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CURCUMIN REDUCES OXIDATIVE DAMAGE BY INCREASING REDUCED GLUTATHIONE AND PREVENTING MEMBRANE PERMEABILITY TRANSITION IN ISOLATED BRAIN MITOCHONDRIA

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Abstract

Mitochondria are critical regulators of energy metabolism and programmed cell death pathways. Mitochondria are also the major site for the production of reactive oxygen species which make this organelle more susceptible to oxidative damage and impairments of mitochondrial functions. Antioxidants have been of limited therapeutic success to ameliorate the toxic effects of oxidative stress in mitochondria. One reason may be the inability of mitochondria to selectively take up antioxidants. In the present study we synthesized mitochondrially targeted curcumin with an aim of delivering this polyphenolic compound to isolated mitochondria. Our observations show the strong anti-oxidative effects of curcumin and mitochondrially targeted curcumin against the lipid peroxidation, protein carbonylation and mitochondrial permeability transition induced by *tert*-butylhydroperoxide. Both curcumin and mitochondrially targeted curcumin significantly enhanced endogenous reduced glutathione level in the mitochondria thus preserving mitochondrial defense system against oxidative stress. We concluded that curcumin and mitochondrially targeted curcumin protected mitochondria against *tert*-butylhydroperoxide by lowering the oxidative damage, increasing the availability of endogenous reduced glutathione and preserving the mitochondrial integrity. Importantly, mitochondrially targeted curcumin was found most effective in ameliorating oxidative stress and preserving mitochondrial integrity than curcumin.

Key words: Mitochondria, curcumin, reactive oxygen species, oxidative stress, membrane permeability transition.

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INTRODUCTION

The mitochondria are cellular power plants essentially play central roles in a number of important metabolic activities, including intermediate metabolic pathways, amino acid biosynthesis, fatty acid oxidation, urea cycle, lipid metabolism, porphyrin synthesis, homeostasis of steroid hormones, homeostasis of cellular calcium ions and apoptosis (45). Similar to all other cells, brain cells are highly depend on the mitochondrial energy source for synaptic activity, promotion of neurogenesis, suppression of pathologic apoptosis, and neuronal molding. Any compromise in this demand leads to catastrophic consequences such as activation of various cascades of dysfunctional metabolism that may impair cellular connectivity, communication and finally to the cell death. Defects in mitochondrial dynamics along with alteration in energy metabolism increase generation of reactive oxygen species (ROS) (6). These abnormally high levels of ROS can increase lipid peroxidation (LPO), oxidize essential proteins and damage nucleic acids thus leading to damage of cellular organelles. The obvious way to intervene oxidative damage is through the use of compounds with antioxidant properties that may reduce the levels of ROS and may prevent the impairment of mitochondria. Despite the clear role of oxidative stress in mitochondrial impairment the use of conventional non enzymatic antioxidants such as vitamin E or vitamin C have little or no protective values (4, 7). One possible explanation for this response may be due to the non-specific nature of antioxidant therapies (32). Consequently, the generalized antioxidants show limited potential and may not locate to mitochondria in sufficient amounts to impact on the oxidative damage. To address this unmet need, investigators have begun to examine the efficacy of antioxidant therapies specifically targeted to mitochondria (31, 32, 51, 53, 56). Lipophilic triphenylphosphonium ion (TPP) when conjugated with antioxidants may serve as an excellent carrier for delivery of antioxidants to mitochondria. Since mitochondria have negative membrane potential therefore the positively charged TPP ion rapidly permeate the mitochondrial lipid bilayer to accumulate several hundred folds within mitochondria (52). The accumulation of antioxidants conjugated with TPP thus may provide protection to mitochondria against oxidative stress more efficiently than non-targeted antioxidants (59).

