytic subunits B1, B2, B5 and a regulatory subunit Rpn13 of proteasome in the cerebral cortex of WNIN/Ob and their lean littermates analyzed by qRT-PCR. Expression values were represented as fold change over lean littermates on an arbitrary scale after normalization with actin. Data represent mean ± SEM of three independent experiments (*$p < 0.05$). Panel C—representative immunoblot of ubiquitin and ubiquitinated proteins and panel D—quantification of immunoblot demonstrating the accumulation of ubiquitin and ubiquitinated protein levels in cerebral cortex of WNIN/Ob. Data represent mean ± SEM of three independent experiments.

3.7. ER stress markers

After observing disturbances in the UPS we decided to examine the status of unfolded protein response (UPR), another important protein quality control system located in the endoplasmic reticulum (ER). We examined gene expression by qRT-PCR of various ER stress markers like GRP78, ATF6, IRE1, XBP1 and CHOP. We found up-regulation of all these five markers at 12 months (Fig. 4B) and except ATF6 and CHOP remaining three markers at 6 months of age (Fig. 4A). Further Western blot for GRP78, total as well as active ATF6∝ (cleaved), and total as well as active XBP1 (spliced) were also performed (Fig. 4C&D). The results clearly showed increased protein levels of GRP78, ATF6∝, XBP1 and CHOP in the cerebral cortex of obese rats when compared to lean rats. The results of Western blot are in accord with real time PCR. So the data clearly indicate the existence of ER stress both at 6 and 12 months of age. We also performed immunohistochemistry besides qRT-PCR for CHOP. CHOP immunoreactivity was visualized in a granular pattern in the nucleus of rat cerebral cortex. Compared to the lean animals, obese animals showed significantly increased immunostaining for CHOP (Fig. 4E).

3.8. Apoptotic and anti-apoptotic markers

Since prolonged ER stress triggers apoptosis, we examined p53 levels by qRT-PCR, Western blot and immunohistochemistry. The results showed significant increase in protein level but not in mRNA level (Fig. 5). Further, we analyzed protein levels of BAX and BCL2, two important mediators that regulate apoptosis by Western blot. Fig. 5C&D reveals increased protein expression of BAX but decreased expression of BCL2 in the cerebral cortex of obese rats. Immunofluorescence analysis for BAX and cleaved caspase-3 was also performed in cerebral cortex sections to confirm the apoptotic cell death. The results showed increased immunostaining of BAX and cleaved fragments of caspase-3 in the cerebral cortex of obese rats when compared to lean rats (Fig. 6).

