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Tetrahydrocurcumin ameliorates homocysteinylated cytochrome-C mediated autophagy in hyperhomocysteinemia mice after cerebral ischemia

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Abstract

High levels of homocysteine (Hcy) known as hyperhomocysteinemia (HHcy), contribute to autophagy and ischemia/reperfusion injury (I/R). Previous studies have shown that I/R injury and HHcy cause increased cerebrovascular permeability; however, the associated mechanism remains obscure. Interestingly, during HHcy, cytochrome-c becomes homocysteinylated (Hcy-cyto-c). Cytochrome-c (cyto-c) transports electrons and facilitates bioenergetics in the system. However, its role in autophagy during ischemia/reperfusion injury is unclear. Tetrahydrocurcumin (THC) is a major herbal antioxidant and anti-inflammatory agent. Therefore, the objective of this study was to determine whether THC ameliorates autophagy during ischemia/reperfusion injury by reducing homocysteinylation of cyto-c in hyperhomocysteinemia pathological condition. To test this hypothesis we employed 8–10 weeks old male cystathionine-beta-synthase heterozygote knockout (CBS^{+/-}) mice (genetically hyperhomocysteinemic mice). Experimental group was: CBS^{+/-}, CBS^{+/-}+THC (25mg/kg in 0.1%DMSO dose); CBS (+/-)/I/R and CBS (+/-)/I/R+THC(25mg/kg in 0.1%DMSO dose). Ischemia was performed for 30 min and reperfusion for 72 hours. THC was injected intra-peritoneally (I.P.) once daily for a period of 3 days after 30 of ischemia. The infarct area was measured using 2,3,5-triphenyltetrazolium chloride staining. Permeability was determined by brain edema and Evans Blue extravasation. The brain tissues were analyzed for oxidative stress, matrix metalloproteinase-9 (MMP-9), damage-regulated autophagy modulator (DRAM), and microtubule-associated protein 1 light chain 3 (LC3) by Western blot. The mRNA levels of S-Adenosyl-L-homocysteine hydrolases (SAHH) and Methylenetetrahydrofolate reductase (MTHFR) genes were measured by Quantitative real-time polymerase chain reaction. Co-immunoprecipitation was used to determine the homocysteinylation of cyto-c. We found that brain edema and Evans Blue leakage were reduced in I/R+THC treated groups as compared to sham operated groups along with reduced brain infarct size. THC also decreased oxidative damage and ameliorated the homocysteinylation of cyto-c in-part by MMP-9 activation which leads to autophagy in I/R groups as compared to sham operated groups. This study suggests a potential therapeutic role of dietary THC in cerebral ischemia.

Keywords

Stroke; Blood Brain Barrier; Redox Stress; Methionine; Curcumin

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INTRODUCTION

Cerebral stroke is a major cause of morbidity and mortality worldwide. Many studies indicate a plethora of risk factors for cerebral stroke (i.e., hypertension, obesity, smoking, and diabetes mellitus) (Manolescu et al., 2010). Nevertheless, cerebrovascular events can occur sometimes in individuals without any of the previously mentioned risk factors. Homocysteine is a thiol-containing amino acid formed during the methionine metabolism via methylation to homocysteine which requires S-Adenosyl-L-homocysteine hydrolases (SAHH), remethylation to methionine, which requires methylenetetrahydrofolate reductase (MTHFR); and transsulfuration to cysteine which requires cystathionine beta synthase (CBS) enzymes (Obeid and Herrmann, 2006). Severe hyperhomocysteinemia (HHcy), which results in abnormally high plasma homocysteine concentration, is usually caused by cystathionine beta synthase (CBS) deficiency (Selhub, 1999; Watanabe et al., 1995). HHcy is a common risk factor for stroke, vascular and possibly neurodegenerative diseases (Beard, Jr. and Bearden, 2011; Kraus et al., 1999).

HHcy causes increased oxidative stress by differential expressions of oxidant and anti-oxidant enzymes (Tyagi et al., 2005b). Oxidative stress has been suggested to be involved in brain edema and post-ischemic neuronal damage (Abe et al., 1988). Ischemia rapidly consumes endogenous antioxidants and produces excessive amounts of toxic free radicals (Chen et al., 2011). The accumulation of these toxic free radicals plays an essential role in blood brain barrier (BBB) disruption through matrix metalloproteinases (MMPs) activation (Gasche et al., 1999; Romanic et al., 1998). MMPs are membrane-bound, zinc-binding proteolytic enzymes essential for the breakdown of extracellular matrix (ECM) components of the basement membrane around cerebral blood vessels and neurons. MMPs are synthesized as pre-proenzymes, secreted from cells as proenzymes, and activated by other proteases and free radicals in the extracellular compartment (Lee et al., 2005). Among these MMPs, MMP-2 and MMP-9 are key enzymes (Romanic et al., 1998). Several reports have suggested that MMP-9 plays a significant role in brain injury after cerebral ischemia (Fujimura et al., 1999; Lee et al., 2004; Rosenberg et al., 1998). Pharmacological inhibition of MMP-9 as well as targeted deletion of the MMP-9 gene in mice resulted in substantial reductions of brain damage after ischemia (Asahi et al., 2000; Wang et al., 2000).

Jakubowski demonstrated that Hcy-induced vascular damage could be related to modification of proteins by a metabolite of Hcy, Hcy-thiolactone (N-homocysteinylation) (Jakubowski, 2007; Jakubowski, 2008). Indeed N-homocysteinylation is elevated in CBS deficient patients and is more toxic than Hcy itself (Perla-Kajan et al., 2008). N-homocysteinylation impairs protein function and leads to cell and tissue damage. In vitro studies have demonstrated homocysteinylation of cyto-c. Cyto-c is a mitochondrial peripheral membrane protein and is essential for mitochondrial function. Cyto-c is very susceptible to the modification by N-homocysteinylation (Perla-Kajan et al., 2007; Zhao et al., 2010). The role of N-Hcy-Cyto-c in autophagy during genetic HHcy is not clear.

Autophagy is a regulated process of degradation and recycling of cellular constituents (Rami et al., 2008). Autophagy may help to promote cell survival (Rami et al., 2008) or may also promote cell death (Reggiori and Klionsky, 2002). There were evidences that autophagy increases in neurons in the periphery of the ischemia area after 6–24h post injury (Rami et al., 2008). In contrast post-ischemic injections of the autophagy inhibitor 3-methyladenine block the ischemia-induced increase in LC3-II and DRAM (autophagy marker). These results point out the need to develop autophagy inhibitors that could be more suitable than 3-methyladenine (3-MA, an autophagy inhibitor) for therapeutic purpose (Rami et al., 2008).