The *tert*-butylhydroperoxide (t-BuOOH) is a short chain analog of lipid hydroperoxides which mimics the toxic effect of peroxidized fatty acids. Peroxy radicals can be generated from t-BuOOH in the mitochondria by its interaction with ferrous iron in a reaction similar to the Fenton reaction (24). Mitochondria are a major target of the cytotoxicity of t-BuOOH (19, 34). Curcumin {1,7-bis(4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione (diferuloylmethane), the main yellow bioactive polyphenolic, non- flavonon, pharmacologically active component (39) of turmeric {Curcuma longa (zingiberaceae)} has been shown to have a wide spectrum of therapeutic actions such as anti- parasitic (48), anti- inflammatory (3), anti-carcinogenic (49) anti- protein-aggregate (8) and antioxidant activities (10). Curcumin, also serves as a potential sensor for some small biologically important molecules (20). Curcumin was used as one of the main ingredients in Ayurvedic preparation for the human embryonic kidney cell viability (54). The antioxidant activity of curcumin protects the brain from free radical induced oxidative damage and improves learning and memory (36). It is thought to be comparatively effective to standard antioxidants such as vitamin E, (60) vitamin C, and more effective than supe-

roxide dismutase (SOD) (46, 47, 55, 57). In the present study, we evaluated the protective effects of curcumin and mitochondrially targeted curcumin (mt-curcumin) against the adverse oxidative damage caused by t-BuOOH.

MATERIALS AND METHODS

Chemicals

Triphenylphosphine, HCl, ethyl acetate, ethanol, sodium hydroxide (NaOH), were purchased from HiMedia Laboratories (Mumbai, India). Potassium dihydrogen orthophosphate (KH_2PO_4), dipotassium hydrogen orthophosphate (K_2HPO_4), metaphosphoric acid, sodium chloride and ethylene diamine tetra acetate (EDTA) were obtained from Merck, India. Streptomycin sulphate, curcumin, trichloroacetic acid (TCA), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) and di-nitrophenyl-hydrazine (DNPH) were obtained from Sigma Chemical Company Inc. (St. Louis, MO, USA).

Synthesis of targeted antioxidant

The mt-curcumin was synthesized by covalent linkage of curcumin with lipophilic TPP. The charge of the lipophillic cation evenly can distribute over a large hydrophobic surface area by which activation energy for their movement across the mitochondrial membrane lowers down thus they easily passes through mitochondrial membrane (26, 43). TPP was reacted with a brominated precursor to obtained phosphonium lipophilic cation. To synthesize targeted derivative, a solution of bromo-antioxidant (curcumin: 5.42 mmol) was refluxed and evaporated the volatiles under vaccum below 100°C. The compound obtained was hygroscopic, dissolved in methanol and precipitated after adding n-hexane. Filtered precipitate was again dissolved in methanol containing triphenylphosphine (3.22 mmol). After refluxing the solution, the volatiles were evaporated under vaccum to obtain targeted derivative of curcumin.

Experimental animal

Adult Swiss albino mice (Mus Musculus albinus) weighing 25-30 g were obtained from College of Veterinary Science and Animal Husbandry Mhow, India. All studies using these mice were approved by the research committee of Vikram University in accordance with the International guidelines for the care and use of laboratory animals. All animals were kept under the same environmental conditions i.e. at a room temperature 25 ± 1°C with 12 hr light / dark cycle.

Mitochondrial preparation

The animals were sacrificed by decapitation. Tissue (brain) were rapidly excised and placed into ice cold isolation medium (0.25 M sucrose). Tissue was weighed finely minced and homogenized (10% w/v) in an isolation medium using glass homogenizer. Mitochondria were isolated from normal mice brain by conventional differential centrifugation method as described (38). Isolation buffer contains 0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HC1 (pH 7.4). The experiments were performed immediately after purification of the mitochondria.

Mitochondrial treatments

Mitochondria were incubated in a reaction mixture containing; 120 mM KCl, 2mM phosphoric acid and 15 mM Tris. The experiment was performed in four groups. The first group served as Control. Mitochondria of the second group were incubated with t-BuOOH (0.4 µM) for 30 minutes. In the third group mitochondria were pre treated with five different concentrations of curcumin (i.e. 100 µM, 200 µM, 300 µM, 400 µM and 500 µM) for 15 minutes and then incubated with t-BuOOH for another 30 minutes. The incubation mixture of the mitochondria of the fourth group was the same except the curcumin was replaced with mt-curcumin.