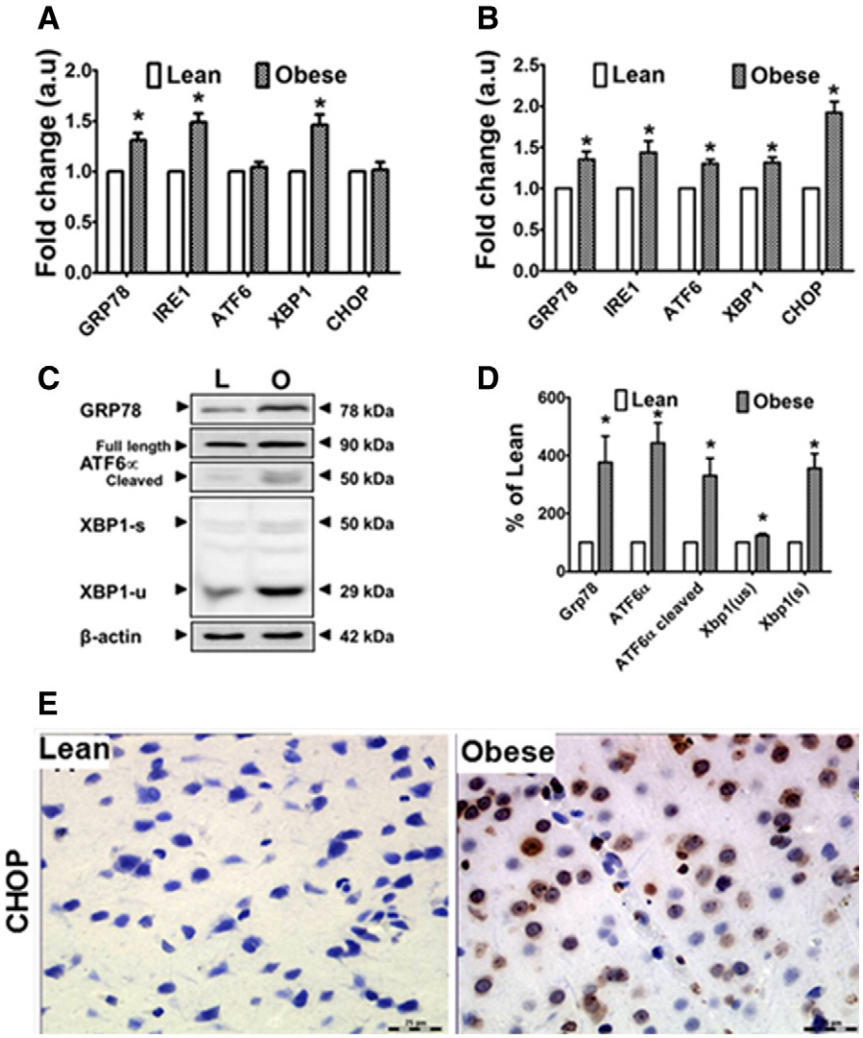

Fig. 4. ER stress markers and CHOP in the cerebral cortex of lean and obese rats. The expression pattern of GRP78, IRE1, ATF6, XBP1 and CHOP at 6 months (panel A) and (panel B) at 12 months in the cerebral cortex of WNIN/Ob and their lean littermates analyzed by qRT-PCR. Expression values were represented as fold change over lean littermates on an arbitrary scale after normalization with actin. Data represent mean ± SEM of three independent experiments. WNIN/Ob rats showed significant upregulation of all ER stress markers and CHOP at 12 months whereas only GRP78, IRE1 and XBP1 at 6 months when compared with their lean littermates ($^*p < 0.05$). Panel C—representative immunoblots of ER stress proteins and panel D—quantification of immunoblots of ER stress protein in cerebral cortex of WNIN/Ob and their littermates at 12-months; expression was normalized for actin and are represented as percent of expression. Data represent mean ± SEM of three independent experiments. GRP78, total ATF6, activated (cleaved) ATF6, total XBP1 as well as activated (spliced) XBP1 protein levels were significantly up-regulated in the cerebral cortex of obese rats when compared with their lean littermates. Panel E—expression of CHOP in cerebral cortex of WNIN/Ob and their lean littermates at 12-months analyzed by immunohistochemical staining. Sections were counterstained with hematoxylin (blue). The results showed increased immunostaining of CHOP in cerebral cortex of obese rats when compared with their lean littermates. All the pictures were taken at 630× magnification. Scale bars, 25 μm.

3.9. Status of UPS, UPR and apoptosis in diet-induced obesity in wild-type rat

To prove further that the neurologic alterations are due to metabolic stress or obesity in WNIN/Ob rat but not because of its genotype, most of the above parameters were analyzed in high-fat diet induced obesity in WNIN rat model. The rats showed increased body weight (540 vs 380 g), and insulin levels (46.33 ± 11.83 vs 24.67 ± 9.07 uU/mL) with unaltered glucose levels, suggesting that these animals have developed IR but not hyperglycemia. Similar to WNIN/Ob rat, we observed up-regulation of UCHL-1 protein, down regulation of UCHL-5, decreased proteasomal activity, activated UPR, accumulation of p53 and BAX (Fig. 7). Further, increased TUNEL positive cells were also observed in obese rat cerebral cortex when compared to control diet fed rats (Fig. 7A) reinforcing the concept of altered UPS may cause neuronal cell death under obese conditions.

