Protective effect of crocin on acrolein-induced tau phosphorylation in the rat brain

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Acrolein, as a by-product of lipid peroxidation, is implicated in brain aging and in the pathogenesis of oxidative stress-mediated neurodegenerative disorders such as Alzheimer's disease (AD). Widespread human exposure to the toxic environmental pollutant that is acrolein renders it necessary to evaluate the effects of exogenous acrolein on the brain. This study investigated the toxic effects of oral administration of 3 mg/kg/day acrolein on the rat cerebral cortex. Moreover, the neuroprotective effects of crocin, the main constituent of saffron, against acrolein toxicity were evaluated. We showed that acrolein decreased concentration of glutathione (GSH) and increased levels of malondialdehyde (MDA), Amyloid-β (Aβ) and phospho-tau in the brain. Simultaneously, acrolein activated Mitogen-Activated Protein Kinases (MAPKs) signalling pathways. Co-administration of crocin significantly attenuated MDA, Aβ and p-tau levels by modulating MAPKs signalling pathways. Our data demonstrated that environmental exposure to acrolein triggers some molecular events which contribute to brain aging and neurodisorders. Additionally, crocin as an antioxidant is a promising candidate for treatment of neurodegenerative disorders, such as brain aging and AD.

INTRODUCTION

Epidemiological studies and experimental research projects have demonstrated that environmental exposure to air pollutants, pesticides and metals, as well as diet, constitutes a major risk factor for a range of human disorders such as neurodegenerative diseases and early aging. These environmental factors contribute to the pathophysiology of neurodisorders resulting from damage to tissue biomolecules by increasing free radical generation and inducing oxidative injuries (Migliore and Coppedè 2009).

Formation of two brain lesion types, neurofibrillary tangles (NFTs) and amyloid plaques, is the main pathological characteristic of Alzheimer's disease (AD) (Gómez-Ramos et al. 2004, Huang and Jiang 2009).

NFTs are composed of aggregated hyperphosphorylated microtubule-associated protein tau in a structure known as Paired Helical Filaments (PHF). Tau protein plays an important role in elongation and stabilization of microtubules, as well as in regulation of axonal transport in neural cells (Gómez-Ramos et al. 2004).

It is established that β-amyloid (Aβ), a main component of senile plaques, is toxic to various neuronal cell types. Moreover it has been shown that the presence of Aβ in the brain is correlated with dementia (Mondragón-Rodríguez et al. 2012).

It has been proposed that oxidative stress is one of the pathogenic mechanisms of tau phosphorylation and formation of neurofibrillary lesions (Carroll et al. 2011).

Many studies have demonstrated that levels of acrolein, a reactive unsaturated aldehyde, increase significantly during the occurrence of neurodegenerative diseases like AD. Acrolein induces oxidative stress-mediated toxicity; it may be associated with the pathogenesis of neurodegenerative disorders by increasing

the phosphorylation levels of tau and Aβ peptide accumulation (Gómez-Ramos et al. 2002, Lovell et al. 2001, Ramassamy et al. 2010). Additionally, the presence of acrolein adducts in neurofibrillary tangles has been reported (Calingasan et al. 1999).

Acrolein is endogenously formed by lipid peroxidation of fatty acid sources in the brain, and may damage important biomolecules such as lipids, proteins and DNA. Moreover, acrolein is capable of amplifying free radical generation (Kehrer and Biswal 2000).

Furthermore, acrolein is a widespread toxic environmental pollutant that is found in automobile engine exhaust and cigarette smoke, and is produced by the burning of organic materials and fat-containing foods (Kehrer and Biswal 2000). Acrolein is a strong electrophile and shows the highest reactivity with nucleophile sites, particularly sulfhydryl groups of proteins (LoPachin et al. 2009). Thus, acrolein adduct disrupts the function of many enzymes and quickly depletes cellular glutathione contents (Stevens and Maier 2008).

Crocin is a glycosylated carotenoid and main constituent of the stigmas of saffron (*Crocus sativus* L.), a commonly-known spice for enhancing food flavour and colour. Saffron is also used in folk medicine for various purposes in different parts of the world (Hosseinzadeh and Nassiri-Asl 2012). Various studies have shown a range of pharmacological effects of saffron and its constituents, crocin, safranal and picrocrocin (Rezaee and Hosseinzadeh 2013, Alavizadeh and Hosseinzadeh 2014). These include antidepressant properties (Hosseinzadeh et al. 2003, 2007, Wang et al. 2010, Vahdati et al. 2014), anti-inflammatory and antinociceptive properties (Hosseinzadeh and Younesi 2002, Sahebari et al. 2011, Amin and Hosseinzadeh 2012, Boskabady et al. 2012), anticonvulsant properties (Hosseinzadeh et al. 2008), antidote effects (Hariri et al. 2011, Razavi et al. 2013a,b), anti-tumour properties (Rastgoo et al. 2013) brain and skeletal muscle ischemia–reperfusion improvement (Hosseinzadeh and Sadeghnia 2005, Hosseinzadeh et al. 2009, Ghadrdoost et al. 2011) and treatment of memory impairment (Hosseinzadeh and Ziaei 2006, Hosseinzadeh et al. 2012). Along with its neuroprotective (Mehri et al. 2012) and antioxidant properties, anti-apoptotic effects of crocin have been also reported in both *in vitro* and *in vivo* studies (Hosseinzadeh et al. 2009, Mehri et al. 2012, Lari et al. 2013).