Sample preparation for assays

After incubation, different groups of mitochondria were assayed using suitable buffers. 0.05 M phosphate buffer was used for LPO assay, 100 mM phosphate buffer (pH 7.4) containing 0.1% digitonin was used for protein carbonyl assay and 0.1 mol/l cold metaphosphoric acid containing 0.02% EDTA and 0.1 m mol/l DTNB was used for reduced glutathione (GSH) assay.

Lipid peroxidation (LPO)

LPO was determined by measuring thiobarbituric acid reactive substance (TBARS) in terms of malonaldehyde equivalent (MDA) using the molar extinction coefficient of 1.56 x 10^5 $min^{-1}.cm^{-1}$ as described (35). Briefly, specimens were homogenized in 0.05 M phosphate buffer pH 7.70, centrifuged at 3,000 x g for 15 min, and the supernatant was used for the assay. Samples of 0.1 ml supernatant were mixed with 0.2 ml of 8.1% SDS, 1.5 ml 20% glacial acetic acid, and 1.5 ml of 0.8% thiobarbituric acid (TBA). Following these additions, tubes were mixed and heated at 95 °C for 60 min in a water bath, and cooled under tap water before mixing with 1 ml distilled water and 5 ml mixture of n-butanol and pyridine (15: 1). The mixture was centrifuged at 2,200 x g for 10 min. The TBARS value was determined by measuring the absorbance of upper organic layer at 532 nm. The results were expressed as nmol TBARS/mg protein.

Protein carbonyl content

Protein carbonyl content was assessed according to Levine et al., (1990) (25) with some modifications. Briefly: Mitochondrial samples obtained from different groups were homogenized in 100 mM phosphate buffer pH 7.40 containing 0.1% digitonin. Mixture was centrifuged at 4000 x g. Supernatant was taken and equally divided in two test tubes and protein was precipitated by adding equal volumes of 20% trichloroacetic acid (TCA). The solution recentrifuged at 2800 x g and supernatant was discarded. 1.5 ml DNPH (10 m mol/l) in 1 mol/l HCl was added to one tube and 0.5 ml DNPH in 2 mol/l HCl was added to the another tube. Both the tubes were vortex mixed for 1 hour. 1.5 ml 20% TCA was added and centrifuged at 11,000 x g. The pellets were washed three times with ethyl acetate: ethanol mixture (1:1 v/v) to remove the excess of DNPH. The final protein pellet was dissolved in 1.25 ml 6 M guanidine hydrochloride and the carbonyl content was evaluated by detecting the absorbance at 370 nm in a Perkin-Elmer UV spectrophotometer. A standard curve was obtained using bovine serum albumin (BSA) and included in each assay to determine linearity and the extent of derivatization. The results of carbonyl content were expressed in terms of nmol carbonyl group/mg protein.

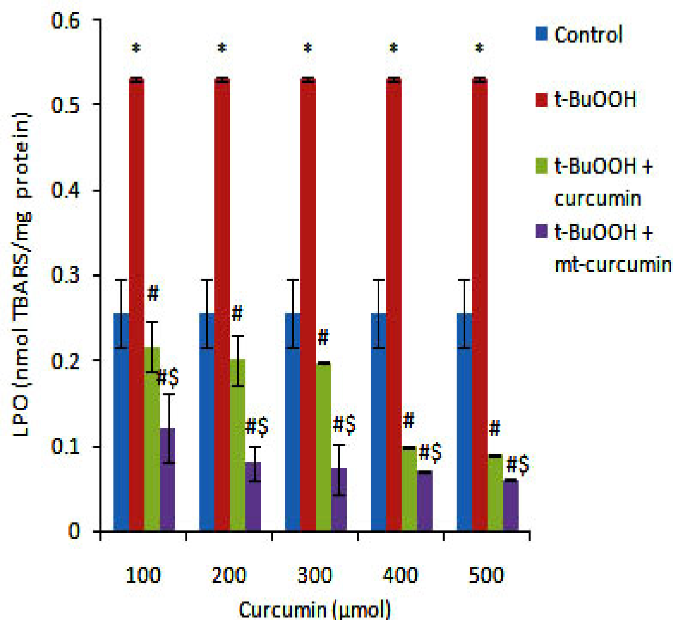

Figure 1. Showing lipid peroxidation (LPO; nmol TBARS/mg protein) in mice brain mitochondria treated with t-BuOOH (0.4 µM), t-BuOOH (0.4 µM) + curcumin (100 µM to 500 µM), t-BuOOH (0.4 µM) + mt-curcumin (100 µM to 500 µM). Mitochondria of control group were treated with vehicle only. *Significant different from control ($p<0.05$; n = 5), #significant difference from t-BuOOH treated mitochondria ($p<0.05$; n = 5), $significant difference from curcumin ($p<0.05$; n = 5).