Tetrahydrocurcumin (THC) is one of the major metabolites of curcumin with potent antioxidant and anti-inflammatory activity (Pan et al., 1999). Curcumin (diferuloyl methane) is a polyphenol compound, extracted from the rhizomes of the *Curcuma longa* plant (turmeric). Structurally, THC and curcumin are similar except THC lacks the double bonds of curcumin (Okada et al., 2001). However, recent attention has been focused on THC because it appears to exert a greater antioxidant activity in both *in-vitro* and *in-vivo* systems (Okada et al., 2001; Pari and Murugan, 2004). Unlike curcumin, THC is stable in all physiological buffers. In recent years, curcumin has been shown to be neuro-protective and also lower blood glucose levels in type 2 diabetes mice (Nishiyama et al., 2005). However, the effect of THC on various aspects of HHcy has not been investigated. Thus we explore in the present study the effect of THC on homocysteinylated cyto-c mediated autophagy in the well characterized genetic model of severe HHcy in mice after cerebral ischemia.

EXPERIMENTAL PROCEDURES

Mice

All experimental procedures were approved and carried out in accordance with the Institutional Animal Care and Use Committee of the University of Louisville. 8–10 weeks old CBS heterozygous knockout mice (CBS+/-) were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility center at University of Louisville. The mice were fed with regular mice chow PMI®LabDiet®St Louis, MO (Cat # 5015) and water ad libitum. Mice genotypes were determined by a polymerase chain reaction of DNA obtained from tail biopsies with a specific set of primers (Kumar et al., 2008).

Focal Cerebral Ischemia

Animals weighing 22–25 g were anesthetized with sodium pentobarbital (70 mg/kg bd wt). Body temperature was controlled using a rectal temperature probe and maintained at 37°C ±0.5°C by a thermostat-controlled heated blanket. Ischemia was induced by occlusion of the middle cerebral artery (MCAO), using a modified intraluminal technique. MCAO was performed with a silicone resin-coated 6-0 nylon monofilament (Ethicon, Titusville, N.J) which was introduced into a small incision of the left common carotid artery and advanced distal to the carotid bifurcation for temporary occlusion of the middle cerebral artery (30 min). Regional cerebral blood flow (CBF) was continuously monitored using a laser doppler probe (Moor Instruments, moor FLPI) to verify ischemia and reperfusion. The occlusion was established when the CBF dropped below 20% of the pre-MCAO flow-value. After 30 minutes of middle cerebral artery occlusion, blood flow was restored by withdrawal of the monofilament. Sham-operated mice were subjected to the same anesthesia and surgical procedure, except MCAO (Longa et al., 1989).

Administration of THC

THC used in the study was purchased from Sabinsa Corporation, USA. (1) CBS+/- heterozygous knockout mice with vehicle treatment; (2) CBS+/- heterozygous knockout mice with THC treatment; (3) CBS+/- heterozygous knockout mice with ischemia/reperfusion injury (I/R); (4) CBS+/- heterozygous knockout mice with ischemia/reperfusion injury with THC treatment. THC was dissolved in 0.1% dimethyl sulfoxide (DMSO; Sigma, USA). 30 mins after MCAO, THC (25mg/kg/day) or vehicle was given for 3 days by intraperitoneal injection.

Assessment of Neurological functional

After recovery from anesthesia and again after 72hrs, neurological function was assessed according to the method of Longa et al. (Longa et al., 1989). Neurological findings were

scored on a 5-point scale. No neurological deficit = 0, failure to extend right paw fully = 1, circling to right = 2, falling to right = 3, did not walk spontaneously and has depressed levels of consciousness = 4.

Determination of infarct volume

Animals were sacrificed 72hrs after MCAO. Brains were quickly removed and frozen at – 20 °C for 10 min. Coronal sections were cut into 2 mm slice using a mouse brain slice matrix (Harvard Apparatus, Holliston, MA, USA). The slices were stained for 30 min at 37°C with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, Taufkirchen, Germany) in PBS to visualize the infarctions (Bederson et al., 1986; Kleinschnitz et al., 2010).

Measurement of Brain water content

The water content of the brain tissue was measured by the wet and dry weight method as described previously (Fukui et al., 2003). Briefly, Brain tissue samples from CBS+/- + vehicle, CBS+/-+THC, CBS+/- + I/R and CBS+/- I/R+ THC mice were immediately weighed to obtain wet weight (*WW*). The tissue was then dried in an oven at 95°C for 72 hrs and weighed again to obtain the dry weight (*DW*). The formula $(WW - DW) / WW \times 100\%$ was used to calculate the water content and expressed as a percentage of wet weight.

Evaluation of BBB Integrity

We investigated the integrity of the BBB in mice by measuring the extravasation of Evans blue (Belayev et al., 1996; Uyama et al., 1988). Evans blue dye (2%, 4 mL/kg body weight) was slowly administered via carotid artery and allowed to circulate for 3 hrs in mice with or without THC treatment. At the end of the experiment, the mice were perfused with saline to wash away any remaining dye in the blood vessels. The dissected brain were weighed and extracted in 1.0ml of 50% trichloroacetic acid solution. After homogenization and centrifugation, the extract was diluted with ethanol (1:3), and its fluorescence was determined at 620 nm (excitation) and 680nm (emission) with a spectrophotometer (Spectra Max 3000, CA, USA). The amount of Evans blue was expressed as was expressed as nm/ ischemic hemisphere by using a standardized curve.

In Situ Zymography

In situ zymography gelatinolytic activity was assessed using a commercially available kit (Molecular Probes) as described previously (Gasche et al., 2001) with some modification. After 30 min of ischemia or 30 min of ischemia followed by 72hrs of reperfusion, the animals were sacrificed, and the brains were frozen in 2-methylbutane with liquid nitrogen. Frozen brain were sectioned with a cryostat to a thickness of 30µm and incubated for 3 hours at 37°C with 400µg/mL DQ gelatin conjugate (Molecular probe), a fluorogenic substrate. After 3hr washed with PBS and fixed in 4% paraformaldehyde in PBS. DQ gelatin cleaved by MMPs resulted in a green fluorescent product (excitation, 495nm; emission, 515nm).

Western Blot Analysis

Change in protein content during ischemia was assessed by Western blot analysis (Tyagi et al., 2010). Briefly, frozen brain tissue washed twice with ice-cold PBS and lysed with ice-cold RIPA buffer (containing 5mM of ethylenediaminetetraacetic acid), which was supplemented with phenylmethylsulfonyl fluoride (1 mM) and protease inhibitor cocktail (1ml/ml of lysis buffer). Protein content of the lysate was determined using the Bicinchronic Acid protein assay kit (BCA, Pierce, Rockford, IL). Equal amounts of protein (60ug) were resolved on 10–15% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane

(PVDF, Bio-Rad) as described (Qipshidze et al., 2010). The membranes were blocked with 5% non-fat dry milk in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 7.4), incubated with respective primary antibodies for 3 hrs at room temperature. After probing with appropriate secondary antibodies for 1.5 hrs at room temperature the blots were analyzed with Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD) as described earlier (Qipshidze et al., 2010). Membranes were stripped and re-probed for GAPDH as a loading control. The protein expression intensity was assessed by integrated optical density (IOD) of the area of the band in the lane profile. To account for possible differences in the protein load, measurements presented are the IOD of each band under study (protein of interest) divided by the IOD of the respective GAPDH band.

