Original Paper

Curcumin Inhibits Heat-Induced Apoptosis by Suppressing NADPH Oxidase 2 and Activating the Akt/mTOR Signaling Pathway in Bronchial Epithelial Cells

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Key Words

Curcumin • Heat • Apoptosis • NADPH Oxidase 2 • Akt/mTOR • Thermal Inhalation Injury

Abstract

Background: Heat causes bronchial epithelial cell apoptosis, which is a known factor contributing to airway damage during thermal injury. Accumulating evidence has shown the effect of curcumin on inhibiting apoptosis. In this study, we investigated whether curcumin suppresses heat-induced apoptosis in bronchial epithelial cells and the underlying mechanism.

Methods: Bronchial epithelial cells 16HBE140 cells were incubated at either 42 °C, 47 °C, 52 °C, or 57 °C for 5 min in a cell incubator and then returned back to normal culture conditions (37 °C). An *in vivo* thermal inhalation injury rat model was established with a heat gun blowing hot air into the airway of rats. 16HBE140 cells and lung tissue were obtained for further study with or without curcumin treatment. Cell viability was determined by measuring the absorbance of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). 2',7'-dichlorofluorescein acetate fluorescence was used as a measure of reactive oxygen species (ROS) production. Levels of Bcl2, Bax, α -ATP, cleaved Poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, gp91^{phox}, p47^{phox}, p67^{phox}, p22^{phox}, p40^{phox}, and Rac were determined by Western blotting. TUNEL staining was used to determine apoptosis. **Results:** Heat treatment triggered the apoptosis of 16HBE140 cells as shown by the increase in apoptosis molecular markers including Bcl-2, Bax, cleaved PARP, and cleaved caspase-3. Administration of curcumin significantly inhibited apoptosis of 16HBE140 cells and suppressed the membrane translocation of NADPH oxidase 2 cytosolic components, as well as ROS production. Downregulation of Akt and mTOR phosphorylation induced by heat was also reversed by curcumin. Furthermore, we demonstrated that NADPH oxidase 2 is upstream of Akt/mTOR in heat-induced apoptosis. The protective role of curcumin on bronchial epithelia apoptosis was also confirmed *in vivo* by a rat inhalation injury model. **Conclusion:** This study demonstrates that one of the critical mechanisms underlying curcumin inhibiting heat-induced apoptosis is through suppressing NADPH Oxidase 2 and activating the Akt/mTOR signaling pathway in bronchial epithelial cells.

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Introduction

Thermal inhalation injury is a common concurrent injury in burn patients and is associated with significant longstanding pulmonary dysfunction and mortality [1]. Previous studies have suggested that bronchial epithelial cells are early targets of thermal inhalation injury [2]. Most studies have focused on the bronchial epithelial inflammation induced by chemical smoke or heat, ignoring that heat can also induce cell apoptosis and death, contributing to the inhalation injury [3]. Heat is a major extracellular stimulus that causes cellular oxidative stress and apoptosis [4]. However, the pathogenesis of heat-induced bronchial epithelia apoptosis remains largely unknown.

Overproduction of reactive oxygen species (ROS) triggers cell apoptosis through various signaling cascades [5, 6]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the primary source of superoxide production and consists of two subunits on the membrane (p22phox and gp91phox) and four subunits in the cytoplasm (p47phox, p67phox, p40phox, and Rac). Upon activation, the cytoplasmic subunits translocate to the membrane and combine with the membrane subunits to trigger NADPH oxidase activation [7]. In the present study, we aim to determine whether ROS derived from NADPH oxidase is involved in heat-induced apoptosis of bronchial epithelial cells both *in vitro* and *in vivo*.

Akt, a serine/threonine kinase, plays an important role in controlling pathophysiological changes [8]. An increasing number of studies have reported that phosphorylated Akt prevents cells from undergoing apoptosis, which is regulated by ROS [9]. The mammalian target of rapamycin (mTOR) serves as a central regulator of cell apoptosis by integrating both intracellular and extracellular signals. Discoveries over the last decade have shown that the Akt/mTOR pathway is significantly associated with cell apoptosis [8]. Currently the relationship between NADPH oxidase, ROS and Akt/mTOR after heat treatment is not clear.

Curcumin, an active polyphenol derived from the rhizomes of *Curcuma longa*, is reported to have antioxidant and anti-apoptotic effects [10]. Recent studies have indicated that long-term curcumin administration significantly inhibits oxidative stress and apoptosis in human neuroblastoma cells [11]. Curcumin has also been reported to regulate the PKC δ /NADPH oxidase/ROS and Akt/mTOR signaling pathways in cancer cells [12, 13]. However, whether curcumin regulates NADPH oxidase/ROS or the Akt/mTOR signaling pathway after heat treatment and the underlying mechanisms are poorly understood.