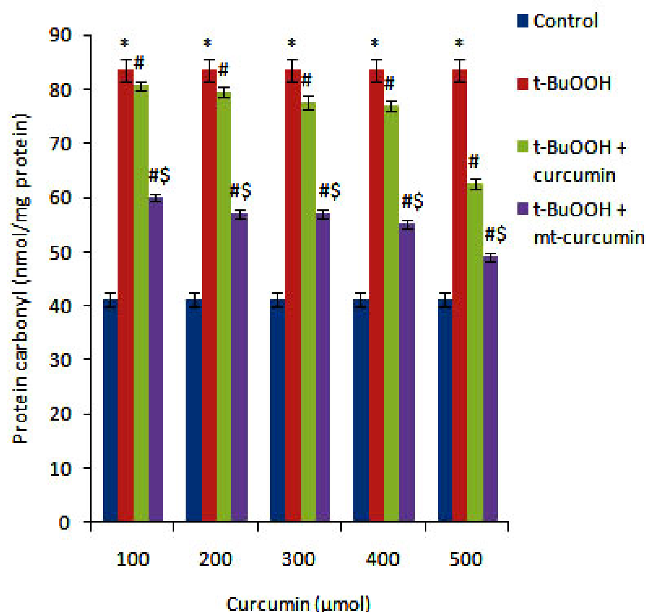

Figure 2. Showing protein carbonyl (nmol /mg protein) in mice brain mitochondria treated with t-BuOOH (0.4 µM), t-BuOOH (0.4 µM) + curcumin (100 µM to 500 µM), t-BuOOH (0.4 µM) + mt-curcumin (100 µM to 500 µM). Mitochondria of control group were treated with vehicle only. *Significant different from control ($p<0.05$; n = 5), #significant difference from t-BuOOH treated mitochondria ($p<0.05$; n = 5), $significant difference from curcumin ($p<0.05$; n = 5).

Reduced glutathione (GSH)

The GSH content of tissue homogenates was quantitated as described (22) involving the spectrophotometric assessment of the formation of 5-thio-2-nitrobenzoate from DTNB in the presence of NADPH and glutathione reductase. Briefly, specimens were homogenized in 0.5 M ice cold metaphosphoric acid and centrifuged at 16,000 x g for 15 min at 4 ºC. The supernatant (0.5 ml) was equilibrated with 4 ml of ice cold 0.1 mM solution of DTNB in 0.1 M phosphate buffer pH 8.0 and the optical density was obtained at 412 nm in a Perkin-Elmer UV-Spectrophotometer. A calibration curve was prepared using GSH as standard.

Determination of membrane permeability transition (MPT; Mitochondrial swelling)

Isolated mitochondria (0.5 mg of protein) were re-suspended in 2 ml Hank's balanced salt solution (HBSS; 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4, 0.49 mM $MgCl_2$, 0.41 mM $MgSO_4$, 132 mM NaCl supplemented with 1.26 mM $CaCl_2$ and 10mM HEPES, pH7.3). Experiment was performed on isolated mitochondria in different test tubes at room temperature with t-BuOOH (0.4 µM), t-BuOOH + curcumin (500 µM) and t-BuOOH + mt-curcumin (500 µM). Mitochondrial swelling was estimated from the changes of light scattering at 540 nm measured in mitochondrial suspensions (0.5 mg of protein in 2 ml) as described (37).

Assay of Total Protein

Total protein content of mitochondrial fraction was measured by Folin-phenol reaction as described by Lowry *et al.* (1951) (27). The standard curve of BSA was included in each assay to determine linearity and measured the extent of derivatization.

Statistical Analysis

All data are expressed as mean ± Standard Error mean (S.E.M.) statistical comparisons were made relative to the appropriate control group by student's t test. The 0.05 level was selected as point of minimal statistical significance in every comparison.