Confocal Microscopy

Hcy level in brain tissue during ischemia was assessed by immunohistochemistry analysis as described earlier (Qipshidze et al., 2010). Samples of brain tissue from all experimental groups were immediately placed onto freezing media and stored at -70°C until they were used. Briefly, sections were post-fixed in 4% of paraformaldehyde, and labeled with Hcy antibody (Abcam Antibodies, Cambridge, MA). After an overnight incubation, sections were washed with PBS and incubated with appropriate secondary antibody conjugated with Texas Red. After washing, sections were mounted with FluoroGel mounting medium (GeneTex, Inc) and visualized with confocal microscopy (Olympus, FluoView 1000, objective 60x). DAPI was used for nuclei staining. Total fluorescence (red) intensity in 5 random fields (for each experiment) was measured with image analysis software (Image-Pro Plus, Media Cybernetics) and expressed in fluorescence intensity units (FIU). The fluorescence intensity values were averaged for each experimental group.

Reverse transcription-polymerase chain reaction

The RT-PCR was performed for mRNA expression of SAHH, MTHFR and GAPDH in all experimental group using ImProm-IITM Reverse Transcription system kit (Promega Corporation, Madison, WI, USA, cat # A3800) as described elsewhere (Mishra et al., 2010). For gene amplification the RT-PCR program was $95^{\circ}\text{C}-7.00\text{ min}$ [$95^{\circ}\text{C}-0.50\text{ min}$, $55^{\circ}\text{C}-1.00\text{ min}$, $72^{\circ}\text{C}-1.00\text{ min}$] $\times 34$, $72^{\circ}\text{C}-5.00\text{ min}$, $4^{\circ}\text{C}-\infty$. The primers for RT-PCR are: MTHFR- Forward $5'-\text{CTACCAGAGCCCAAGACAGC}-3'$; reverse $5'-\text{ATTGCAGTTGCTCCTTGTCC SAHH-Forward } 5'-\text{CGATTCTGTCACCAAGAGCA}-3'$; reverse $5'-\text{TGAAGGAGTTGCTCATCAGC}-3'$; GAPDH Forward $5'-\text{TGTTGCTGTAGCCGTATTCA}-3'$; reverse $5'-\text{CAACAGCAACTCCCCTCTT}-3'$. The RT-PCR product was electrophoresed on 1% agarose gel in TAE with 0.008% ethidium bromide. All of the data were analyzed using GAPDH as loading control.

Quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) for SAHH and MTHFR genes was performed using an Mx3000p QPCR method as described previously (Tyagi N. et. al.2010). The first-strand cDNA reaction (0.5 μl) was subjected to real-time PCR amplification using gene-specific primers. The sequences of the primers used are as follows: SAHH: $5'-\text{ACTGAGAAGCAGGCCAGTA}-3'$ (forward) and $5'-\text{CACTGTCCAGGCTACTGCAA}-3'$ (reverse) and MTHFR: $5'-\text{CCATCCTCAGACCCTGTTGT}-3'$ (forward) and $5'-\text{CGTCCACGATGTGGTAGTTG}-3'$ (reverse); GAPDH $5'-\text{CAACAGCAACTCCCCTCTT}-3'$ (forward) and $5'-\text{TGTTGCTGTAGCCGTATTCA}-3'$ (reverse). Real-time PCR assays were performed in 25- μl reactions, consisting of $2 \times (12.5 \mu\text{l})$ Brilliant SYBR Green QPCR Master Mix (Stratagene), 400 nmol/L primers (0.5 μl each from the stock), 11 μl of water, and 0.5 μl of template. The thermal conditions consisted of an initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing, and extension at 56°C for

1 min, and, for a final step, a melting curve of 95°C for 15 s, 56°C for 15 s, and 95°C for 15 s. All reactions were performed in triplicate to reduce variation. Data normalization was accomplished using GAPDH as an endogenous control, and the normalized values were subjected to a $2^{-\Delta\Delta C_t}$ formula to calculate the fold change between the control and experimental groups.

Co-immunoprecipitation

Briefly, tissue extract from all groups (150mg protein) were pre-incubated for 1 h with 40 μ l of Protein A–Sepharose beads at 4°C. Samples were centrifuged for 2 min at 1500 g, and the supernatants were further incubated with the rabbit anti-cyto-c antibody (Abcam Antibodies, Cambridge, MA) (Damdimopoulos et al., 2002). Protein A–Sepharose (40 μ l) was added to each sample and the mixture was incubated for 3 h, at 4 °C. Protein A–Sepharose was pelleted by centrifugation at 1500g for 2 min and washed three times in PBS. The samples were boiled in SDS/PAGE loading buffer, in the presence of 5% (v/v) 2-mercaptoethanol, for 4 min and centrifuged at 1500g for 2 min. The resulting supernatants were subjected to SDS/PAGE (12% gel), and Western blotting was performed with specific antibodies against cyto-c and homocysteine (rabbit polyclonal). Membranes were re-probed with antibody against the GAPDH.

Statistical Analysis

Results are expressed as mean \pm SD. Statistical analysis used Student *t* test or ANOVA to compare quantitative data with normal distribution and equal variance. A *p* value <0.05 was considered statistically significant unless otherwise specified.

RESULTS

Effect of THC on cerebral infarction and neuroscore

To determine the neuroprotective effect of THC against ischemia/reperfusion insult, we measured the Infarct volume and neuroscore with or without THC administration. The infarct volume and neuroscore were significantly increased in CBS+/- ischemic group as compared to sham-operated group (CBS+/-) (Fig.1A, B). While the infarct volume and neuroscore were significantly decreased in THC treated mice as compared to CBS+/- ischemic mice (Fig.1A, B). As shown in Fig. 1A, a remarkably decreased pale-colored region was observed in the THC-treated mice brain as compared with CBS+/- ischemia/reperfusion. These observations indicate that THC can prevent the ischemia/reperfusion induced brain injury.

Effect of THC on blood brain barrier integrity after focal cerebral ischemia

Increased vascular permeability and disruption of the blood brain barrier (BBB) could be initiating factors for the development of cerebral injury and critical factors for the determination of post-ischemic severity. To elicit the effect of THC administration on BBB permeability, we quantified the extravasation of Evans blue in the brain as an indicator of BBB breakdown. In the sham operated group (CBS+/-), the content of Evans blue was unchanged (Fig. 2A). The levels of Evans blue were significantly increased in CBS+/- ischemic group as compared to sham-operated group (CBS+/-) (Figs. 2A). While the extravasation of Evans blue were significantly decreased in THC treated mice as compared to CBS+/- ischemic mice (Fig.2A). Increased vascular permeability results in edema formation, which is a major complication in strokes. Brain water content, as the index of cerebral edema, was higher in the CBS+/- ischemic group as compared to sham-operated group (CBS+/-). Administration of THC produced a significant decrease in post-ischemic cerebral edema (Fig. 2B) as compared to CBS+/- ischemic group. These results indicate that

THC could improve post-ischemic brain injury by decreasing the vascular permeability and edema.