In the present study, we hypothesized that curcumin suppressed heat-induced apoptosis by regulating NADPH/ROS and the Akt/mTOR signaling pathway.

Materials and Methods

Cell culture and treatment

16HBE140 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin in a CO₂ incubator at 37 °C. Then, cells were incubated at 42 °C, 47 °C, 52 °C, or 57 °C for 5 min, moved to 37 °C and harvested after 12 h, 1 day, 2 days, and 5 days, following previous reports [14]. Curcumin (Sigma-Aldrich, Shanghai, China) was added to the culture medium 4 h before or 1 h after the heat treatment. In some circumstances, DPI (10 μ M Sigma-Aldrich, Shanghai, China), gp91ds-tat (10 μ M Sigma), sgp91 ds-tat (5 μ M, Sigma), IFG-1 (100 μ M, Sigma), or Mhy1485 (50 μ M, Sigma) were added 4 h before the heat treatment.

Animals and thermal inhalation injury

All procedures were approved by the Animal Ethical Committee of Chongqing Medical University and were carried out in accordance with the approved guidelines. The thermal inhalation injury model was carried out as previously described [15]. Curcumin (10 mg/kg body weight) was administered intragastrically two days before the thermal inhalation and administered twice daily after the surgery. Both the model and sham groups were given a gavage of distilled water at the same volume as the drug.

Cell viability assay

Cell viability was determined by measuring the absorbance of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in living cells in accordance with the manufacturer's instructions [16]. Briefly, cells were seeded into 96-well microtiter plates containing 100 μ L of medium at the previously described temperatures (37 °C, 42 °C, 47 °C, 52 °C, and 57 °C) for 5 min. Then, the cells were harvested at 12 h, 1 day, 2 days, and 5 days after treatment, and 10 μ L of 5 mg/mL MTT (Thermo Fisher Scientific, Fremont, USA) was added to the medium in each well. The plates were incubated for an additional 4 h at 37 °C. After 4 h, the medium was discarded, and 100 μ L of acid isopropanol was added to dissolve the formazan product. The absorbance at 540 nm was measured by a Bio-Tek EPOCH Microplate Reader (Bio-Tek Instrument, USA).

Oxidative stress determination

To evaluate the ROS production, 2',7'-dichlorofluorescein diacetate (DCF-DA) (Thermo Fisher Scientific, Fremont, USA) was used to measure the changes in ROS levels [17]. Briefly, cells were cultured in glass-bottomed dishes and incubated with 5 μ M/L H2DCFDA in the dark for 30 min. Cells were washed thrice times with pre-warmed PBS and then resuspended in PBS. The relative levels of fluorescence were determined by confocal microscopy at the excitation wavelength of 488 nm and emission wavelength of 525 nm. Images were obtained at 10 frames per second using a 4 $\times$ objective (10 nm). 4-hydroxynonenal and nitrotyrosine ELISA Kits were purchased from Cell Biolabs, Inc. (San Diego, CA) and used according to the manufacturer's instructions. The data were normalized by protein levels. All assays were performed in triplicate.

Preparation of the membrane fraction

To separate the cellular membrane, cells were lysed in lysis buffer containing 250 mM sucrose, 20 mM HEPES (7.4), 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, and 1 mM DTT. The cell membrane was separated by ultracentrifugation at 100,000 *g* for 2 h. The membrane fraction was obtained by suspending the pellet with RIPA lysis buffer.

Western blot analysis

16HBE140 cells were harvested and then lysed in RIPA buffer containing a mixture of protease inhibitors (Thermo Scientific, Fremont, CA). The lung tissue was collected at different time points and washed with PBS three times. Aliquots of the lung lysates (30 μ g protein/well) and cell lysates (20 μ g protein/well) were separated on 4–20% Bis-Tris gels. Western blotting was carried out as previously described [15]. Antibodies against Bcl2, p53, p21, p27, p29, p39, p40, p42, p44, p46, p48, p51, p53, p55, p57, p61, p63, p65, p67, p69, p70, p71, p72, p73, p75, p77, p79, p80, p81, p82, p84, p85, p86, p87, p88, p89, p90, p91, p93, p94, p95, p97, p98, p99, p100, p101, p102, p103, p104, p105, p106, p107, p108, p109, p110, p111, p112, p113, p114, p115, p116, p117, p118, p119, p120, p121, p122, p123, p124, p125, p126, p127, p128, p129, p130, p131, p132, p133, p134, p135, p136, p137, p138, p139, p140, p141, p142, p143, p144, p145, p146, p147, p148, p149, p150, p151, p152, p153, p154, p155, p156, p157, p158, p159, p160, p161, p162, p163, p164, p165, p166, p167, p168, p169, p170, p171, p172, p173, p174, p175, p176, p177, p178, p179, p180, p181, p182, p183, p184, p185, p186, p187, p188, p189, p190, p191, p192, p193, p194, p195, p196, p197, p198, p199, p200, p201, p202, p203, p204, p205, p206, p207, p208, p209, p210, p211, p212, p213, p214, p215, p216, p217, p218, p219, p220, p221, p222, p223, p224, p225, 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(Promega, WI, USA) according to the manufacturer's instruction. The positive cells were counted on 10 random sections. The number of positive stained cells was expressed as a percentage of the total number of cells counted in the 10 tissue sections.