Based on the neurotoxic effects of acrolein and high antioxidant activity of crocin, the present study was designed to investigate the effects of exogenous acrolein on the formation of early neurodegenerative markers, including accumulation of Aβ and hyperphosphorylated tau *in vivo*. To this end, rats were gavaged with high doses of acrolein for two weeks, and possible correlations between induced oxidative stress and MAPK as well as Akt pathways in amyloidogenesis and phosphorylation of tau protein were investigated. In addition, the neuroprotective effects of crocin against acrolein-induced toxicity were examined.

METHODS

Materials

Mouse monoclonal p-Tau (Ser396, PHF-13), mouse monoclonal Tau (Tau46), mouse monoclonal p-Akt (Ser473), rabbit polyclonal Akt, rabbit monoclonal p-GSK-3β (Ser9), rabbit monoclonal GSK-3β, mouse monoclonal p-SAPK/JNK (Thr183/Tyr185), rabbit polyclonal SAPK/JNK, mouse monoclonal p-p44/42 MAPK (Erk1/2, Thr202/Tyr204), rabbit polyclonal Erk1/2, mouse monoclonal p-p38 MAPK (Thr180/Tyr182), rabbit polyclonal p38 MAPK, mouse beta actin, rabbit beta actin, anti-rabbit and anti-mouse IgG labelled with HRP were provided by Cell Signaling, and mouse monoclonal p-Tau (pT231) was obtained from Abcam (USA). Polyvinylidene difluoride (PVDF) membrane (#162-0177) and Bradford protein assay kit (#500-0002) were purchased from Bio-Rad (USA). BCA protein assay kit and ECL detection reagent kit (#32106) were obtained from Pierce (USA). Chemiluminescent BetaMark™x-42, #SIG-38952-kit was purchased from Covance (USA). Complete protease inhibitor cocktail (#P8340), Acrolein (Fluka, ≥95.0%) and all chemicals were purchased from Sigma (Sigma-Aldrich Corp. St Louis, USA).

Preparation of Crocin

Stigmas of *C. sativus* L., collected from Ghaen, Khorasan province, in the north-east of Iran, were purchased from Novin Saffron (Mashhad, Iran) and evaluated in accordance with ISO/TS 3632-2. Crocin was extracted from saffron and purified as previously described by Hadizadeh et al. 10 g of Saffron stigmas powder were briefly suspended in 25 mL ethanol 80% at 0°C and shaken for 2 min., then centrifuged at 4 000 g for 10 min, after which the supernatant was separated. This extraction was repeated six times. The resulting solution was kept at −5°C in a thick-walled

glass container for 24 days in darkness for crystallization. The obtained crocin crystals were separated from the solution (Hadizadeh et al. 2010).

Animals

Male Wistar rats weighing 250–300 g were used in all experiments. The animals were obtained from Animal House at the School of Pharmacy, Mashhad University of Medical Sciences, Iran. The animals were maintained under standard 12 h light-dark cycles and 25±2°C with free access to food and water. All animal experiments were approved by the Ethical
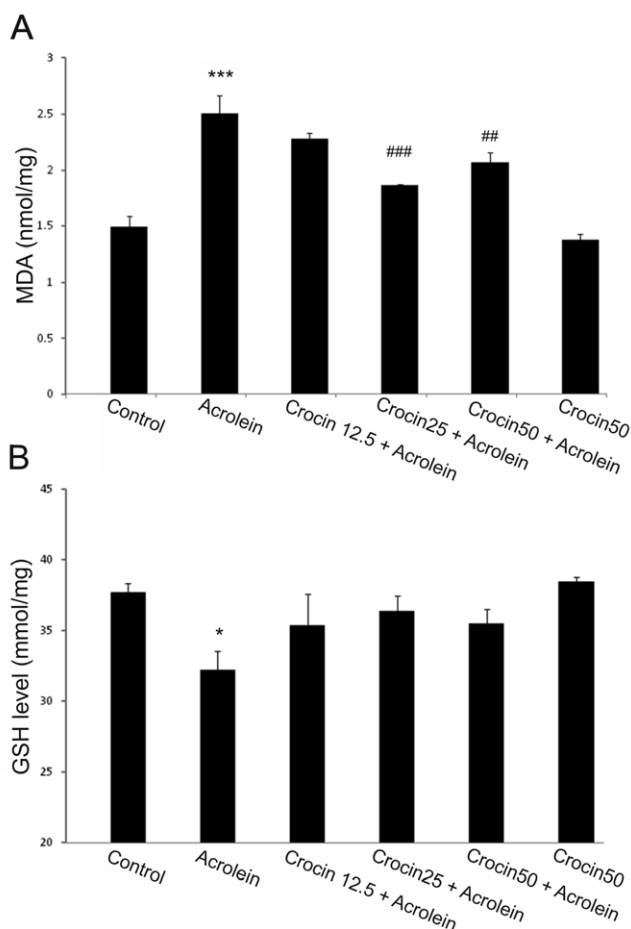

Fig. 1. Effect of acrolein and crocin treatment on MDA levels (A) and GSH contents (B) in rat cerebral cortex. Acrolein was administered *via* gavage and crocin was intraperitoneally injected into rats for two weeks. Data are expressed as mean±SEM from six separately prepared samples. ***$P<0.001$ and *$P<0.05$ indicate statistically significant vs. control group, ###$P<0.001$ and ##$P<0.01$ indicate statistically significant vs. acrolein group.