4. Discussion

The epidemiological studies suggest that prevalence of overweight and obesity has reached epidemic proportions worldwide [1], and in the past 20 years, the rates of obesity have tripled in developing countries as well [2,3]. Several serious medical conditions are now associated with obesity and it is no longer considered as just a problem of overweight. It is now aptly referred as a metabolic syndrome and is associated with several degenerative diseases, including neurodegenerative conditions. In this study we describe neuronal damage in a spontaneously developed obese rat model, WNIN/Ob rat and provide the molecular basis in the form of altered UPS. This was further substantiated by carrying out studies in a high fat diet rat model.

The status of neurons in the prefrontal cerebral cortex was observed under TEM. The results clearly visualized neurodegeneration in the obese rats. Characteristics of cellular degeneration were witnessed in obese rats. Further, degenerative and shrunken neurons with disappeared nucleus and ER were observed more often in obese rats. In accordance with the TEM results, TUNEL assay indicated more TUNEL-positive neuronal cells in obese rats than in lean rats.

Alterations in the various components of UPS such as UCHs (DUBs), Ub and proteasomal activity in the cerebral cortex of obese rats clearly indicate disturbance in the UPS. This system is in place to degrade not only misfolded and damaged proteins, but is also essential in regulating a host of cell signaling pathways involved in proliferation, adaptation to stress, regulation of cell size, and cell death [18]. UCHs play an important role in regulating the functions of UPS and their involvement in various pathological conditions is well evident. For instance UCHL-1 is associated with many neurodegenerative diseases (Parkinson's disease, Alzheimer's disease, Huntington disease, dementia and retinitis) but its role is largely unknown [19]. In addition to neurodegeneration UCHL-1 is shown to be involved in 15 different cancers so far [20–26]. UCHL-3 is associated with Parkinson's disease, cervical carcinoma, colon cancer, ataxia, breast cancer, cervicitis, retinitis, neuronitis and obesity [27,28]. Proteasome interacting protein UCHL-5 is least studied UCH so far and is found to be associated with cervical carcinoma, hepatocellular carcinoma, cervicitis and malaria [29].

Results of the present study showed up-regulation of UCHL-1 and UCHL-3 in the cerebral cortex of obese rats from 6 months of age, while UCHL-5 is down regulated from 35 days onwards. We observed increased levels of free Ub in the cerebral cortex and this can be due to increased levels of UCHL-1 that generate free monomeric Ub from Ub-proproteins and its activity has been shown to be required for normal synaptic function [30]. Initially UCHL-1 was thought to be neuron specific but later its occurrence is proved in almost all tissues. In the present study, we observed elevated p53 at protein level but not at transcript level in the cerebral cortex of obese rats. Previous studies demonstrated that UCHL-1 could activate the p14ARF-p53 signaling pathway by deubiquitinating p53 and p14ARF as well as ubiquitinating MDM2, which might be through its two opposing enzyme activities, hydrolase and ligase, further resulting in its tumor suppressive role [31]. UCHL-1 induces G0/G1 cell cycle arrest and apoptosis through stabilizing p53 and is identified as a cancer-specific methylated gene, and silenced by promoter methylation in multiple tumors suggesting that the UCHL-1