Statistics

Statistical analysis was performed using Graphpad prism (Version 6; CA, USA) a Student's t test, one way analysis of variance (ANOVA) and the least significant difference (LSD) test. The data are presented as the mean $\pm$ standard deviation (SD) of three independent experiments for each group. All tests were two tailed, and the level of significance was set at $P < 0.05$.

Results

Heat inhibited cell viability and induced apoptosis in 16HBE140 cells

To test the effect of different temperatures on the 16HBE140 cells, cells were incubated at 37 °C, 42 °C, 47 °C, 52 °C, or 57 °C for 5 min and then recovered at 37 °C. The MTT assay showed a decline in cell viability after a 5-min exposure to elevated temperatures (47 °C, 52 °C, and 57 °C) at 12 h, 1 day, 2 days, and 5 days post-treatment. Treatment at 42 °C had no obvious effect on cell viability. However, treatment at 47 °C or 57 °C resulted in low cell viability throughout all of the time points tested (Fig. 1A). The air temperature in inhalation injury has been reported to be approximately 48.3 °C at the bronchi [19]. Thus, treatment at 47 °C was close to the pathophysiological situation of inhalation injury [20]. We observed that after 47 °C treatment for 5 min, the cell viability decreased from day 1 to day 4 and increased back to control levels at day 5 (Fig. 1B). Fig. 1C, as shown by TUNEL staining, a significantly increased number of cells underwent apoptosis 12 h after heat treatment, with the highest level on day 1 and a return to control level on day 5. The expression of apoptosis-related proteins, including Bcl2, p53, cleaved PARP, and cleaved caspase-3, was markedly increased as well (Fig. 1D).

Protective effects of curcumin against heat-induced apoptosis in vitro

Curcumin is a plant-derived compound and shown to protect cells from apoptosis through different pathways [11]. We proceeded to test whether curcumin exerted a protective effect on heat-induced apoptosis in 16HBE140 cells. The cells were treated with curcumin (10 or 20 $\mu\text{mol/L}$) 4 h before or after heat treatment. One day after heat treatment, cell viability and apoptosis were examined. As shown in Fig. 2A and 2B, cell viability was increased, and the number of TUNEL-positive cells decreased significantly in both the curcumin pre- and post-treatment groups compared to that of the control group. In addition, Western blot analysis showed that cells treated with curcumin showed significantly decreased expression of p53, cleaved PARP, and cleaved caspase-3 and increased expression of Bcl2 compared to that of the control group (Fig. 2C).

Curcumin decreased heat-induced ROS generation by inhibiting membrane translocation of NADPH oxidase 2 cytosolic subunits

Because ROS was involved in the heat-induced apoptosis and NADPH oxidase 2 is the primary source of ROS, we tried to determine whether curcumin treatment alleviated heat-induced apoptosis via inhibiting NADPH oxidase 2/ROS production. DCF-DA fluorescence staining revealed that intracellular ROS increased after heat treatment, peaked on day 1 and returned to normal on day 5 (Fig. 3A). Levels of 4-hydroxynonenal and nitrotyrosine, which are oxidative modification proteins partially produced by superoxide or ROS formation, were also examined by ELISA. Both 4-hydroxynonenal and nitrotyrosine showed a similar changing pattern as that of ROS (Fig. 3B). Moreover, we found that heat-induced ROS generation and the expression of superoxide signal proteins were markedly decreased by curcumin administration, pre- or post- heat treatment (Fig. 3C and 3D). Furthermore, as shown by Western blot analysis, heat treatment significantly increased the expression