Committee Acts of Mashhad University of Medical Sciences (# 89609).

Pilot study

Four groups of animals (*n*=6) treated with different doses of acrolein (1, 3, and 5 mg/kg/day) and A control group, which received distilled water by gavage for two weeks.

In the group that received 5 mg/kg/day acrolein, three animals died within the first 72 h. Only one rat with 3 mg/kg/day acrolein died prior to the end of the experiment.

MDA levels in cerebral cortex tissues of the acrolein treated groups were measured and compared with those of the control group. We found that the level of MDA was significantly higher in rats which had received 3 mg/kg/day compared with the control group, but did not considerably change compared to those with a dose of 1 mg/kg/day. Based on these pilot experiments, a dose of 3 mg/kg of acrolein was chosen for this study.

Main study

36 rats were randomly divided into six groups of six rats each: (1) Control group: treated with distilled water; (2) Acrolein group: treated with fresh aqueous solutions of 3 mg/kg/day acrolein; Acrolein-Crocin groups were treated with: (3) 3 mg/kg/day Acrolein + 50 mg/kg/day Crocin, (4) 3 mg/kg/day Acrolein + 25 mg/kg/day Crocin, (5) 3 mg/kg/day Acrolein + 12.5 mg/kg/day Crocin; (6) Crocin group: treated with 50 mg/kg/day Crocin. A freshly prepared daily dose of acrolein solution or distilled water was administered to the animals orally, or they received crocin intraperitoneally, for a period of two weeks.

After two weeks of treatment, the animals were decapitated, and their brains were immediately removed and washed using an ice-chilled physiological normal saline solution. The cerebral cortex was isolated, flash frozen and stored at −80°C until further use.

Measurement of lipid peroxide levels in cerebral cortex tissue

To estimate lipid peroxidation in the cerebral cortex, MDA levels in different experimental groups were measured. Cerebral cortex tissues were homogenized (10% w/v) in cold 1.15% KCl solution. After centrifu-

gation, total protein concentrations were measured in supernatants using a Bradford protein assay kit. 0.5 ml of supernatants was mixed with 3 ml of phosphoric acid (1%) and 1 ml of thiobarbituric acid (0.6%). Then, the mixtures were incubated in a boiling water bath for 45 min. Samples were cooled down to room temperature and 4 ml of n-butanol was added to the mixtures, vortexed for 1 min, and finally centrifuged at 5 000 g for 10 min. Intensity of pink colour, generated by thiobarbituric acid reactive substances (TBARS) in n-butanol phase, was determined spectrophotometrically at 532 nm using a Synergy H4 Hybrid Microplate Reader (BioTek, USA) and reported as nmol of MDA/mg protein (Yavuz et al. 1997).

Measurement of reduced glutathione (GSH)

Cerebral cortex GSH was measured using a colorimetric technique according to the method described by Moron and colleagues (Moron et al. 1979). Tissues were briefly homogenized in 0.1 M of ice cold phosphate buffer (pH 7.4) and centrifuged. Total protein concentrations of supernatants were determined as mentioned above. Tissue homogenates (0.5 ml) were mixed with an equal volume of trichloroacetic acid (10% TCA) and vortexed. After centrifugation at 3 000 g for 10 min the supernatants were collected, and 0.5 ml of supernatants were mixed with a reaction buffer containing 2.5 ml of phosphate buffer (0.3 M, pH 8.4) and 0.5 ml of 0.01 M, DTNB [5, 5′ dithiobis-(2-nitrobenzoic acid)]. Absorbance was then measured within 4–5 min at 412 nm using a spectrophotometer. Results of GSH level measurements were expressed as nmol/mg protein.

Measurement of Aβ levels

Total Aβ (soluble and insoluble) in cerebral cortex tissues were extracted according to tissue preparation protocol of the commercially-available ELISA kit, while total protein concentration of samples were determined using a BCA protein assay kit.

Levels of $A\beta_{1-42}$ were determined per manufacturer's instructions. Each sample extract (100 μl) was placed into a precoated well in triplicate and incubated overnight at 4°C for 18h. After washing with a washing buffer, 100 μl of mixed chemiluminescent substrates (A and B) was added to each well, and the emitted light was immediately measured using a Synergy H4 Hybrid Reader (Biotek, USA).

Total $A\beta_{1-42}$ concentrations were calculated using a standard curve and expressed as pg/mg total protein.