RESULTS

Isolated brain mitochondria were used for the evaluation of protective effect of curcumin and mt-curcumin against well known mitochondrial oxidant, t-BuOOH. First we tested the pro-oxidant effect of t-BuOOH on mitochondrial LPO. As shown in Fig. 1 our results showed a significant ($p < 0.05$) increase in LPO (from 0.256 ± 0.04 nmol TBARS/mg protein in control to 0.53 ± 0.002 nmol TBARS/mg protein in t-BuOOH treated mitochondria) when brain mitochondria were incubated with 0.4 µM t-BuOOH for 30 min. Pretreatment of mitochondria with different concentrations of curcumin and mt-curcumin resulted in significant reduction of LPO in mitochondria compared to t-BuOOH treated group. Pretreatment of mitochondria with mt-curcumin was more effective in reducing the levels of LPO against t-BuOOH when compared with pretreatment of mitochondria with curcumin ($p < 0.05$).

Next we tested the pro-oxidative effect of t-BuOOH on protein carbonyl in brain mitochondria. Fig. 2 shows a significant ($p < 0.05$) increase (83.62 ± 2.00 nmol/mg protein) in protein carbonyl in mitochondria treated with t-BuOOH in comparison to control (41.18 ± 1.23 nmol/mg protein). Pretreatment of mitochondria with different concentrations of both curcumin and mt-curcumin caused reduction in protein carbonyl content as compared with t-BuOOH treated mitochondria. The mt-curcumin was found more effective in ameliorating the pro-oxidant ef-

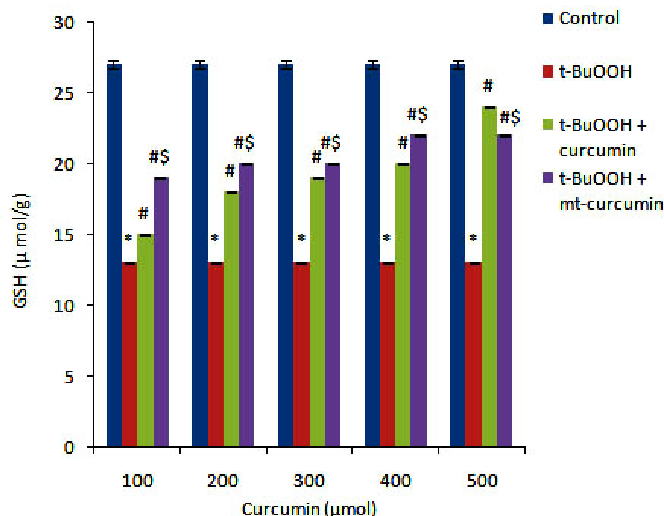

Figure 3. Showing reduced glutathione (GSH; µmol /g) in mice brain mitochondria treated with t-BuOOH (0.4 µM), t-BuOOH (0.4 µM) + curcumin (100 µM to 500 µM), t-BuOOH (0.4 µM) + mt-curcumin (100 µM to 500 µM). Mitochondria of control group were treated with vehicle only. *Significant different from control (p<0.05; n = 5), [#]significant difference from t-BuOOH treated mitochondria (p<0.05; n = 5), [$]significant difference from curcumin (p<0.05; n = 5).