Fig 1. Time-dependent heat-induced apoptosis and reduced viability of bronchial epithelial cells. (A) Cell viability was assessed by MTT detection at different temperatures and different time points. (B) Viability was gradually decreased in the cells incubated at 47 °C from 12 h to 1 day post-heat treatment and gradually returned to normal levels on day 5. (C) Representative images and corresponding quantification of TUNEL staining at different time points after 47 °C treatment. (D) Western blot and corresponding quantification of expression of apoptosis-related proteins. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, no significant difference. Comparison to con.

of Rac, p47^{phox}, p40^{phox}, and p67^{phox} in the cellular membrane on day 1, and the expression returned to basal levels on day 5 (Fig. 3E). The expression of cytosolic subunits decreased to almost control levels in both the curcumin treatment group as well as the NADPH oxidase 2 inhibitor DPI and orgp91ds-tat treatment groups. A similar trend was observed in the MPG treatment group, which is known as a ROS scavenger (Fig. 3F). DPI, gp91ds-tat, or MPG treatment also markedly inhibited apoptosis, as indicated by the decreased expression of Bax, cleaved PARP, and cleaved caspase-3 (Fig. 3G). To further explore the role of NADPH oxidase 2 in heat-induced apoptosis, NADPH oxidase 2 siRNA (Nox2 siRNA) was used to downregulate the expression of NADPH oxidase 2 (Fig. 3H). As shown by Western blot

Fig. 2. Protective effects of curcumin against heat-induced apoptosis *in vitro*. (A) 16HBE140 cells after curcumin treatment were analyzed for cell viability by MTT assay. Representative images and corresponding quantification of TUNEL staining 1 day after curcumin treatment. (C) Western blot analysis confirmed that pre- or post-treatment with curcumin attenuated the levels of apoptosis markers. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Compared to con or DMSO treatment.

analysis, downregulation of Mcl-1 and Bcl-2 led to significant decreases in apoptosis markers, suggesting an inhibition of apoptosis (Fig. 3I). These results demonstrated that inhibition of the membrane translocation of NADPH oxidase 2 cytosolic components and Ros production is involved in the anti-apoptosis effect of curcumin in heat treatment.

Curcumin protected cells from heat-induced apoptosis by activating the Akt/mTOR signaling pathway

Akt/mTOR signaling is vital in cell apoptosis[21]. We proceeded to investigate whether Akt/mTOR was involved in the mechanism by which curcumin inhibits heat-induced apoptosis. The results demonstrated that the levels of phosphorylated AKT at both the Thr308 and Ser473 sites significantly decreased on day 1 and day 2 and were recovered on day 5 (Fig. 4A). Considering that Akt at Thr308 binds to the phosphoinositide-dependent protein kinase 1 (PDK1) and Ser473 binds to the second mTOR complex 2 (mTORC2), we tested the expression of PDK1 and mTOR after heat treatment. Western blot showed that levels of PDK1 phosphorylated mTOR (Ser2448) and mTOR (Ser2481) significantly decreased on day 1 and day 2 (Fig. 4A). As we expected, curcumin pre- or post-heat treatment markedly upregulated phosphorylated Akt at the Ser473 and Thr308 sites, as well as phosphorylated mTOR at the Ser2448 and Ser2481 sites, along with expression of PDK1. These effects of curcumin were similar to those of IGF-1, a specific Akt activator (Fig. 4B and 4D). In addition, IGF-1 or Mhy1485 abolished the apoptosis induced by heat treatment as indicated by the expression of apoptosis markers (Fig. 4C).

As both NADPH oxidase 2 and Akt/mTOR have been demonstrated to be involved in the mechanism of curcumin inhibiting cell apoptosis induced by heat, we proceeded to explore whether there was crosstalk between these two signaling pathways. Treatment with the NADPH oxidase inhibitor DPI or the gp91^{phox} inhibitor gp91ds-tat significantly upregulated the phosphorylation level of both PDK1/Akt and mTOR, as shown by Western blot analysis

Fig. 3. Curcumin decreases heat-induced ROS generation by inhibiting membrane translocation of NADPH oxidase 2 cytosolic components. (A) ROS production was detected by DCFDA fluorescence at different time points after heat treatment. (B) Expression levels of 4-hydroxynonenal (4-HNE) and nitrotyrosine (NT) were tested by ELISA at different time points after heat treatment. (C) ROS production was detected by DCFDA fluorescence after curcumin treatment. (D) 4-HNE and NT production was detected by ELISA after curcumin treatment. (E) Representative Western blot showing the trend of the expression of NADPH oxidase subunits in cells incubated at 47 °C at different time points post-treatment. (F) Western blot analysis showing that curcumin treatment, NADPH oxidase inhibitor DPI treatment, gp91ds-tat treatment, and ROS scavenger MPG treatment reversed the heat-induced increase in the membrane-bound cytosolic subunits. (G) Western blot analysis showing the expression of NADPH oxidase subunits after DPI, gp91ds-tat, and ROS scavenger MPG treatments attenuated the expression of apoptosis markers. (H) PCR analysis was performed to analyze the expression levels of Nox2. (I) Western blot analysis showing that Nox2 siRNA attenuated the expression of apoptosis markers. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, no significant difference. Compared to con or DMSO treatment.