Western blotting

Each frozen cerebral cortex tissue sample (200 mg) were crashed in liquid nitrogen. Tissues were suspended in an ice-cold homogenization buffer containing 50 mM Tris–HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 10 mM sodium-β glycerophosphate, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 0.1% (v/v) 2-mercaptoethanol, 2% (w/v) sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 μl complete protease inhibitor cocktail using a Polytron homogenizer (IKA®T10, Germany). Then, lysates were sonicated on ice using a probe sonicator (UP100H, Germany). After centrifugation at 16 000 g for 10 min at 4°C, supernatants were collected and transferred to clean microtubes and the protein concentrations were determined using a Bio-Rad protein assay kit. Supernatants were mixed with equal volumes of 2X SDS buffer containing 100 mM Tris-base, 20% v/v Glycerol, 4% w/v SDS, 10% v/v 2-mercaptoethanol and 0.2% w/v bromophenol blue, heated in a boiling water bath for 10 min. and stored at −80°C.

Equal amounts of total proteins from each sample were loaded onto an SDS polyacrylamide gel. After electrophoresis, the protein bands were transferred to a
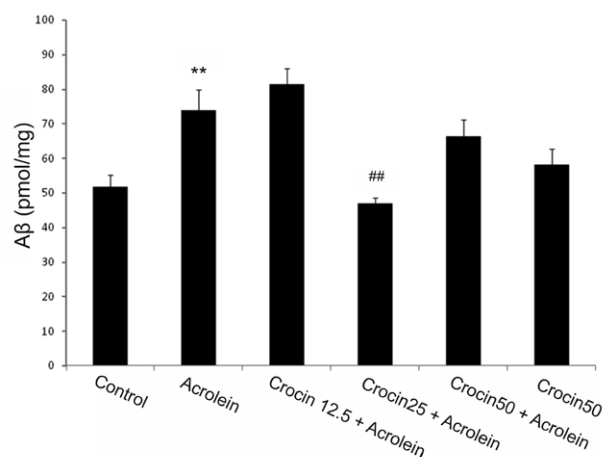

Fig. 2. Effect of acrolein and crocin treatment on Aβ level in rat cerebral cortex. The level of Aβ1-42 was assessed by ELISA. Each value expressed as mean±SEM from six separately prepared samples. **$P<0.01$ indicates statistically significant vs. control group, ##$P<0.01$ indicates statistically significant vs. acrolein group.

PVDF membrane. The membranes were blocked for 3 h in TBST (Tris-buffered saline with 0.5% Tween 20) containing 5% BSA powder (bovin serum albumin). Next, blots were probed overnight at 4°C with specific antibodies. Membranes were washed three times for 5 min and incubated with HRP-conjugated secondary antibodies for 1–2 h. Immuno-labelled bands were visualized using an ECL detection reagent kit and Alliance 4.7 gel doc (UK).

Intensities of the protein bands were analysed by optical densitometry using UVband image analysis software (UVITEC, Cambridge, UK). All phospho and corresponding total bands were normalized against corresponding β actin intensities.

Statistical analysis

The experimental results are presented as mean±SEM. The statistical differences between groups were determined by one-way ANOVA analysis of variance, followed by Tukey-Kramer test for multiple comparisons to calculate significance. $P<0.05$ was considered statistically significant.
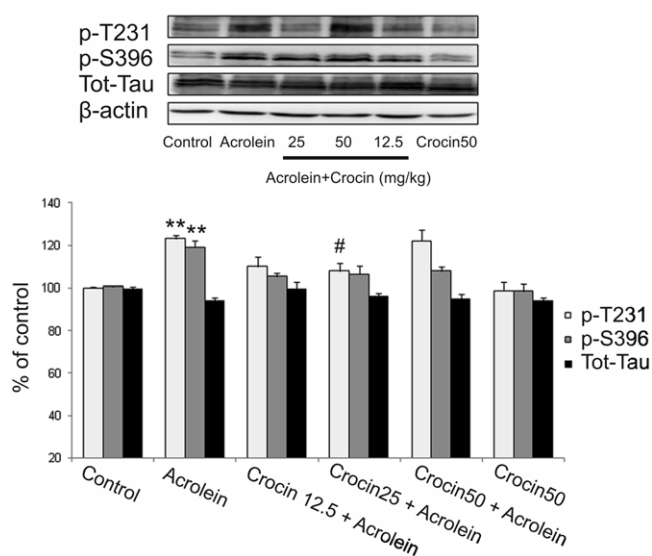

Fig. 3. Effect of acrolein and crocin treatment on tau phosphorylation in rat cerebral cortex. The levels of phospho-tau were detected by Western blotting using specific phospho-tau (pT231), Ser396 (PHF-13) and total tau (Tau-46) antibodies. Quantitative data were presented as densitometry values normalized against β-actin and expressed as mean±SEM percent of control group from six independent experiments.**$P<0.01$ indicate statistically significant vs. control group. #$P<0.05$ indicates statistically significant vs. acrolein group.