Effect of THC on Homocysteine Metabolism

Because stress decreases MTHFR and increases SAHH echelons thus causes an increase in total Hcy levels in tissue. We measured mRNA expression of Hcy metabolic enzymes MTHFR and SAHH. Induction of ischemia significantly decreases the expression of MTHFR whereas SAHH expression was increased in CBS+/- ischemic group as compared to sham-operated group (CBS+/-) (Fig.3A, B,C,D). Supplementation with THC restored expression of SAHH and MTHFR in ischemic brain (Fig. 3A, B,C,D). Impairment in Hcy metabolism causes an increased in total Hcy levels. To assess the effect of THC on total Hcy levels during cerebral ischemia, we evaluated total Hcy levels in brain tissue by immunohistochemistry in all the groups. Total Hcy levels were robustly increased in ischemia groups as compared to sham operator groups (Fig.3E, F). The THC treatment significantly reduced total Hcy level in CBS+/- I/R+THC group as compared to CBS+/- I/R groups. These results suggest the inhibitory action of THC on Hcy levels (Fig.3).

Effect of THC on Oxidative Stress

To test whether elevated levels of homocysteine caused oxidative stress during ischemia/reperfusion injury. We measured protein expression of p47phox (oxidant enzyme) and catalase (antioxidant enzyme). Protein level of p47phox was significantly up-regulated in CBS+/- ischemic group as compared to the sham operator groups (CBS+/-) whereas protein level of catalase was significantly down-regulated in CBS+/- ischemic group as compared to sham operator groups (Figs.4A, B). Treatment with THC mitigates these effects. These results suggested that during brain injury, increased total Hcy level caused elevation in oxidative stress and THC treatment attenuated these effects (Fig.4).

Effect of THC on Homocystenylation of Cytochrome-c

To determine whether an increase in Hcy levels cause protein N-homocystenylation of cyto-C during ischemia/reperfusion injury and induce autophagy. In the ischemic brain, the levels of cyto-c homocystenylation were increases as compared to sham-operated group. THC treatment inhibits the homocystenylation of cyto-c (Fig.4C). N-linked Hcy thiol group was blocked by the treatment with THC (Fig.4).

Effect of THC on MMPs

MMP-9 is a known marker for cerebral injury. To assess total MMPs activity, *in-situ* DQ-gelatin zymography was performed in fresh brain slices from all groups. There was a marked increase in gelatinolytic activity in the ischemic group as compared to sham operator groups (CBS+/-) (Figs.5A, B, C, and D). Gelatinolytic activity was reduced in the THC-treated groups (Figs.5A, B, C,D). To verify the gelatinolytic activity result, we performed Western blot in all groups (Figs.5E, F) Interestingly, MMP-9 protein expression was markedly increased in CBS+/- ischemia groups as compared to sham operator groups (CBS +/-) (Figs.5E,F). These results suggested that THC administration significantly inhibited the induction of MMP-9 (Fig.5).

Anti-autophagy effect of THC

Ischemia/reperfusion injury induces autophagy. Our results showed LC3-II levels and DRAM protein expression was increased in CBS+/- ischemic group as compared to sham operator groups (Figs.6). Administration of THC inhibited the conversion of LC3-I to LC3II (Figs.6A, C) and also decreased the protein expression of DRAM in CBS+/- ischemic group as compared to sham operator groups (Figs.6B,D). These results suggested that protective

effects of THC are associated with the antiautophagic effect after cerebral ischemia/reperfusion injury, in part, by homocystenylation of cyto-c and activation MMP-9.

DISCUSSION

The aim of this study was to investigate the neuroprotective effects of tetrahydrocurcumin, a polyphenolic non-enzymatic antioxidant agent, against Hcy induced neurotoxicity. The goal was to establish an animal model of genetically hyperhomocystemia. Previously Jiang et al. Showed the neuroprotective effect of curcumin during cerebral ischemia/reperfusion injury in rat (Jiang et al., 2007; Thiagarajan and Sharma, 2004).

Results of the present study shows that THC decreased infarct volume and improved neurological outcomes after ischemic/reperfusion injury (I/R) (Fig.1) in genetically hyperhomocystemia mice. Tetrahydrocurcumin diminishes homocystenylation of cyto-c by decreasing oxidative stress, MMP-9 and provides neuronal protection through autophagy mechanisms. Disruption of the blood brain barrier occurs under various pathological conditions such as I/R injury. Which causing to an increased vascular permeability with subsequent development of brain edema (Utepergenov et al., 1998). Protection of the blood-brain barrier has become an important experimental therapeutically target during I/R injury (Veltkamp et al., 2005). In the present study, our results indicate that THC inhibited the neurotoxicity of Hcy by decreasing water content of the brain and prevented the absorbance of Evans blue dye after I/R injury, suggesting that THC protects blood-brain barrier integrity by reducing endothelial cells damage (Fig.2) (Jiang et al., 2007).

In the body Hcy is formed from methionine and can be metabolized to cystathionine or methylated to methionine by three enzymes MTHFR, SAHH and CBS. CBS enzyme is the first enzyme of the tran-sulfuration pathway (Kleinschnitz et al., 2010) and directly involved in the removal of Hcy from the methionine cycle (Finkelstein, 1998). Deficiency of CBS enzyme caused homocystinuria (Kleinschnitz et al., 2010). In order to understand the role of THC treatment in expression of Hcy metabolic pathway genes in brain tissue, we measured the mRNA expression of MTHFR and SAHH. QRT-PCR results revealed down-regulation of MTHFR and up-regulation of SAHH genes in CBS+/- +I/R group which was mitigated with THC supplementation (Fig 3A,B,C, D). These results suggest that THC retain transulfuration and remethylation pathways, thus reduce the total Hcy level (Fig.3E,F).

It is well known that Hcy causes cell detachment and cell death by creating oxidative stress (Tyagi et al., 2005b; Tyagi et al., 2006). Thus, the generation of excessive free radicals (ROS) during I/R injury in genetic HHcy condition plays a major role in brain injury associated with stroke. Our result showed that tetrahydrocurcumin treatment significantly decreased oxidative stress by down-regulating oxidant enzyme (phox47) and up-regulating anti-oxidant enzyme (catalyse) expression after I/R injury. Our results are consistent with earlier observations regarding the antioxidant role of THC (Fig.4).