Fig. 4. Curcumin exerts a protective effect against heat-induced apoptosis by activating the Akt/mTOR signaling pathway. (A) Expression levels of Ser473 and Thr308 phospho-Akt, total Akt, Ser2448 and Ser2481 phospho-mTOR, PDK1 and total mTOR proteins are shown at different time points after heat treatment. (B) Western blot analysis showing a modest increase in both Akt and mTOR phosphorylation and PDK1 in response to curcumin pre-treatment and post-treatment. (C) Western blot analysis showing that IGF treatment and Mhy1485 attenuated the overexpression of apoptosis markers (compared to DMSO treatment). (D) Western blot analysis showing the change in Akt and mTOR expression after DPI, gp91ds-tat, sgp91ds-tat and Akt activator IGF treatment (vs. DMSO treatment). (E) Western blot analysis showing the change in Akt and mTOR expression after Nox2 siRNA treatment compared to control siRNA treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, no significant difference.

(Fig. 4D). As expected, knockdown of Nox2 with siRNA had a similar effect as the NADPH oxidase inhibitor (Fig. 4E). These results suggested that NADPH oxidase 2 is upstream of Akt/mTOR signaling in heat-induced apoptosis, and the NADPH oxidase 2/Akt/mTOR signaling pathway was involved in the inhibition of heat-induced apoptosis by curcumin.

Anti-apoptosis activity of curcumin in a rat model of thermal inhalation injury

Heat-induced bronchial epithelia apoptosis and the anti-apoptosis effect of curcumin were also tested *in vivo* by taking advantage of a rat model of thermal inhalation injury. In the

Fig. 5. Curcumin exhibits anti-apoptosis activity in a rat model of thermal inhalation injury. (A) The trend of apoptosis was observed by Western blot analysis and the corresponding quantification after thermal inhalation injury. (B) Curcumin significantly decreased the expression of Bax, cleaved PARP, and cleaved caspase-3 (compared to DMSO treatment). (C) TUNEL staining in lung tissue from rats of the four groups. (D) Western blot analysis showing the change in Akt and mTOR phosphorylation and PDK1 expression in the lung tissue after curcumin treatment of thermal inhalation injury. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ns, no significant difference.

animal model, the temperature of the air from the heat gun is approximately 800 °C and is cooled down to approximately 48 °C at the bronchi, which is consistent with the temperature used *in vitro*. The lung tissue was harvested for Western blot and TUNEL staining. The expression of apoptosis-related proteins in lung tissue increased on day 1, peaked on day 2, and then gradually decreased on day 7 after heat treatment (Fig. 5A). Curcumin strikingly

Fig. 6. Curcumin exerts a protective effect against heat-induced apoptosis by activating the Akt/mTOR signaling pathway.

decreased the expression of Bax, cleaved PARP, and cleaved caspase-3, as determined by Western blot (Fig. 5B). Consistently, immunohistochemistry staining indicated that treatment with curcumin significantly reduced apoptotic cells after heat treatment (Fig. 5C). As shown in Fig. 5D, heat reduced the expression of phosphorylated Akt at the Ser473 and Thr308 sites, phosphorylated mTOR at the Ser2448 and Ser2481 sites and PDK1. However, curcumin treatment reversed the decrease back to normal level. These results demonstrated that the Akt/mTOR signaling pathway was inhibited after thermal inhalation and activated after curcumin treatment *in vivo*.

Discussion

This study investigated the effect of curcumin in inhibiting heat-induced apoptosis and obtained several major findings. First, treatment with curcumin significantly alleviated heat-induced apoptosis and increased cell viability. Second, curcumin administration, both pre- or post- heat treatment, markedly inhibited the membrane translocation of NADPH oxidase 2 cytosolic components, which resulted in a reduction in ROS generation and alleviated apoptosis after heat treatment. Third, the Akt/mTOR signaling pathway was implicated in the inhibition of heat-induced bronchial epithelia apoptosis by curcumin. Lastly, NADPH oxidase 2 was found to be downstream of Akt/mTOR signaling in heat-induced apoptosis. Briefly, these results demonstrated that curcumin alleviated heat-induced apoptosis by inhibiting NADPH oxidase 2 activation and upregulating the Akt/mTOR signaling pathway.