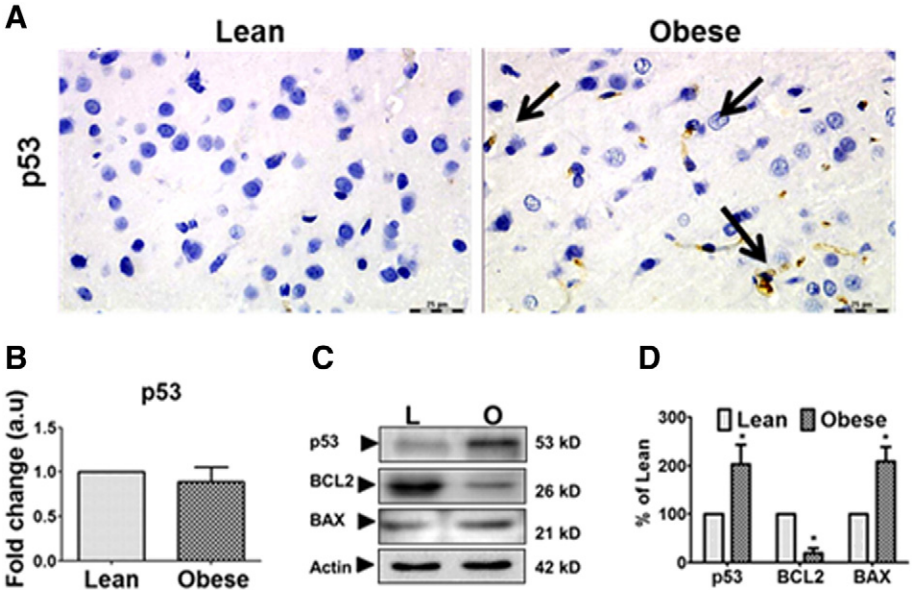

Fig. 5. Apoptotic regulators in the cerebral cortex of 12 month old lean and obese rats. Panel A—expression of p53 in cerebral cortex of WNIN/Ob and their lean littermates analyzed by immunohistochemical staining. Sections were counterstained with hematoxylin (blue). The results showed increased immunostaining of p53 in cerebral cortex of obese rats when compared with their lean littermates. All the pictures were taken at 630× magnification. Scale bars, 25 μm. Panel B—the expression of p53 at 12 months in the cerebral cortex of WNIN-Ob and their lean littermates analyzed by qRT-PCR. Expression values were represented as fold change over lean littermates on an arbitrary scale after normalization with actin. Data represent mean ± SEM of three independent experiments. Panel C—representative immunoblots of p53, BAX, BCL2. Panel D—quantification of immunoblots for p53, BAX and BCL2 in cerebral cortex of 12 month WNIN/Ob and their lean littermates; expression was normalized for actin expression and is represented as percent of lean. Data represent mean ± SEM of three independent experiments. The results showed significant increase in protein levels of p53, BAX and BCL2 with no change in mRNA levels of p53 in obese rats when compared with their lean littermates ($p < 0.05$).

is a functional tumor suppressor and potential tumor marker for cancer [32].

The activated p53 triggers gene expression of BAX along with many other target genes that are involved in DNA repair and cell cycle arrest. BAX forms a homo dimer and releases cytochrome c from the mitochondria, which results in caspase-9 activation that in turn activates the effector caspase-3. In the present study we clearly observed increased levels of BAX, activated caspase-3 and decreased levels of BCL2 protein. Thus our results: increased levels of UCHL-1, p53, BAX and cleaved caspase-3 clearly showing UCHL-1 mediated apoptosis in the cerebral cortex of WNIN/Ob rats leading to neuronal cell death. Therefore, we hypothesize that UCHL-1 mediated apoptotic mechanism for neurodegeneration through stabilizing p53 as p53-mediated neuronal cell death is well established [33].

Although, UCHL-3 is unchanged in several neurodegenerative diseases, photoreceptor cell apoptosis in the retinal degeneration of UCHL-3-deficient mice is reported in one study [34]. Interestingly, we observed retinal degeneration in WNIN/Ob rats [11]. The cellular localization and function of UCHL-3 remain unknown in the brain; however, recent studies have shown its association with obesity. UCHL-3$^{-/-}$mice displayed a reduction of adipose tissue mass and were protected against high-fat diet-induced obesity and insulin resistance [35].