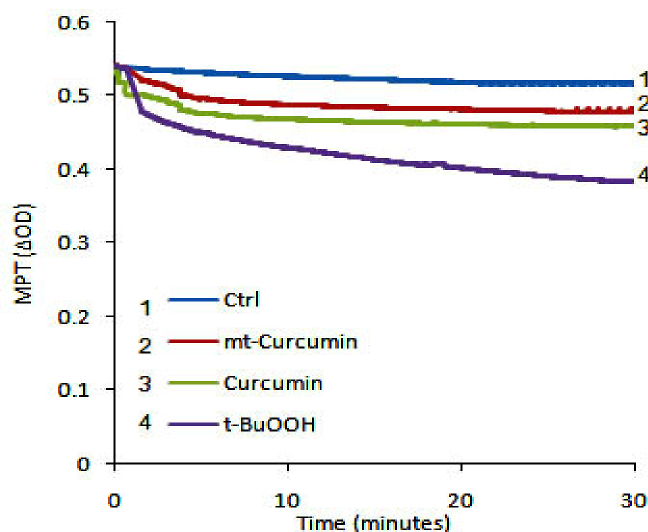

Figure 4. Showing mitochondrial permeability transition (MPT; ΔOD) in mice brain mitochondria treated with t-BuOOH (0.4 µM), t-BuOOH (0.4 µM) + curcumin (500 µM), t-BuOOH (0.4 µM) + mt-curcumin (500 µM). Mitochondria of control group were treated with vehicle only. Each treatment is the mean of atleast 5 observations (n = 5).

fect of t-BuOOH than curcumin ($p < 0.05$).

Mitochondrial GSH serves as a major antioxidants and detoxifying enzymes and maintain the appropriate mitochondrial redox environment to avoid or repair oxidative modifications leading to mitochondrial dysfunction and cell death. As shown in Fig. 3 the treatment of t-BuOOH caused a significant ($p < 0.05$) lowering of GSH levels (13 ± 0.07 µmol /gm) in the mitochondria compared to control (27 ± 0.29 µmol /gm). Pre-incubating mitochondria with different concentrations of curcumin and mt-curcumin increased the level of GSH compared with t-BuOOH treatment. The mt-curcumin was found more effective in preserving the GSH in mitochondria than curcumin ($p < 0.05$).

Mitochondrial oxidative stress augments MPT which is frequently the decisive event for cell survival or cell death. Fig. 4 shows an increase in the MPT by t-BuOOH treatment as observed by the decrease in the absorption. The pre-incubation of mitochondria with curcumin and mt-curcumin prevents the MPT induced by t-BuOOH. The mt-curcumin was more effective in prevention of MPT than curcumin.

DISCUSSION

Increased ROS and oxidative stress has been associated with brain mitochondrial damage which in turn affect many cellular processes in the cell. Curcumin possesses both phenolic and β-diketone functional groups shows significant antioxidant and free radical scavenging activities (42). In addition, the curcumin also enhances the activities of anti-oxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase (41). In mitochondria, the curcumin has been shown to reduce ROS and prevent oxidative damage (61). The efficacy of curcumin to enter into mitochondria and provide protection against oxidative damage can be amplified by targeting this flavonoid to mitochondria. In the present study we have prepared mt-curcumin by conjugating curcumin with lipophilic cation, TPP and then delivered it to mitochondria isolated from mice brain. Since mitochondria have negative membrane potential this conjugated anionic compound can easily passes through mitochondrial membranes and can provide protection to mitochondria. Our observations showed that both curcumin and mt-curcumin significantly protected the oxidative damage and increased the mitochondrial antioxidant defense. However, mt-curcumin was found most efficacious in reducing oxidative burden from isolated mitochondria.

Curcumin interacts with oxidative cascade by scavenging or neutralizing free radicals, inhibiting LPO and maintains cell membrane integrity and their function (1). As formation of lipid peroxides are presumptive markers for oxidative damage (14) so in this study first we tested the effect of t-BuOOH on LPO in brain mitochondria. Our results showed a significant increase in the LPO by t-BuOOH. The incubation of t-BuOOH treated mitochondria with curcumin significantly decreased the levels of LPO. Preincubation of mitochondria with mt-curcumin caused further reduction of LPO. The present study shows the efficacy of both curcumin and mt-curcumin in reducing the LPO induced by t-BuOOH. We obtained the greater efficacy of mt-curcumin than curcumin in reducing the levels of LPO. In previous studies (44) also the efficacy of mt-curcumin was analyzed in mitochondria isolated from spinal cord. Data showed reduction in LPO in the spinal cord mitochondria by mt-curcumin against nicotine toxicity (44). In addition, mitochondria isolated from cardiac cells of rat also show reduced LPO level by curcumin given prior to experimental ischemia (30 min) and reperfusion (60 min) (I/R) (15). Curcumin was also found to ameliorate indomethacin drug-induced LPO in mitochondria isolated from small intestine (50).