It is known that Hcy induces cerebral arteriolar stiffness and finally brain dysfunction (Nappo et al., 1999). The mechanism involves 1) Hcy toxicity is the modification of proteins by a Hcy metabolite i.e Hcy- thiolactone (HTL); 2)protein modification by HTL is N-homocystenylation. The N-homocystenylation has detrimental effects on protein structure and function (Jakubowski, 2006) causing vascular diseases. In this study, we examined the effect of HHcy on homocystenylation of cyto-c during I/R injury (Fig.5). Our findings demonstrate that THC restrain homocystenylation of cyto-c by decreasing oxidative stress, which in turn initiate MMP-9 activation.

Increasing evidence has indicated that MMP-2 and MMP-9 level increases in brain injury (Mun-Bryce and Rosenberg, 1998; Strickland et al., 1996; Wang and Tsirka, 2005; Zhao et

al., 2007). In HHcy conditions, early appearance of MMP-2, MMP-9 has been reported in cardiovascular diseases and neurovascular diseases (Lominadze et al., 2006; Sen et al., 2007). MMPs, especially MMP-9, have a critical function in proteolytic degradation of the BBB in the pathological process. The lack of MMP-9 gene showed less edema than their WT littermates in a model of ICH (Tejima et al., 2007; Xue et al., 2006), as well as in cerebral ischemia (Asahi et al., 2001). In this study, we showed that increased gelatinolytic activity as well as protein expression in CBS+/- ischemic mice was significantly reduced by THC treatment. The results suggest that MMPs are also implicated in I/R injury induced brain dysfunction, at least in part, through MMP-9 activation. These data collectively support the neuro-protective effect of THC (Fig.6). Previous studies have demonstrated that THC down regulates the MMP-2 and MMP-9 expression (Yodkeeree et al., 2008; Yodkeeree et al., 2009) during cancer cell invasion. Cellular organelles are the prime target for Hcy toxicity. However, the effect of HHcy on autophagy particularly in ischemic condition and its consequence on brain function is not ever been questioned. However, the role of autophagy under HHcy conditions was controversial. It has been reported that Hcy induced apoptosis (Tyagi et al., 2005a; Tyagi et al., 2006) or autophagy/mitophagy (Dennis and Mercer, 2009). Uchiyama's group reported that in a mouse ischemia-hypoxia model, many damaged neurons showed some features of autophagic/lysosomal cell death (Adhami et al., 2006; Adhami et al., 2007; Ventruti and Cuervo, 2007). In contrast, one study showed that autophagy is associated with neuroprotection (Ventruti and Cuervo, 2007). Thus, the role of autophagy in cell death and survival in cerebral ischemia remains to be defined. In this study, we have demonstrated that THC ameliorates autophagy in genetically HHcy mice after cerebral ischemia/reperfusion injury. Our results indicated that autophagy occurred with increase in the protein level of DRAM and degradation of LC3-I to LC3-II (Fig.7). Autophagy may represent a novel mechanism by which permanent ischemic stroke induces neuronal death, and inhibition of the activation and maturation of autophagy may help reduce ischemic injury. In conclusion, if hyperhomocysteinemia is one of the pathological reasons for neurodegenerative disorders (stroke), THC may be an effective prophylactic agent in the prevention of oxidative stress by Hcy. Furthermore, data of this study suggest that antioxidant property of THC may be responsible for protection against Hcy oxidative stress, possibly by increasing the endogenous defenses against oxidative stress and hence improves brain functions. Further studies are required to reveal the exact mechanism of Hcy in cell death process (apoptosis or autophagy) and the neuroprotective properties of THC must be studied in more detail.

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Rational

Cystathionine-beta synthase deficient homozygous mice suffer from severe growth retardation and a majority of them are dead by 5 weeks of age. Histological examination showed that the hepatocytes of homozygotes were enlarged, multinucleated, and filled with microvesicular lipid droplets. Plasma homocysteine levels of the homozygotes were approximately 40 times normal. Heterozygous mutants have an approximately 50% reduction in cystathionine beta-synthase mRNA and enzyme activity and have twice normal plasma homocysteine levels. Thus, the heterozygous mutants are promising for studying the *in vivo* role of elevated levels of homocysteine.

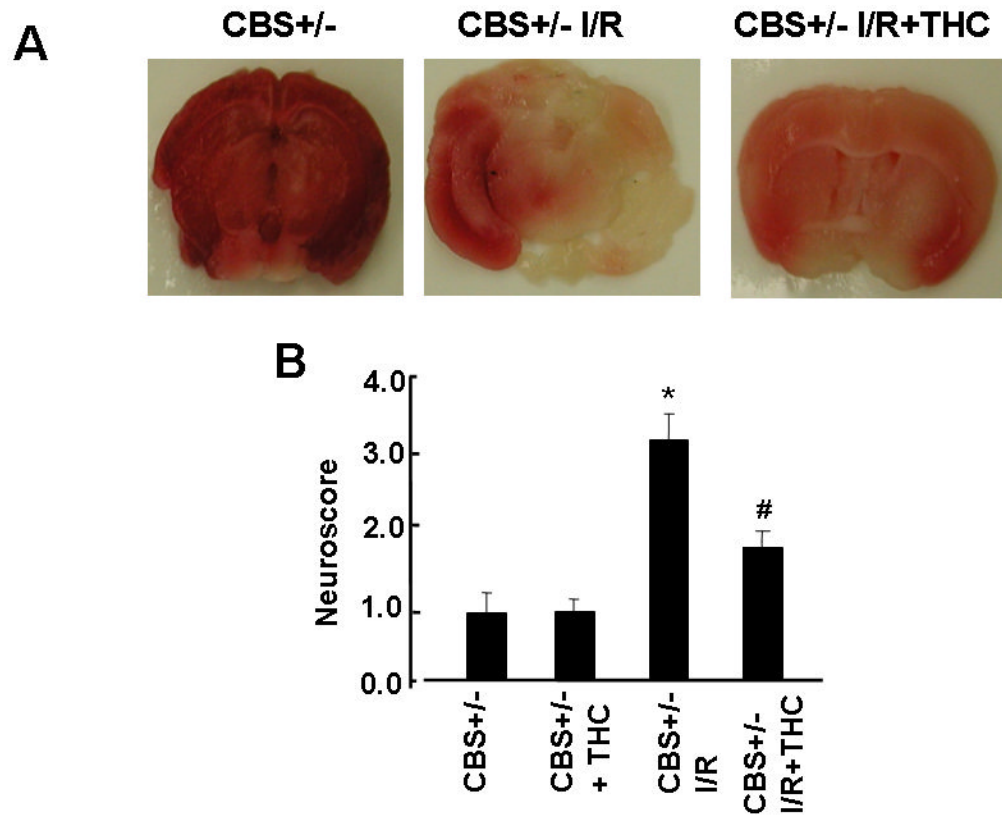


Figure 1. Neuroprotective effect of THC against ischemia/reperfusion insult

A: Representative coronal sections stained with 2% TTC after 45 min of middle cerebral artery occlusion and 72 hrs of reperfusion showing infarction. Dark colored region indicates no-ischemic portion of brain and pale-colored region indicates ischemic portion of the brain.

