Sodium tanshinone IIA sulfonate attenuates tumor oxidative stress and enhances apoptosis in an intermittent hypoxia mouse model

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Abstract

Objective The present study was designed to determine the effect of sodium tanshinone IIA sulfonate (TSA) on tumor oxidative stress and apoptosis in a mouse model of intermittent hypoxia (IH) which was considered a novel feature of obstructive sleep apnea. **Materials and methods** Mice were randomly assigned to control (normoxia) group (CTL), control plus TSA (CTL+TSA) group, IH group, and IH plus TSA (IH+TSA) group. The IH exposure lasted for 5 weeks. TSA was intraperitoneally injected in the CTL+TSA and IH+TSA group. Malondialdehyde (MDA) and superoxide dismutase (SOD) were detected for tumor oxidative stress levels. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and western blotting of Bax, Cleaved Caspase-3 were conducted for evaluating tumor apoptotic levels. The expression levels of nuclear factor erythroid 2-related factor 2 (Nrf2) and NF- κ B were also evaluated by western blotting. **Results** Compared with the CTL group, mice exposed to the IH had higher MDA and lower SOD levels, and the TUNEL-positive cell rate, Bax and Cleaved Caspase-3 expressive levels were decreased in the IH group. The oxidative stress indexes were suppressed and the apoptotic levels were upregulated after treatment with TSA under the IH condition. The lower Nrf2 and higher NF- κ B levels can be reversed by treatment with TSA under the IH condition. **Conclusions** The IH contributes to high oxidative stress and low apoptosis in tumor-bearing mice. TSA appears to improve IH-induced oxidative stress and apoptosis via Nrf2/NF- κ B signaling pathway.

Background

Sodium Tanshinone IIA sulfonate (TSA) is a naturally occurring compound, which was extracted from Chinese herb *Salvia miltiorrhiza*. Previous studies have indicated its antioxidant and anti-inflammation effects in that TSA can reduce reactive oxygen species (ROS) production and alleviate proinflammatory cytokines [1, 2]. Robust evidence showed TSA has been extensively used for cardiovascular diseases, and inflammatory disease such as chronic hepatitis [3, 4]. Recently, studies *in vitro* found that TSA exhibits anticancer activity in many kinds of cancer cells, such as lung cancer, leukemia, liver cancer, and gastric cancer [5-8]. Its anticancer effect may be partly attributed to its antioxidant and pro-apoptotic properties[6, 9]. Except cell models, an *in vivo* study demonstrated the anticancer effect of TSA[9].

Obstructive sleep apnea (OSA) has a high global prevalence (15%-24%)[10-12]. One of the significant pathophysiological features of OSA is intermittent hypoxia (IH) which contributes to systematic inflammation, oxidative stress, endothelial dysfunction, and apoptosis [13, 14]. During the last decade, a considerable amount of literature has shown increased cancer incidence and mortality in OSA populations[15, 16]. Even in experimental studies, our prior study [17] and other animal studies [18-20]demonstrated that IH appears to induce tumor growth, invasion, and metastasis.

From the aforementioned information, we hypothesized that oxidative stress and apoptosis may play an important role in the pathogenesis of IH accelerating cancer progression. Hereby, the main issues addressed in the present study are to access the effect and underlying molecular mechanism of TSA on tumor oxidative stress and apoptosis in an IH mouse model mimicking OSA.

Methods

Animals and subgroups

Forty-eight 7-week male C57BL/6 mice were purchased from the Chinese Academy of Science Laboratory Animals Center in Shanghai, China. All mice were housed in standard cages with 12:12-h light-dark cycle, and free access to water and food. Mice were randomly assigned to the following four groups (n=12 in each group): control (normoxia) group (CTL), control plus TSA group (CTL+TSA), IH group, and IH+TSA. The body weight of mice in each group was measured each week. This protocol was approved by the ethics committee in Zhongshan Hospital, Xiamen University (approved number: 2017-015), and conducted in accordance with the Guide for the Care and Use of Laboratory Animals [21]. All measures were taken to minimize the number of animals used and to reduce the pain of animals.

intermittent hypoxia exposure

The protocol of IH exposure was performed according to our previous study[22, 23]. Briefly, the mice in the IH and IH+TSA groups (n=24) were subjected to a self-made plexiglass chamber with one-way valves, and a programmable instrument regulated the times of the three gases (oxygen, nitrogen, compressed air) flowing to the chamber, making the oxygen concentration in the chamber fluctuating from 21% to nadir 6-8%. The cycle time of hypoxia and re-oxygenation is 120 seconds. The IH exposure was conducted from 8 AM to 4 PM daily for 5 consecutive weeks.