Protein carbonyls are used as a marker of protein oxidation that formed as a consequence of the oxidative stress. In the present study, the effects of curcumin and mt-curcumin on t-BuOOH dependent carbonyl content of brain cells were investigated. As shown in Fig. 2 an increase in protein carbonyl level was found when isolated mitochondria were subjected to t-BuOOH exposure. Mitochondria pretreated with both curcumin and mt-curcumin inhibits

oxidative damage marked by a decrease in the level of carbonylated proteins. The mt-curcumin showed more efficacy of preventing protein carbonylation than mitochondria incubated with curcumin. Earlier reports have indicated that curcumin may reduce oxidative stress conditions and delay age associated increase in protein carbonyl levels in brain cells (9). Curcumin also found to maintain mitochondrial redox and reduce mitochondrial protein carbonylation after 4-HNE treatment (40). The potassium dichromate induced increase in protein carbonyl content in mitochondria isolated from rat liver cells was also prevented by curcumin (12). GSH is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) and its level is necessary for maintaining cellular redox potential and in cellular defenses against oxidative stress (29). In mitochondria GSH is found mainly in reduced form and represents a minor fraction of the total GSH pool (13, 16). Mitochondrial GSH also has an important role in maintaining the mitochondrial integrity (28). The GSH depletion under stressed condition can increase mitochondrial damage and cause defect in mitochondrial energy conservation (17). In the present study t-BuOOH treatment caused a significant depletion of GSH in the mitochondria. Our previous studies shows that the decreased level of GSH level in t-BuOOH treated brain cells may be due to its enhanced utilization during detoxification of t-BuOOH (2). Prior treatment of various concentrations of both curcumin and mt-curcumin caused the gradual restoration of GSH content in the mitochondria. Notably, the mt-curcumin was found most effective in restoring GSH level than curcumin (Fig. 3). Our observations are clearly consistent with previous studies showing the effectiveness of mt-curcumin in defending the mitochondria from lethal effects of oxidative stress. In an in vitro study, the curcumin was found to restore the GSH when cells are treated with sodium fluoride (33). Similarly, potassium dichromate induced decrease of GSH was effectively prevented by curcumin pretreatment in rat hepatic mitochondria (12). Curcumin also augment levels of GSH in mitochondria isolated from cortical neurons (61) and from cardiac cells of rat during experimental ischemia and reperfusion (15).

Increased oxidative stress can compromise mitochondrial membrane potential that can induce the MPT, leading to mitochondrial swelling (18, 23). One of the mechanism by which oxidative stress may promote MPT is by oxidation of thiol groups on the adenine nucleotide translocator (ANT) (58). Swelling of mitochondria can be measured in terms of decrease in optical density at 540 nm by the changes of volume of the mitochondrial matrix. A decrease in mitochondrial swelling is marked by an increase in absorbance whereas an increase in mitochondrial swelling is marked by a decrease in absorbance at 540nm. In the present study we found a significant increase in MPT as demonstrated by a decrease in the optical density by incubation of mitochondria with t-BuOOH. However, preincubation of mitochondria with both curcumin and mt-curcumin decreased MPT in isolated mitochondria treated with t-BuOOH. The mt-curcumin was found most effective in preventing MPT than curcumin. Our results are consistent with the previous results obtained by Morin et al. (2001) (30). These studies showed that t-BuOOH induced mitochondrial swelling can be protected by curcumin. Also curcumin can prevent isoprenaline induced opening of the MPT and mitochondrial swelling in cardiac cells (21). In addition, curcumin reduce potassium dichromate induced MPT (12) and prevents mitochondrial dysfunction in an aging model (11).

Although the exact mechanisms how curcumin and mt-curcumin attenuate mitochondrial oxidative damage and prevents MPT are unknown, the present and previous studies have shown that curcumin has the potential to inhibit LPO and effectively intercept and neutralize ROS and reactive nitrogen species (5). We conclude that both curcumin and mt-curcumin are able to reduce the oxidative damage in isolated mitochondria of brain but targeting mitochondria with mt-curcumin is more effective in reducing mitochondrial damage and preventing MPT than curcumin. Thus mt-curcumin in future may prove as a therapy for treatment of conditions associated with mitochondrial oxidative stress and dysfunctions subject to the confirmation of results in in vivo conditions.

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