B: Neuroscore of vehicle and THC-treated mice measured 3day after middle cerebral artery occlusion. * $P < 0.05$ values significantly different from CBS+/- mice, # $P < 0.05$ values significantly different from CBS+/- I/R mice; $n = 4$ animals/group.

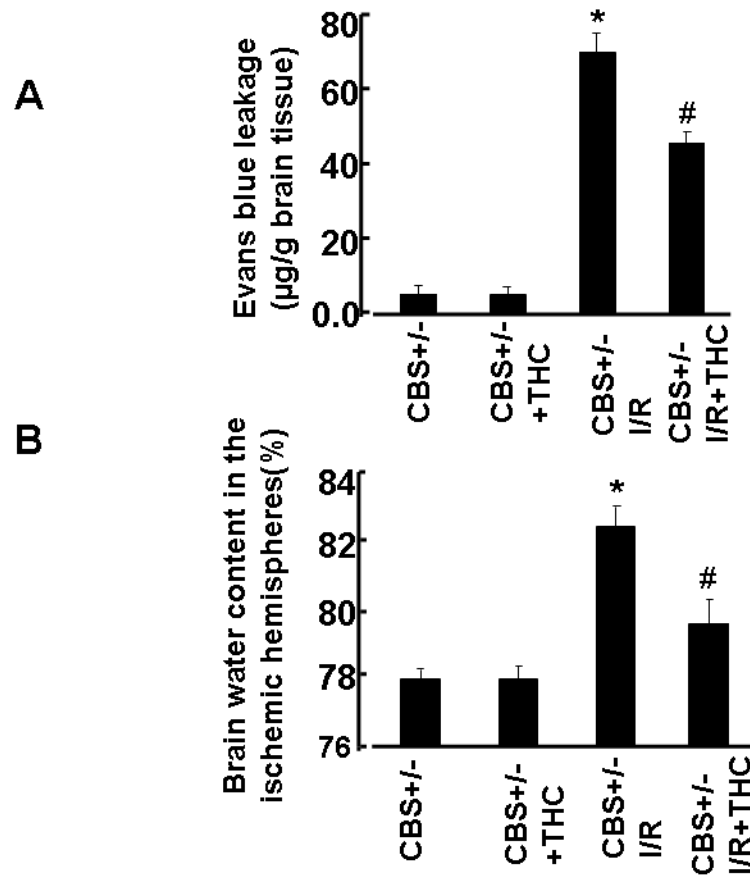
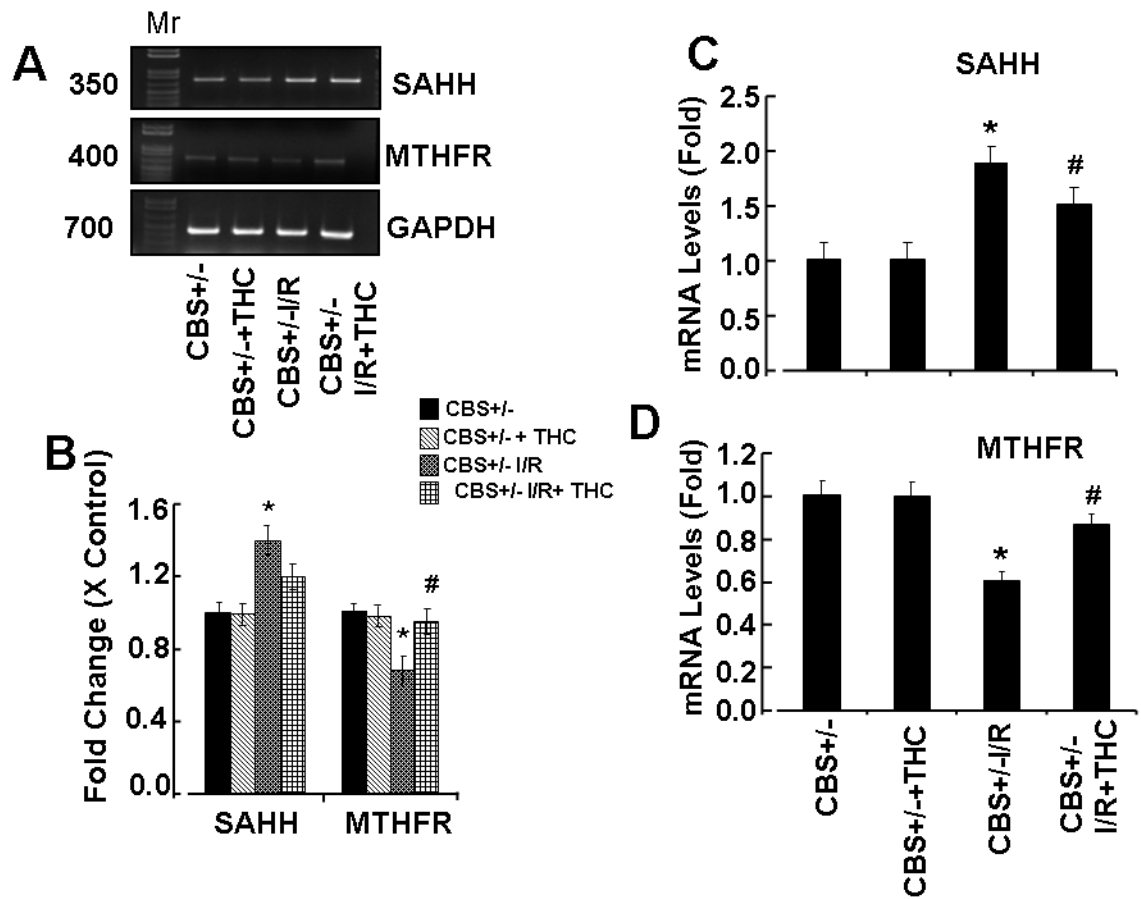


Figure 2. Effect of THC on blood-brain barrier integrity

A: Quantitative analysis of the extravasation of Evens blue dye showing integrity of the blood–brain barrier after 45min of MACO and 72hrs of reperfusion. **B:** Brain edema formation after 45 min of MACO and 72 hrs of reperfusion. * $P < 0.05$ values significantly different from CBS+/- mice, # $P < 0.05$ values significantly different from CBS+/- I/R mice; $n = 4$ animals/group.