UCHL-5 was down regulated both at gene and protein levels in the cerebral cortex of WNIN/Ob rats from 35 days onwards. The conserved DUB, UCHL-5 is found on proteasome in organisms ranging from fission yeast to humans. Rpn13, also known as adhesion regulating molecule 1 (ADRM1), was recently identified as a novel 19S proteasome cap-associated protein, which recruits UCHL-5 to the 26S proteasome [36]. We observed up-regulaton of Rpn13 in the cerebral cortex; this might be due to compensatory effect to decreased levels of UCHL-5. Deubiquitination by UCHL-5 is activated by proteasomal binding, which enables UCHL-5 to process polyubiquitin chains. Overproduction of Rpn13 in human embryonic kidney 293T cells increased the cellular levels of ubiquitin conjugates and decreased the degradation of short-lived proteins. Furthermore, transfection of the C-terminal half of Rpn13 slows proteolysis and induces cell death, probably by acting as a dominant-negative form [37]. So the decreased UCHL-5 and increased Rpn13 levels could be the reason for decreased proteasome activity and increased ubiquitin conjugates observed in the cerebral cortex of obese rats.

The UPR is characterized by the induction of chaperones, degradation of misfolded proteins and attenuation of protein translation by the help of ER stress sensors. Three specialized ER stress-sensing proteins involved in the canonical mammalian UPR pathway: protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1α (IRE1α) and activating transcription factor 6 (ATF6) are found to be up-regulated in the cerebral cortex of 12 months old WNIN/Ob rats. We further showed up-regulation of spliced (activated) form of XBP1, cleaved (activated) ATF6$\propto$ and major ER chaperone-Bip/GRP78 at protein level by immunoblotting, strongly suggests existence