RESULTS

Effects of crocin on acrolein-induced oxidative stress biomarkers

Figure 1 shows that levels of MDA (67.4%↑, $P<0.001$) significantly increased, whereas GSH content (14.6%↓, $P<0.05$) decreased in the brain cortex tissues of acrolein-treated animals as compared with control rats. Co-administration of crocin with acrolein significantly reduced MDA concentration at doses of 25 mg/kg (25.4%↓, $P<0.001$) and 50 mg/kg (17.3%↓, $P<0.01$), whereas they resulted in non-significant increases in GSH levels (up to 13%) at all crocin doses when compared to corresponding levels in acrolein-treated rats. MDA and GSH levels in the cerebral cortex of rats that were only treated with 50 mg/kg crocin were the same as control group.

Effects of crocin on acrolein-increased Aβ concentrations in rat cerebral cortex

Subacute oral toxicity of acrolein increased $A\beta_{1-42}$ levels in the cerebral cortex (43.3%↑, $P<0.01$) compared to the untreated group. The intraperitoneal injection of crocin at a dose of 25 mg/kg attenuated levels of $A\beta_{1-42}$ following exposure to acrolein (36.5%↓, $P<0.01$) as compared with acrolein-treated rats. The 50 mg/kg crocin dose led to insignificant decrease (21%↓) in $A\beta_{1-42}$ levels. Also, the 12.5 mg/kg crocin dose did not lead to considerable changes in $A\beta_{1-42}$ levels compared with the acrolein-treated group (Fig. 2).

Effects of crocin on acrolein-induced tau phosphorylation in rat cerebral cortex

Aβ peptide contributes to tau hyperphosphorylation, which may subsequently lead to neurotoxicity and loss of neurons (Huang and Jiang 2009).

To assess the effect of acrolein and crocin on tau phosphorylation state in rats' cerebral cortex, antibodies that detect total tau protein (tau 46) as well as Ser396 (PHF-13) and T231 phosphorylated tau (pT231) were used.

Figure 3 shows that acrolein significantly increased the amount of p-Ser396 (19%↑, $P<0.01$) and p-T231 (23%↑, $P<0.01$) as compared with the control group, while there was no significant effect on total tau levels. Co-treatment with 25mg/kg crocin significantly (14%↓, $P<0.05$) reversed the phosphorylation state of T231 as

compared with the acrolein-treated group; 12.5 and 50 mg/kg crocin did not show significant effects on decreasing p-T231 as compared with the acrolein group, while co-treatment with 50 mg/kg crocin led to significant differences from the control group. However, crocin did not lead to a significant decrease in the phosphorylation of Ser396 [12.5 mg/kg (13%↓) and 25mg/kg (12%↓)] compared with the acrolein group.

Effects of crocin on acrolein-induced Akt pathway activation

To study the contribution of the Akt/GSK-3β signalling pathway in tau phosphorylation following exposure to acrolein, we examined the activation of this pathway by measuring the phosphorylation state of Ser473 residues on Akt. Additionally, GSK-3β as a downstream substrate of Akt plays a primary role in the hyperphosphorylation of tau protein. GSK-3β is activated through increased Tyr216 and/or decreased Ser9 phosphorylation (Huang and Jiang 2009, Zhao et al. 2011).

We observed significant increases in phosphorylated Akt (ser 437) levels in the brain of acrolein-treated rats compared with control rats (30%↑, $P<0.01$), while total Akt remained unchanged in all groups. Levels of p-Akt in all crocin treatment groups significantly decreased as compared to the acrolein-treated group; Acrolein + 12.5 mg/kg crocin (33.8%↓, $P<0.001$), Acrolein + 25 mg/kg crocin (35.3%↓, $P<0.001$) and Acrolein + 50 mg/kg crocin (25.2%↓, $P<0.05$) (Fig. 4A).

GSK-3β activation was analysed by measuring phosphorylation of Ser9 using western blot analysis. No alteration in the levels of either phosphorylated GSK-3β at Ser9 or total GSK-3β (Fig. 4B) were observed among acrolein-treated, control and crocin groups.

Effects of crocin on acrolein-induced MAPK pathway activation

To understand the involvement of MAPK signalling in increased tau phosphorylation, we determined the activation of ERK1/2, SAPK/JNK and SAPK/p38 after exposure to acrolein.

We observed that levels of p-ERK1/2 (39.7%↑, $P<0.01$), p-JNK (29.7%↑, $P<0.001$) and p-p38 (25%↑,