Thermal injury of the airway caused by heated air is a leading cause of burn-related death [21]. However, the hot air is cooled down to approximately 48.3 °C by the time it reaches the bronchi airway because over 90% of the heat is released in the upper airway [22]. It has been reported that high temperatures (those above 43 °C) can cause pathological changes and affect cell functions [23]. Therefore, the experimental condition of 47 °C for 5 min was employed to treat cells in all *in vitro* experiments (designated as heat treatment), which is relevant to the pathological situation in thermal injury. Our results demonstrated that the heat treatment condition could induce apoptosis in 16HBE140 cells.

Apoptosis is one of the most important pathogenic mechanisms underlying heat-induced bronchial epithelial cell damage [24]. Reactive oxygen species (ROS) are well-known oxygen radicals that lead to oxidative stress and apoptosis [25]. ROS are rapidly produced and accumulate when cells suffer from injury, and ROS overloading leads to irreversible cellular apoptosis [26]. Consistent with these reports, we also observed that heat treatment induced ROS production, which resulted in apoptosis. Furthermore, curcumin was found to be able to reduce ROS production, thus inhibiting cell apoptosis.

This study demonstrates the role of NADPH oxidase 2 cytosolic subunits in mediating heat-induced apoptosis. NADPH oxidase 2 is an enzymatic compound that catalyzes ROS bio-synthesis. In our study, cells treated with heat showed upregulated p47phox and p67phox levels in the cell membrane. Both inhibition of NADPH oxidase 2 with an inhibitor and knockdown with siRNA suppressed heat-induced cell apoptosis. In addition, curcumin treatment markedly decreased p47phox and p67phox expression and NADPH oxidase 2 activation, thus inhibiting apoptosis. Collectively, these results indicate that curcumin protects bronchial epithelial cells from heat-induced apoptosis by regulating the membrane translocation of NADPH.

This study has demonstrated that Akt/mTOR signaling plays a part in heat-induced apoptosis. Akt/mTOR has been identified to play a principal role in the anti-apoptotic signaling pathway in different cell apoptosis models caused by extracellular or intracellular signaling [27, 28]. In the present study, we found that activation of Akt/mTOR signaling was inhibited by heat. Curcumin has been reported to have potent anti-proliferative and antimetastasis effects on various cancers by suppressing Akt/mTOR signaling [13]. However, in our study, curcumin treatment markedly upregulated phosphorylated Akt and mTOR. This inconsistency may be due to different cell types and treatments, suggesting a cell- and tissue-specific effect of curcumin in the regulation of the Akt/mTOR signaling pathway, although the underlying mechanism still needs further investigation. However, the mechanism by which curcumin activates Akt/mTOR in heat-induced apoptosis still needs to be illuminated. Curcumin has been reported to have a broad range of molecular targets. Therefore, some other pathways in addition to the Akt/mTOR pathway may be involved in curcumin suppressing heat-induced cell apoptosis [21].

mTOR activation in bronchial epithelial cells has been reported to be essential for lung injury, which is consistent with our results that mTOR activation plays an important role in the apoptosis of the heat-treated cells [20]. Furthermore, in our study, treatment with a NADPH oxidase 2 inhibitor or knockdown of NADPH oxidase 2 induces activation of Akt/mTOR signaling, indicating that NADPH oxidase 2 is upstream of Akt/mTOR. Of note, S6K1, S6 and 4E-BP1 are downstream of mTOR, regulating cell motility. S6K1 and S6 have been considered to regulate apoptosis, the cell cycle and metabolism [32]. 4EBP1 plays an important role in protein synthesis, cell growth and cell proliferation [33]. Thus, the effect of curcumin inhibiting heat-induced apoptosis may be mediated by these molecules.

Collectively, this study has demonstrated that curcumin inhibits heat-induced apoptosis, and the underlying mechanism is through regulation of the NADPH oxidase 2/Akt/mTOR signaling pathway (as demonstrated in Fig. 6). Thus, regulation of this pathway in pulmonary epithelial cells may be a potential therapeutic strategy for preventing lung injury induced by thermal inhalation.

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Disclosure Statement

The authors declare no conflicts of interests.

References

- Chen J, Yan H, Luo G, Luo Q, Li X, Zhang J, Yuan Z, Peng D, Peng Y, Hu J, Wu J: Characteristics of burn deaths from 2003 to 2009 in a burn center: A retrospective study. *Burns Trauma* 2013;1:80-86.