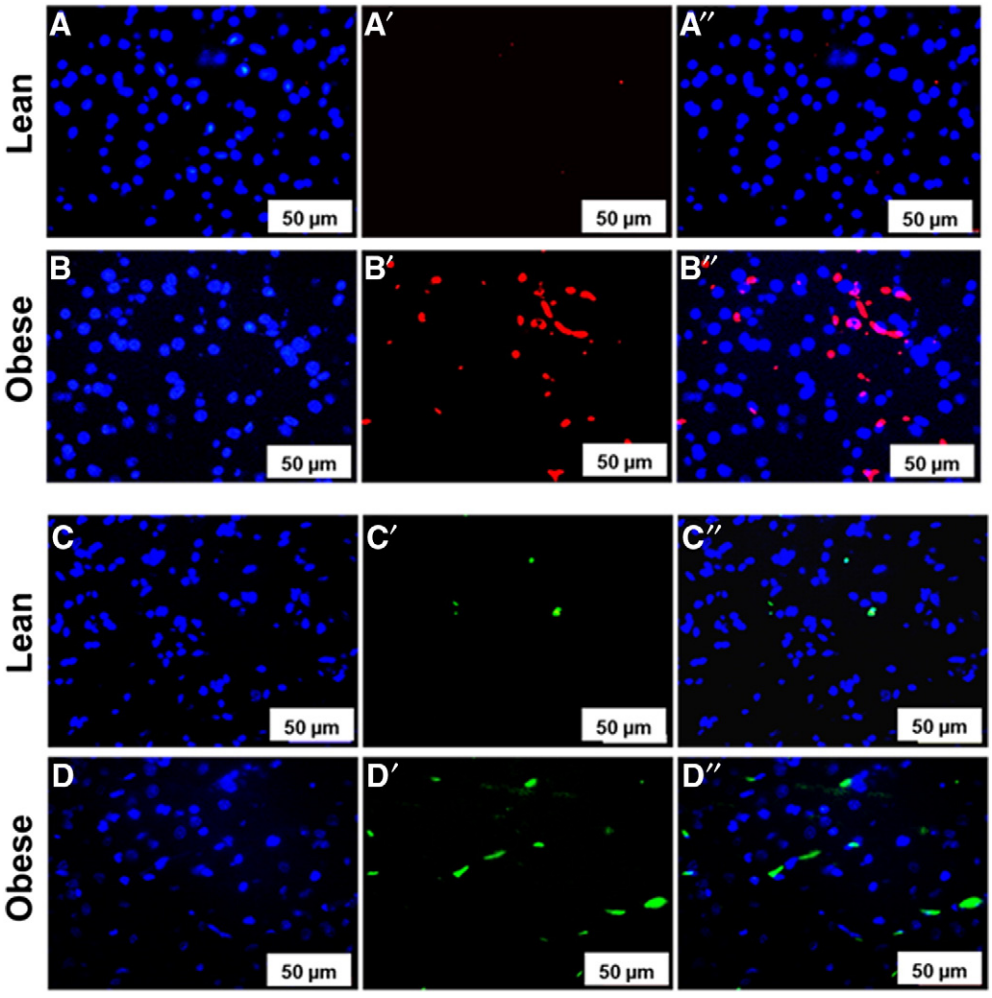

Fig. 6. Immunofluorescence of BAX and cleaved caspase 3 in the cerebral cortex of 12 month old lean and obese rats. Paraffin tissue sections of cerebral cortex from 12 month lean (A, C) and obese rats (B, D) were labeled with antibodies to BAX (red; A, B) and cleaved caspase-3 (green; C, D). Sections were counterstained with nuclei stain DAPI (blue).All the pictures were taken at 630× magnification. Scale bars, 50 μm. The results suggest increased cell death as examined by increased immunostaining of BAX and cleaved caspase-3 in 12 month obese rats when compared with their lean littermates.

of ER stress in the cerebral cortex of these rats. All these ER stress markers except ATF6∝ and CHOP were up-regulated in the cerebral cortex even at 6 months of age which clearly indicates that UPR remained triggered for prolonged period. Studies have suggested that initial participation of the UPR in neurodegenerative disorders is probably cytoprotective. However, when activation of the UPR is sustained over an extended period of time, apoptotic pathways are up-regulated [38]. This is evident in obese rats as there was up-regulation of CHOP,