$P<0.01$) significantly increased in the cerebral cortex after treatment with acrolein. Figure 5 shows two bands at the positions of 42 kDa and 44 kDa for ERK1/2 and two bands at the positions of 46 kDa and 54 kDa for JNK. The sum of densities of the two bands was measured. Our data showed that levels of total protein for these three kinases remained unchanged in
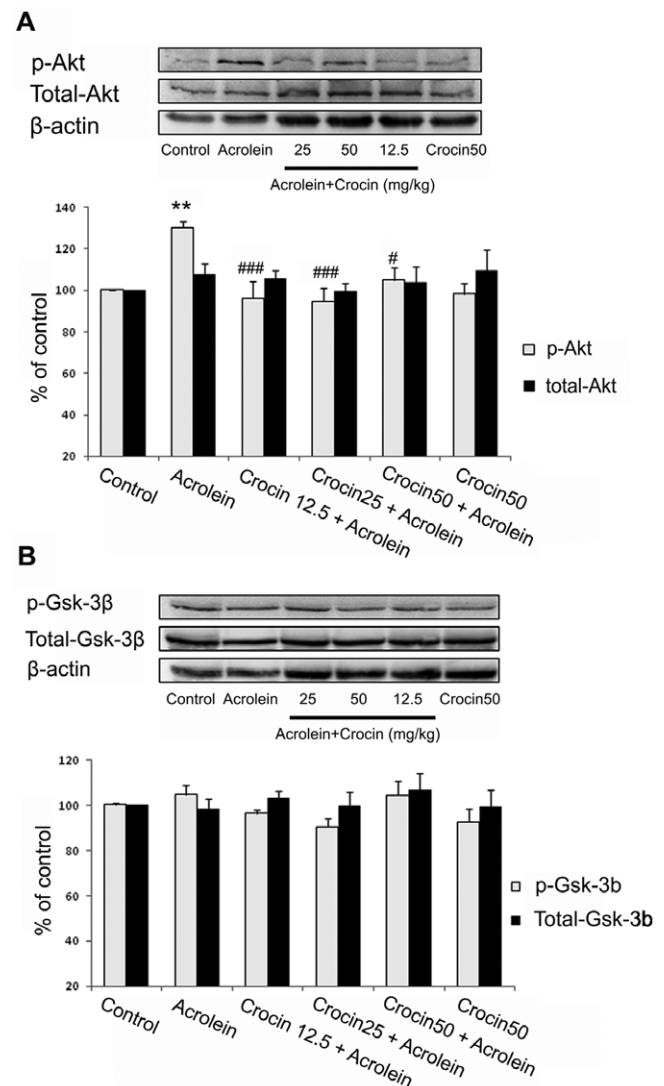

Fig. 4. Effect of acrolein and crocin treatment on phosphorylation of Akt (A) and GSK-3β (B) in rat cerebral cortex detected by Western blotting. The protein levels of p-Ser473 and total Akt, p-Ser9 and total GSK-3β were analysed. Semi-quantitative data were presented as densitometry values normalized against β-actin and are expressed as mean±SEM % of control group from six independent experiments. **$P<0.01$ indicates statistically significant vs. control group, ###$P<0.001$ and #$P<0.05$ indicate statistically significant vs. acrolein group.
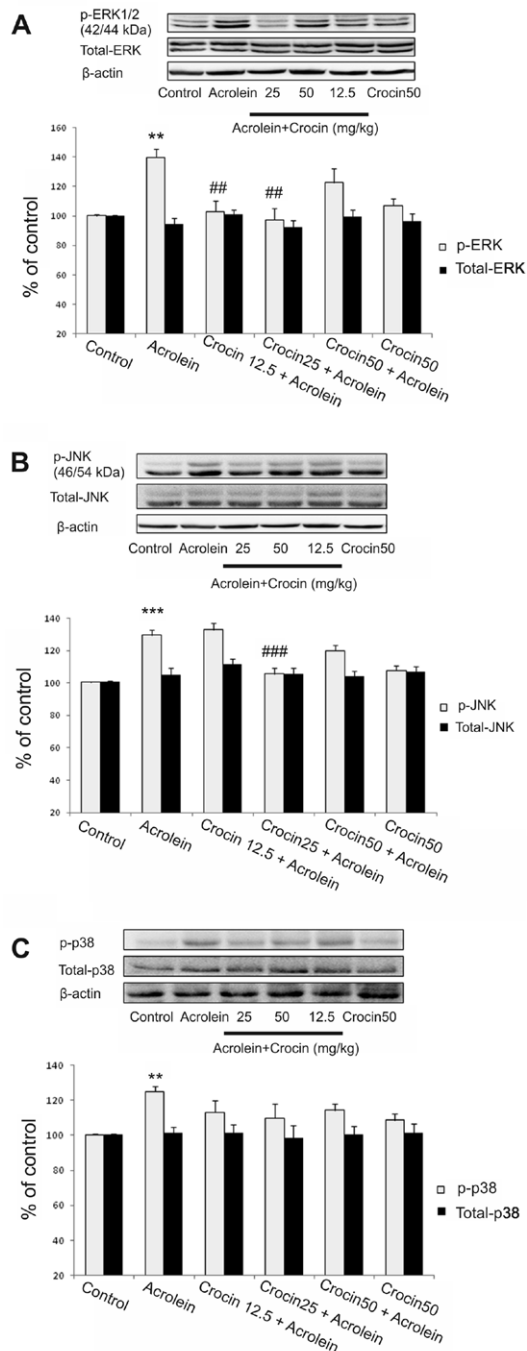
Fig. 5. Effect of acrolein and crocin treatment on MAPKs pathway in rat cerebral cortex. Western blot analysis was carried out to determined protein levels of ERK1/2 and p-ERK (A) JNK and p-JNK (B), p38 and p-p38 (C). Quantitative data were presented as densitometry values normalized against β-actin and expressed as mean±SEM % of control group from six independent experiments. ***$P<0.001$ and **$P<0.01$ indicate statistically significant vs. control group, ###$P<0.001$ and ## $P<0.01$ indicate statistically significant vs. acrolein group.