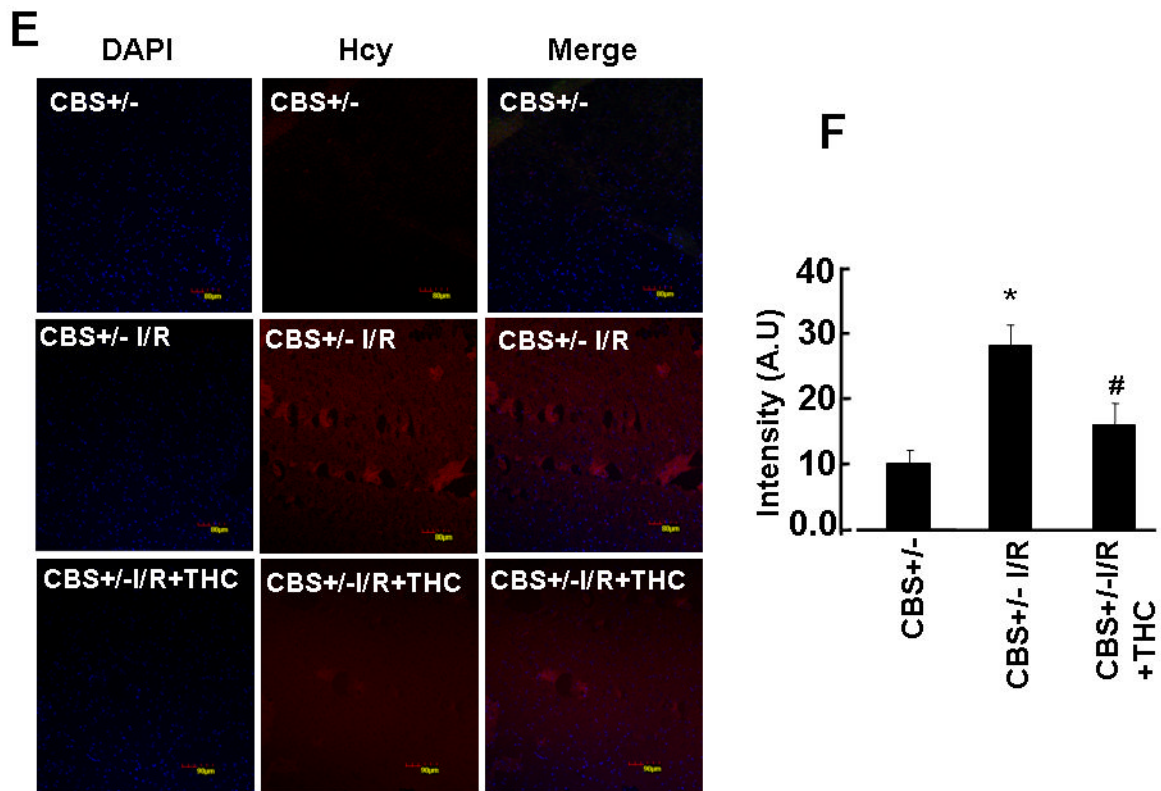


Figure 3. Effect of THC on Hcy metabolism and levels

A: Represented semi quantitative mRNA expression for SAHH, MTHFR and with GAPDH control. **B:** Densitometric analysis of SAHH and MTHFR mRNA expressions as represented in the bar diagram. **C & D:** Total mRNA was isolated from the brain of all experimental groups and the mRNA levels of MTHFR and SAHH was measured by QRT-PCR. * $P < 0.01$ values significantly different from *CBS+/-* mice, # $P < 0.01$ values significantly different from *CBS+/-I/R* mice; $n = 4$ animals/group. **E:** Immunostaining results demonstrate Hcy expression in the brain sections from *CBS+/-*, *CBS+/- + THC*, *CBS+/- I/R* and *CBS+/- I/R+THC* group. Cellular nuclei in all experiments were stained with 4',6-diamidino-2-phenylindole (DAPI). **F:** Bar diagram represents quantification of immunostaining using image pro software. * $P < 0.05$ values significantly different from *CBS+/-* mice, # $P < 0.05$ values significantly different from *CBS+/- I/R* mice; $n = 3$ animals/group.

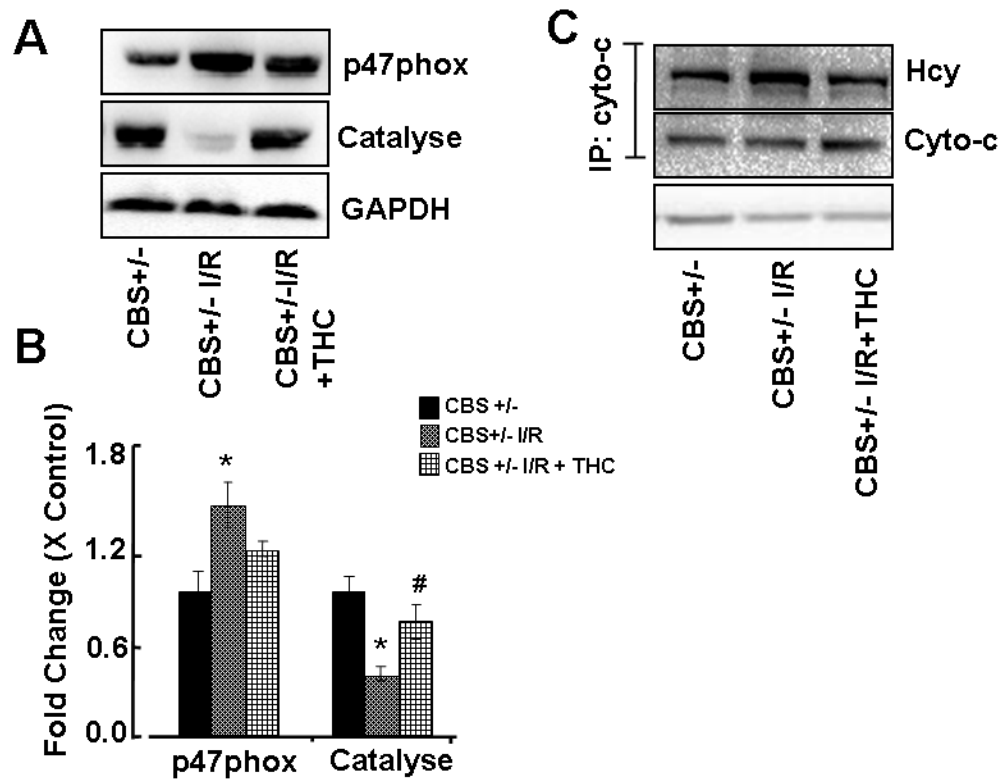


Figure 4. Effect of THC on oxidative stress

A: Representative immunoblots for p47^{phox}, catalyse and GAPDH control. **B:** Densitometric analysis of p47^{phox} and catalyse protein expressions using Image Lab software as shown in the bar diagram. **C:** Brain tissue lysate was immunoprecipitated with anti-cyto-C antibody. Immunoprecipitated samples were subjected to Western blot with anti-Hcy antibody and with anti-cyto-C antibody. GAPDH was used as a loading control. * $P < 0.05$ values significantly different from CBS^{+/-} mice, # $P < 0.05$ values significantly different from CBS^{+/-} I/R mice; $n = 4$ animals/group.