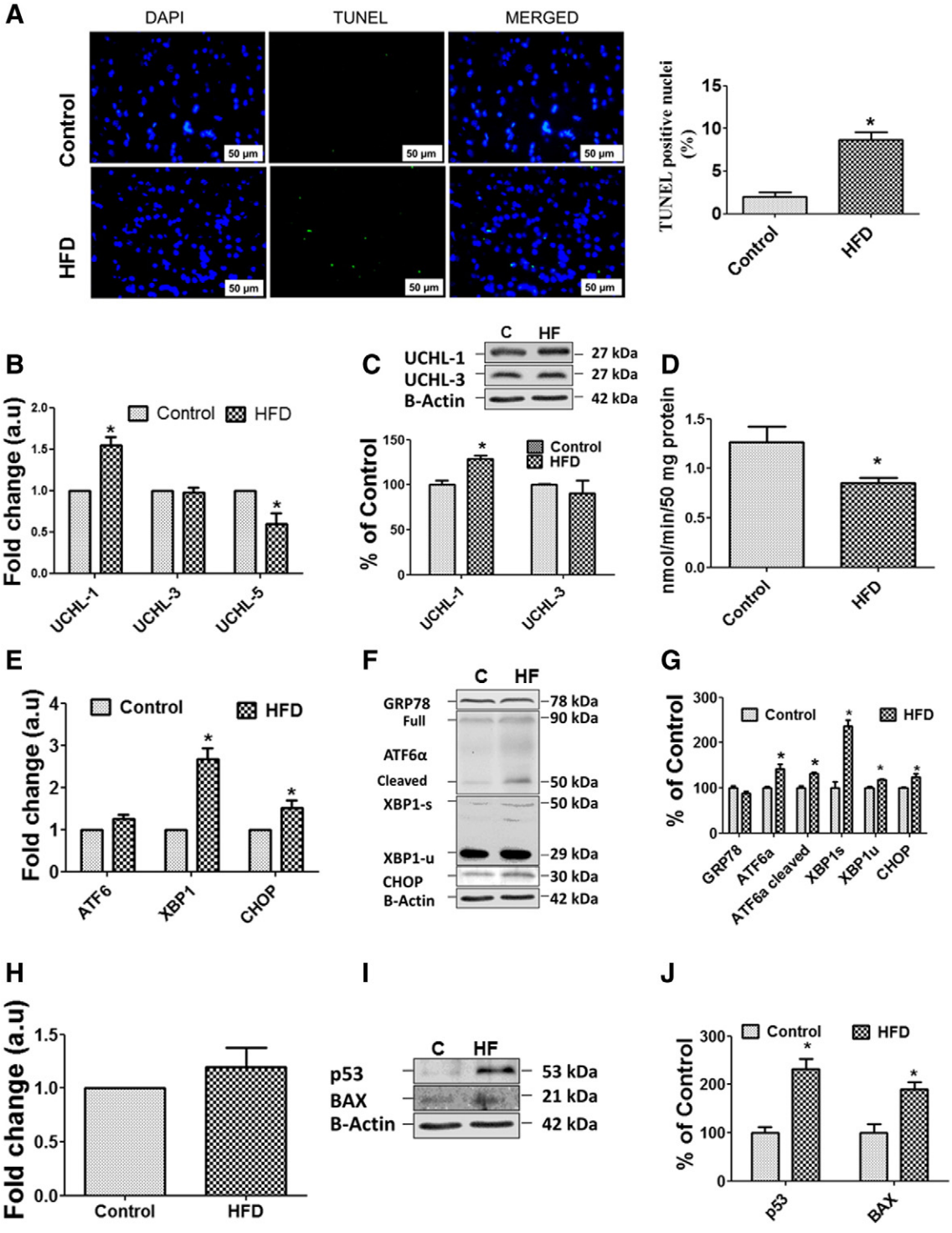

Fig. 7. Status of UPS, UPR and apoptosis in the cerebral cortex of diet induced obese rat. Panel A—apoptosis was measured by TUNEL assay. Representative fluorescence microscopic images (630× magnification) of the cerebral cortex of control and obese rats. Bar graph shows % TUNEL-positive cells (mean ± SE; n = 5; $^*p < 0.05$). Panel B—the expression pattern of UCHs in the cerebral cortex of control and obese rats was analyzed by qRT-PCR. Expression values were represented as fold change over control on an arbitrary scale after normalization with actin. Data represent mean ± SEM of three independent experiments. Panel C—representative immunoblots of UCHs and quantification in cerebral cortex; expression was normalized for actin and are represented as percent of control. Data represent mean ± SEM of three independent experiments ($^*p < 0.05$). Panel D—the chymotrypsin-like proteasomal activity in the cerebral cortex. Data represent mean ± SEM of three independent experiments. Panel E—the expression pattern of ATF6, XBP1 and CHOP analyzed by qRT-PCR. Panel F—representative immunoblots of ER stress markers and panel G—quantification of immunoblots of ER stress markers. Panel H—the expression of p53 was analyzed by qRT-PCR. Panel I—representative immunoblots of p53 and BAX and panel J—quantification of immunoblots for p53 and BAX.