all groups. These findings suggest that JNK, ERK1/2 and p38 may all play a role in mediating the effects of acrolein on tau phosphorylation state in the brain cortex. Co-administration of acrolein with 12.5 mg/kg (36.7%↓, $P<0.01$) and 25 mg/kg (42.4%↓, $P<0.01$) crocin significantly reduced the effect of acrolein on levels of p-ERK1/2 as compared with the acrolein-treated group. Our data showed that co-administration of acrolein with 25 mg/kg of crocin attenuated levels of p-JNK (23.9%↓, $P<0.001$) as compared to the acrolein group. Co-treatment with crocin did not significantly reduce level of p-p38 compared to acrolein. Overall, the protective effects of crocin were not dose-dependent.

DISCUSSION

The present study showed that acrolein significantly increased cerebral cortex MDA levels as an important indicator of lipid peroxidation, and decreased GSH content as the most abundant cell antioxidant and the first line of protection against the destructive effects of oxidative stress. It has been indicated that oral exposure to acrolein could induce ROS generation and consequently provoke oxidative stress injuries in the cerebral cortex.

Oxidative stress is one of the earliest noticeable events contributing to the pathophysiology of various neurodisorders of the central nervous system, including neuron aging, AD and PD, and it plays a key role in the initiation of neurodegeneration through stimulating cell signalling pathways by mediating elevated Aβ levels (Su et al. 2008, Sultana and Butterfield 2010).

There is a vicious cycle between oxidative stress and Aβ, in which Aβ accumulates due to oxidative stress, and oxidative stress is induced by Aβ (Su et al. 2008).

Moreover, Aβ$_{1-42}$ as the main amyloid component of senile plaques easily aggregates and plays an important role in amyloidogenesis, causing neurobehavioral impairments in AD (Thomas et al. 2005).

Our data showed that levels of Aβ$_{1-42}$ were elevated in the acrolein-treated group, and that oral administration of acrolein could induce oxidative stress, amyloidogenesis, and consequently may damage cortical neural cells.

Crocin, as an antioxidant, could attenuate brain MDA levels and restore GSH content in co-administration with acrolein. Moreover, the same treatment

decreased the concentration of $A\beta_{1-42}$ as compared with acrolein-treated rats.

It has already been reported that crocin could pass through the blood–brain barrier and significantly reduce the infarct volume in the cerebral infarction mice model. In addition, crocin could prevent neuron death induced by ischemic stress (Ochiai et al. 2007).

In fact, the neuroprotective potentials of crocin are mainly related to its capacity to function as an antioxidant by acting as a free radical scavenger (Assimopoulou et al. 2005), restoring or maintaining intracellular GSH homeostasis, and enhancing its biosynthesis (Ochiai et al. 2007).

Moreover, a previous *in vitro* study showed that crocin may interact with $A\beta$ peptides and inhibit amyloid fibrillogenesis (Ghahghaei et al. 2012). In addition, a clinical safety evaluation of crocin in healthy volunteers showed a relatively safe and normal profile within the trial period (Mohammadpour et al. 2013). These findings suggest that crocin could be a promising candidate in the prevention and treatment of oxidative stress-related neurodegeneration disorders.

It is believed that $A\beta$ is a key mediator in the pathogenesis of neurodegeneration and PHF formation by inducing abnormal phosphorylation of tau protein (Lee et al. 2001). The accumulation of $A\beta$ induces tau hyperphosphorylation through two signal transduction pathways including activation of receptors on neuronal membranes and oxidative stress induction. The $A\beta$ peptides can directly bind to the related membrane receptors, such as $\alpha7$ nicotinic acetylcholine receptor ($\alpha7$nAChR), which could subsequently induce phosphatidylinositol 3-kinase PI3K/Akt pathway. Activation of PI3K/Akt induces the phosphorylation of GSK-3β which is involved in the hyperphosphorylation of tau. Furthermore, reactive oxygen species (ROS) mediates tau hyperphosphorylation by activation of Mitogen Activated Protein Kinases (MAPK) pathway, including Extracellular Signal-Regulated Kinases (ERK), stress-activated protein kinases c-Jun N-terminal kinase (SAPK/JNK) and p-38 kinase (p38) (Ferrer et al. 2005, Huang and Jiang 2009). In fact, phosphorylation of GSK-3β at Ser9 by p-Akt inhibits GSK-3β activity. The regulation of phosphorylation inputs to GSK-3β may be impaired by neurodegeneration, and is associated with NFTs formation in neurodegenerative disease (Griffin et al. 2005).

To assess the effects of acrolein on phosphorylation of tau protein we chose Ser 396 and Thr 231 residues,

because these sites are targets of GSK-3β, and are also related to NFT formation in neurodegeneration disease (Rankin et al. 2007).

Western blot analysis revealed that p-Ser473Akt levels increased in the cerebral cortex of acrolein-treated rats, but levels of the downstream Akt target, p-Ser9 GSK-3β, remained unchanged.