- 2 Roberts GT, Ghebeh H, Chishti MA, Al-Mohanna F, El-Sayed R, Al-Mohanna F, Bouchama A: Microvascular injury, thrombosis, inflammation, and apoptosis in the pathogenesis of heatstroke: a study in baboon model. *Arterioscl Throm Vas* 2008;28:1130-1136.
- 3 Gu ZT, Wang H, Li L, Liu YS, Deng XB, Huo SF, Yuan FF, Liu ZF, Tong HS, Su L: Heat stress induces apoptosis through transcription-independent p53-mediated mitochondrial pathways in human umbilical vein endothelial cell. *Sci Rep* 2014;4:4469.
- 4 Isaksen TB, Yost MG, Hom EK, Ren Y, Lyons H, Fenske RA: Increased hospital admissions associated with extreme-heat exposure in King County, Washington, 1990-2010. *Rev Environ Health* 2015;30:51-64.
- 5 Cheng CH, Yang FF, Liao SA, Miao YT, Ye CX, Wang AL, Tan JW, Chen XY: High temperature induces apoptosis and oxidative stress in pufferfish (*Takifugu obscurus*) blood cells. *J Therm Biol* 2015;53:172-179.
- 6 Lee SJ, Yang ES, Kim SY, Kim SY, Shin SW, Park JW: Regulation of heat shock-induced apoptosis by sensitive to apoptosis gene protein. *Free Radical Bio Med* 2008;45:167-176.
- 7 Kleniewska P, Piechota A, Skibska B, Goraca A: The NADPH oxidase family and its inhibitors. *Arch Immunol Ther Ex* 2012;60:277-294.
- 8 Thompson SM, Callstrom MR, Jondal DE, Butters KA, Knudsen BE, Anderson JL, Lieber KR, Smith SC, Lee JS, Thorgeirsson SS, Grande JP, Roberts LR, Woodrum DA: Heat Stress-Induced PI3K/AKT/mTORC2-Dependent AKT Signaling Is a Central Mediator of Hepatocellular Carcinoma Survival to Thermal Radiation Induced Heat Stress. *PloS one* 2016;11:e0162634.
- 9 Kim SY, Gul R, Rah SY, Kim SH, Park SK, Im MJ, Kwon HJ, Kim UH: Molecular mechanism of ADP-ribosyl cyclase activation in angiotensin II signaling in murine mesangial cells. *Am J Physiol Renal Physiol* 2008;294:F982-989.
- 10 Dai C, Li D, Gong L, Xiao X, Tang S: Curcumin Ameliorates Furazolidone-Induced DNA Damage and Apoptosis in Human Hepatocyte L02 Cells by Inhibiting ROS Production and Mitochondrial Pathway. *Molecules* DOI:10.3390/molecules21081061.
- 11 Zheng KM, Zhang J, Zhang CL, Zhang YW, Chen XC: Curcumin inhibits apoptosis-induced apoptosis via upregulating heme oxygenase-1 expression in SH-SY5Y cells. *Acta Pharmacol Sin* 2015;36:544-552.
- 12 Huang SL, Chen PY, Wu MJ, Tai MH, Ho CT, Yang JH: Curcuminoids Modulate the PKCdelta/NADPH Oxidase/Reactive Oxygen Species Signaling Pathway to Suppress Matrix Invasion during Monocyte-Macrophage Differentiation. *J Agr Food Chem* 2013;61:1033-1048.
- 13 Jiao D, Wang J, Lu W, Tang X, Chen Y, Chen H, Chen QY: Curcumin inhibited HGF-induced EMT and angiogenesis through regulating c-Myc-dependent PI3K/Akt/mTOR signaling pathways in lung cancer. *Mol Ther Oncolytics* 2016;3:1-10.
- 14 Roti Roti JL: Cellular responses to hyperthermia (40–46°C): Cell killing and molecular events. *Int J Hyperther* 2008;24:3-15.
- 15 Dong ZW, Chen Y, Zhang JC, Zhou T, Chen Y, Chen Y, Tsang LL, Chan HC, Peng YZ: CFTR-regulated MAPK/NF-kappaB signaling in pulmonary inflammation in thermal inhalation injury. *Sci Rep* 2015;5:15946.
- 16 Li W, Liu Y, Lin D, Li X, Lu H, Bi X, Wang M: Low Expression of Polo-like Kinase 1 is Associated with Poor Prognosis in Liver Cancer. *Cancer Transl Med* 2015;1:196-200.
- 17 Chung Y, Wang H, Xing H, Carroll RS, Johnson MD: miR-505 Downregulates 6-Phosphofructo-2-Kinase/Fructose-2,6-bisphosphatase 4 to Promote Cell Death in Glioblastoma. *Cancer Transl Med* 2015;1:158-165.
- 18 Smith W, Freeman MR, Long E, Thompson MA, Pabelick CM, Prakash YS: Cigarette Smoke and Estrogen Signaling in Human Airway Smooth Muscle. *Cell Physiol Biochem* 2015;36:1101-1115.
- 19 Murillo MM, Carmona-Cuenca I, Del Castillo G, Ortiz C, Roncero C, Sanchez A, Fernandez M, Fabregat I: Activation of NADPH oxidase by transforming growth factor-beta in hepatocytes mediates up-regulation of epidermal growth factor receptor ligands through a nuclear factor-kappaB-dependent mechanism. *Biochem J* 2007;405:251-259.
- 20 Rong YH, Liu W, Wang C, Ning FG, Zhang GA: Temperature distribution in the upper airway after inhalation injury. *Burns* 2011;37:1187-1191.
- 21 Hao Y, Cheung CS, Yip WC, Ko WH: Nobiletin Stimulates Chloride Secretion in Human Bronchial Epithelia via a cAMP/PKA-Dependent Pathway. *Cell Physiol Biochem* 2015;37:306-320.
- 22 Suzuki T, Hirayama T, Aihara K, Hirohata Y: Experimental studies of moderate temperature burns. *Burns* 1991;17:443-451.