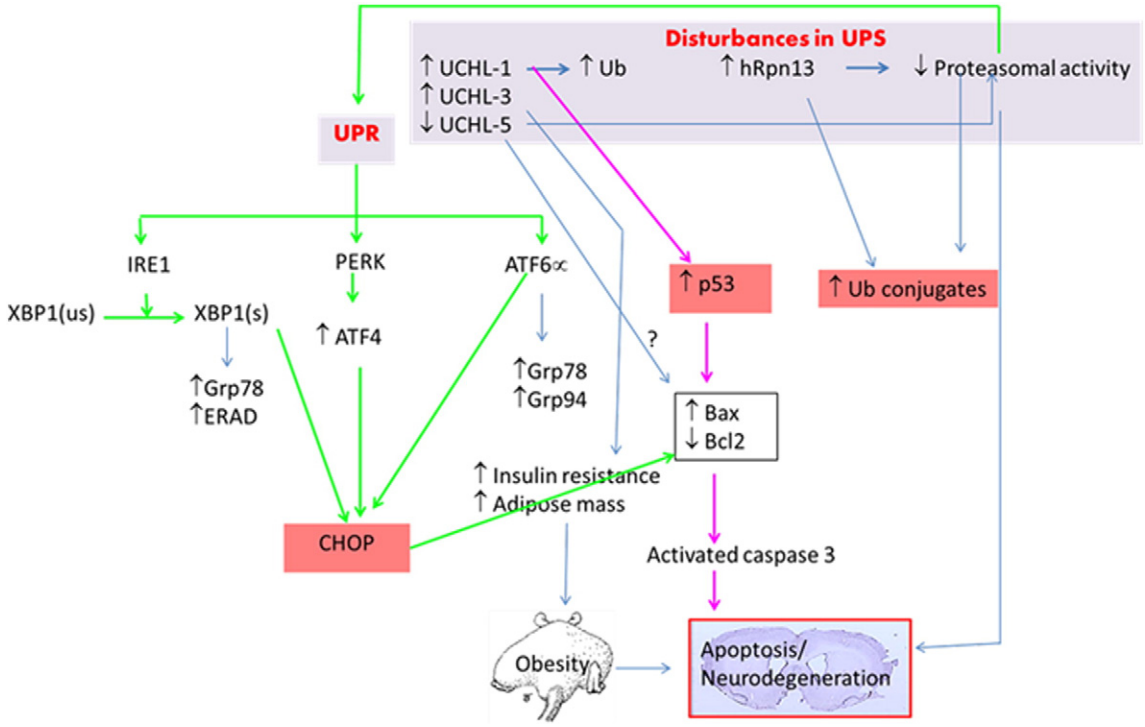

Fig. 8. Proposed scheme depicting altered UPS in WNIN/Ob rats leads to neuronal cell death by two independent pathways. These two pathways: UCHL-1 mediated apoptosis through stabilizing p53 (pink arrows) and activation of ER stress through decreased proteasomal activity (green arrows) are connected to apoptosis/neuronal cell death by activation of Bax.

both at transcript and protein levels. Apoptosis in response to ER stress is mediated largely by CHOP which is downstream of the PERK, XBP1 and ATF6 pathways [39].

The importance of proteasomal activity to neuronal homeostasis is highlighted by previous studies demonstrating that proteasome inhibition alone is sufficient to induce neuron death in vitro [40]. In the present study we observed a significant decline in the proteasomal chymotrypsin-like activity in the cerebral cortex of obese rats when compared to their respective lean rats both at 6 and 12 months of age. Increased proteasome-mediated degradation of un/misfolded proteins merges with the UPR as an adaptive ER homeostatic mechanism [41–43], so that deteriorated proteasomal activity observed in WNIN/Ob rats may potentially lead to ER stress due to lack of removal of damaged proteins. Very recently, Otoda et al. showed that decreased proteasomal activity leads to ER stress in the liver of obese rat [44]. To substantiate that the neurologic alterations are associated with obesity but not just the WNIN/Ob genotype we conducted studies in diet-induced obese rat model and observed similar changes in the high fat diet induced obese rat model. Although, there was a similar trend with regard to the alterations in UPS in high-fat fed rats, degree of changes, particularly TUNEL positive cells is not the same with that of WNIN/Ob rats. This indicates that the contribution of genotype to altered UPS in WNIN/Ob cannot be ruled out.

Based on all these evidences it can be concluded that neuronal cell death in WNIN/Ob rat is due to the unbalanced ubiquitin proteasome system as summarized in Fig. 8. The up-regulated UCHL-1 may stabilize p53 which drives apoptosis through BAX and caspase-3. The decreased proteasomal activity fails to clear the misfolded and unfolded proteins targeted by ER for degradation and thereby eliciting ER stress. Persistent ER stress induces apoptosis through CHOP. Hence, together the results obtained in two different obese rat models strongly suggests that altered UPS leads to neuronal cell death probably in two independent ways: (i) UCHL-1 mediated apoptosis through stabilizing p53 and (ii) through triggering of ER stress. This is the first report of its kind that provides a role for UPS in neuronal damage or cell death in obesity. However, further studies are warranted to establish conclusively the involvement of altered UPS in neuronal damage under metabolic syndrome conditions.

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