However, a previous study on human neuroblastoma cells indicated that acrolein induces hyperphosphorylation of tau protein at ser396/404 as a consequence of GSK-3β and p38 stress-activated protein kinas activation (Gómez-Ramos et al. 2002).

However, in our study, alterations in GSK-3β activity were not involved in tau phosphorylation following exposure to acrolein. It was previously indicated that the contribution of GSK-3 was not the only pathway for phosphorylation of tau *in vivo* (Salkovic-Petrisic et al. 2006). Our data showed that acrolein significantly increased phosphorylation of tau protein at both Ser 396 and Thr 231 residues.

It has been demonstrated that activation of the MAPK signalling pathway in response to oxidative damage (Ferrer et al. 2005) and activation of GSK-3β in response to $A\beta$ peptide accumulation contribute to pathological tau hyperphosphorylation *in vivo* (Muñoz-Montaño et al. 1997).

We examined the implication of MAPK proteins in acrolein-induced tau over-phosphorylation. Our findings showed that acrolein induced production of reactive oxygen species which led to phosphorylation and activation of JNK, p38 and ERK1/2.

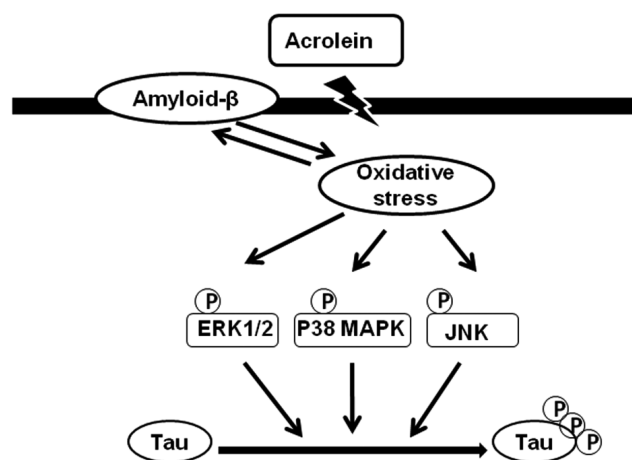Among kinases, c-Jun N-terminal kinases (JNK)

Fig. 6. Diagram showing acrolein-induced signalling in tau hyperphosphorylation.

and p38 are two important members of stress-activated protein kinase (SAPK) pathways and are the main mediators that magnify stress signals to the nucleus. A number of studies have indicated that Aβ induces JNK activation in different types of neuronal cells, and that activated JNK is implicated in the phosphorylation of multiple sites of tau (Su et al. 2008).

Similar to SAPKs, activation of the ERK pathway resulted in elevated levels of phosphorylated tau proteins at multiple sites. In addition, ERK may have an important role in mediating Aβ-induced tau phosphorylation *in vivo* (Zhu et al. 2002).

Our study found that acrolein led to severe induction of oxidative stress and Aβ concentration. Our data showed that treatment with acrolein activates ERK, JNK and p38 in brain tissue. These findings indicated that the MAPK pathway, but not the Akt/GSK-3β pathway, was involved in phosphorylation of tau in acrolein intoxication (Fig. 6). Furthermore, crocin reduced tau phosphorylation by attenuation of active forms of ERK and JNK kinases without any effects on levels of p-GSK-3β.

In a previous brain proteome study we also demonstrated that oral administration of acrolein could alter the levels of several proteins involved in vital neural cell processes, including energy metabolism, antioxidant systems, cell communication and transport. Interestingly, a number of differentially expressed proteins, such as α/β-synuclein, Rho GDP-dissociation inhibitor 1(RGDIR1) and Serine/threonine-protein phosphatase 2B catalytic subunit alpha (PP2BA), are known to be associated with human neurodegenerative disease. α/β-synuclein is a marker of PD, and PP2BA is the main protein phosphatase involved in tau dephosphorylation. Moreover, RGDIR1 is the cell signalling protein which regulates recycling of GTPases and cell membrane trafficking (Rashedinia et al. 2013).

These results coincide with recently published studies demonstrating that chronic oral exposure to acrolein (2.5 mg/kg/day) induces oxidative stress and neuronal damage in the rat brain, as well as AD-like pathologies including neurobehavioral and mild cognitive declination. Furthermore, it has been shown that acrolein could potently modulate the Aβ metabolism, increase levels of amyloid precursor protein and induce p38 and JNK activation in the hippocampal neuronal cell line (Huang et al. 2013a,b).

In the present study, we have reported for the first time that oral exposure to acrolein could induce Aβ elevation and tau hyperphosphorylation in brain tissue.

CONCLUSIONS

We have demonstrated that oxidative stress induced by acrolein increased amyloid levels in the rat cerebral cortex and elevated the activated forms of JNK, p38 and ERK1/2 MAPKs; all of these events taken together may increase tau phosphorylation. Our findings suggest that the molecular mechanisms of exogenous acrolein-induced neural injuries have some similarities to neurodegeneration pathology.

Furthermore, the reduction of tau phosphorylation and Aβ concentration *via* modulation of MAPKs expression is probably involved in the neuroprotective mechanism of crocin. Moreover, crocin may provide a promising approach for the treatment of neurodegenerative diseases such as AD.

ACKNOWLEDGMENTS

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