- 23 Wang JP, Yu YC, Chen SP, Liang HC, Lin CW, Fang K: The collective nuclear migration of p53 and phosphorylated S473 of Akt during ellipticine-mediated apoptosis in human lung epithelial cancer cells. *Mol Cell Biochem* 2015;407:123-133.
- 24 Lv YG, Liu J, Zhang J: Theoretical evaluation of burns to the human respiratory tract due to inhalation of hot gas in the early stage of fires. *Burns* 2006;32:436-446.
- 25 Wang L, Wu G, Qin X, Ma Q, Zhou Y, Liu S, Tan Y: Expression of Nodal on Bronchial Epithelial Cells Influenced by Lung Microbes Through DNA Methylation Modulates the Differentiation of T-Helper Cells. *Cell Physiol Biochem* 2015;37:2012-2022.
- 26 Moench R, Grimmig T, Kannen V, Tripathi S, Faber M, Moll EM, Chandraker A, Lissner R, Germer CT, W Gasser AM, Gasser M: Exclusive inhibition of pi3k/akt/mtor signaling is not sufficient to prevent pd mediated effects on glycolysis and proliferation in colorectal cancer. *Oncotarget* 2016;7:68659-68700.
- 27 Han SG, Castranova V, Vallyathan V: Comparative cytotoxicity of cadmium and mercury in a human bronchial epithelial cell line (BEAS-2B) and its role in oxidative stress and induction of heat shock protein 70. *J Toxicol Environ Health A* 2007;70:852-860.
- 28 Redza-Dutordoir M, Averill-Bates DA: Activation of apoptosis signalling pathways by reactive oxygen species. *Biochim Biophys Acta* 2016;1863:2977-2992.
- 29 Xu L, He S, Yin P, Li D, Mei C, Yu X, Shi Y, Jiang L, Liu F: Punicalagin induces Nrf2 translocation and HO-1 expression via PI3K/Akt, protecting rat intestinal epithelial cells from oxidative stress. *Int J Hyperthermia* 2016;32:465-473.
- 30 Ramakrishnan V, Gomez M, Prasad V, Kimlinger T, Painuly U, Mukhopadhyay S, Haug J, Bi L, Rajkumar SV, Kumar S: Smac mimetic LCL161 overcomes protective ER stress induced by thapsigargin and cyclosporin A, synergistically causing cell death in multiple myeloma. *Oncotarget* 2016;7:56253-56266.
- 31 Sintara K, Thong-Ngam D, Patumraj S, Klaikeaw N, Chaitanya T: Curcumin suppresses gastric NF-kappaB activation and macromolecular leakage in Helicobacter pylori infected rats. *World J Gastroenterol* 2010;16:4039-4046.
- 32 Hu Y, Lou J, Mao YY, Lai TW, Liu LY, Zhu C, Zhang S, Li X, Zhang F, Li W, Ying SM, Chen ZH, Shen HH: Activation of MTOR in pulmonary epithelial cells protects LPS-induced acute lung injury. *Autophagy* 2016;1-14.
- 33 Yu G, Zeng X, Wang H, Hou Q, Tang Y, Qiu W, Li X: 14,15-epoxyeicosatrienoic Acid suppresses cigarette smoke extract-induced apoptosis in lung epithelial cells by inhibiting endoplasmic reticulum stress. *Cell Physiol Biochem* 2015;36:474-486.