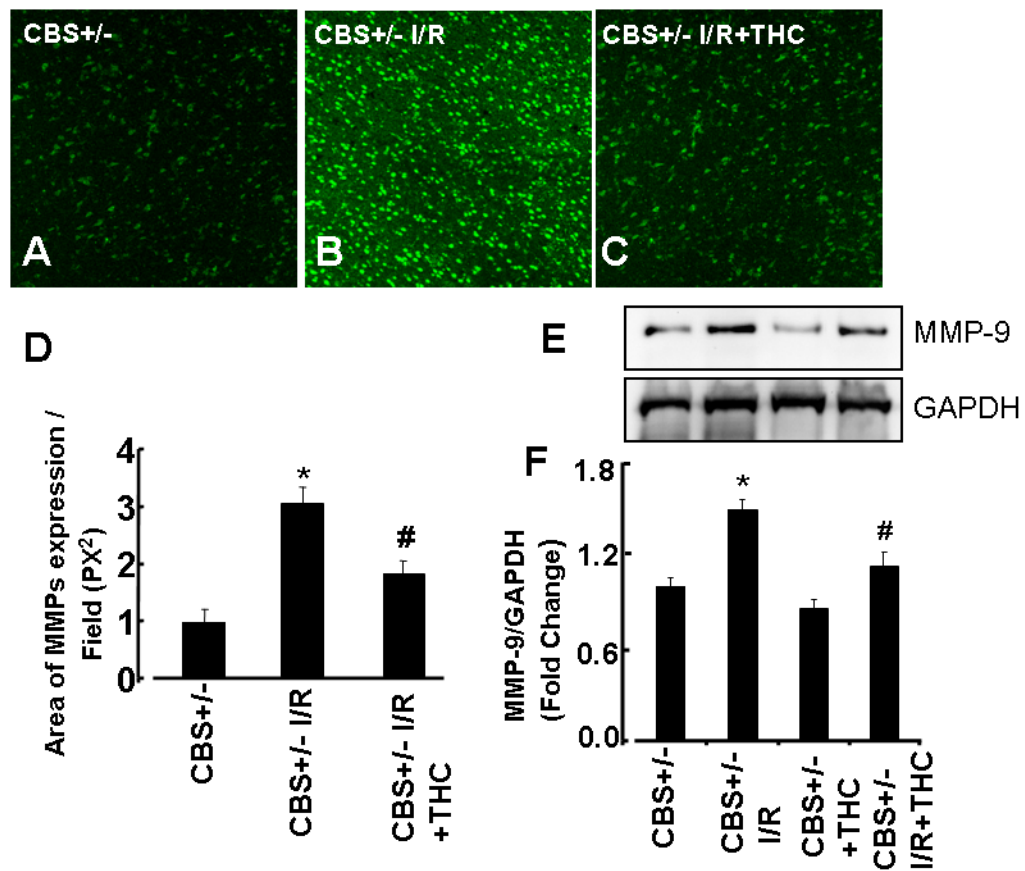


Figure 5. Effect of THC on MMP-9

A, B, and C: Representative photomicrographs of gelatinase activity. **D:** Bar graph of changes in integrated optical density (IOD) in activity of MMP-9. The micrographs were taken under the identical set of conditions for all groups. **E:** Representative immunoblots for MMP-9 protein and GAPDH control. **F:** Densitometric analysis of MMP-9 protein expressions using Image Lab software as shown in the bar diagram. * $P < 0.05$ values significantly different from CBS+/- mice, # $P < 0.05$ values significantly different from CBS +/- I/R mice; $n = 3$ animals/group.

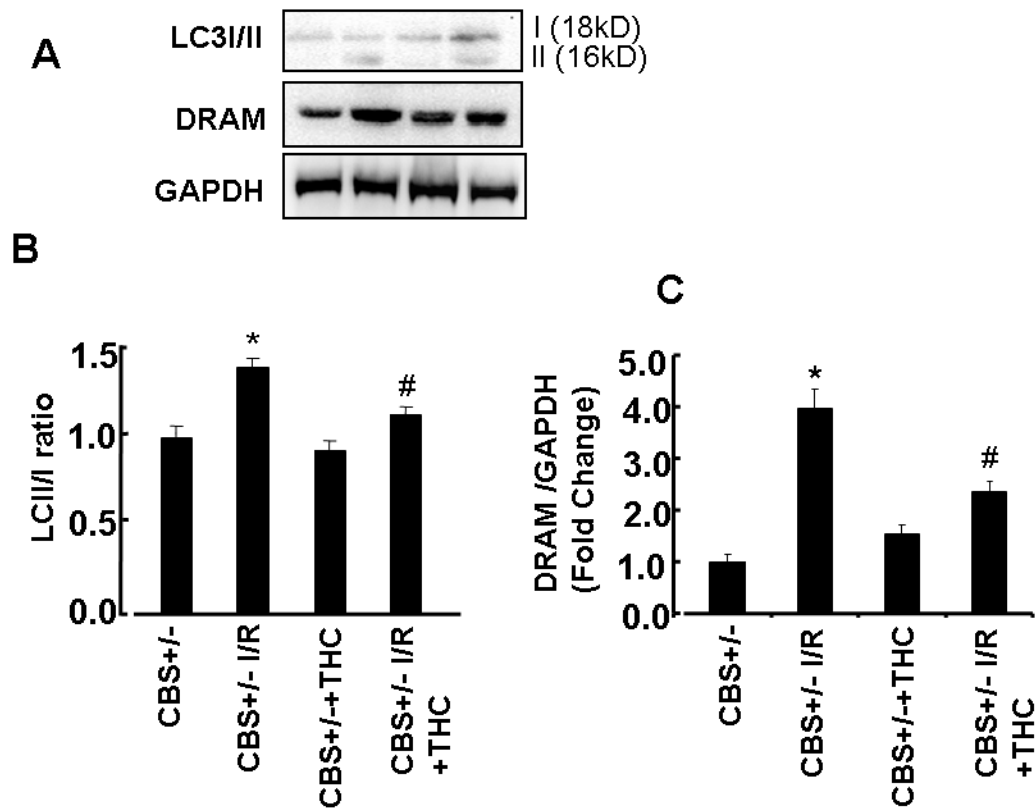


Figure 6. Effect of THC on autophagy

A: Representative immunoblots for cleaved LC3 (LC3-II), DRAM and control GAPDH protein control. **B:** Densitometric analysis of MMP-9 protein expressions using Image Lab software as shown in the bar diagram. * $P < 0.05$ values significantly different from CBS+/- mice, # $P < 0.05$ values significantly different from CBS+/- I/R mice; $n = 4$ animals/group.