Cells culture, tumor induction and tumor measurement

Lewis lung carcinoma (LLC) cells (CoBioer Biosciences Co., Ltd. Shanghai, China) were cultured in accordance to the instruction of the manufacturer. Briefly, LLC cells were maintained at high glucose DMEM and supplemented with 10% fetal bovine serum (GIBCO, USA). A total of 1×10^6 LLC cells with 100 μ l PBS were injected into the right flank of each mouse after one week of IH exposure. When the tumor was palpable, the width (W) and length (L) was detected with an electric caliper every week. Tumor volume (V, mm³) was calculated as $W^2 \times L / 2$.

Drug administration

When tumor volume growing to about 200mm³ (nearly 5-7 days after LLC injection), mice in the CTL+TSA and IH+TSA groups received TSA (purchased from Shanghai No.1 Biochemical & Pharmaceutical Company, Shanghai, China) intraperitoneally injection (10mg/kg) daily according to previous studies[24-27], while those in the CTL and IH groups intraperitoneally injected saline with equal volume.

Tissue preparation

After 5 weeks of experimentation, all mice were deeply anesthetized with pentobarbital and exsanguinated by cardiac puncture and plasma obtained. Tumors were excised, weighted, either frozen in liquid nitrogen and stored at -80°C for further analysis, or fixed in buffered 10% formalin for histological examination.

Oxidative stress measurement

Malondialdehyde (MDA) and superoxide dismutase (SOD), two of the most commonly used indicators of oxidative stress were assayed with relevant kits according to the instructions of manufactures (Beyotime, Beijing, China). Briefly, frozen tumor tissues were homogenized in cold PBS (10% W/V). After lysis for 15min on ice, homogenates were centrifuged and then supernatants were obtained for further detection. MDA levels in the homogenates were determined spectrophotometrically by measuring the presence of thiobarbituric acid-reactive substances. SOD was detected by an assay kit which employs a thiazole salt for the detection of superoxide anions by producing a colored product. The levels of MDA and SOD were reported as the absorbance at a wavelength of 535nm and 560nm, respectively. The enzyme activity is reported as units/mg of protein.

Western blotting

Tumor tissues were homogenized with RIPA buffer (Beyotime, Beijing, China) containing protease and phosphatase inhibitor in a glass homogenizer. After centrifuged, the supernatants were extracted and detected the total protein concentrations with a bicinchoninic acid protein assay (Beyotime, Beijing, China). Equal amounts of boiled protein (40ug/lane) were subjected to 12% sodium dodecyl sulfate-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Boston, MA, USA). After blocking with 5% (W/V) skim milk, the membrane was incubated with the following antibodies at 4°C overnight: rabbit anti-Bax (1:1000, Cell Signaling Technology, USA), rabbit anti-Cleaved Caspase-3 (1:1000, Cell Signaling Technology, USA), rabbit anti-nuclear factor erythroid 2-related factor 2 (Nrf2) (1:2000, Abcam, USA), rabbit anti-NF-κB (1:1000, Cell Signaling Technology, USA) and mouse anti-β-actin (1:2000, Santa Cruz Biotechnology, USA). After a rinse with Tris-buffered saline+Tween-20 for 3 times, the PVDF membranes were incubated with goat anti-rabbit or mouse IgG-HRP at 37°C for 1 hour. The membranes were developed and exposed using an enhanced chemiluminescence kit (Clarity™ Western ECL Substrate, Bio-Rad, USA). The experiment was repeated in triplicate of each mouse. The band densities on the membranes were estimated using the Image J analysis software (National Institutes of Health, Bethesda, MD, USA).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

Tumor tissues were fixed in buffered 10% formalin for 24 hours, dehydrated in graded alcohol series, cleaned with xylene, and then embedded in paraffin. Embedded tissues were sectioned to 5μm per slices. The sections were washed in PBS and incubated with TUNEL reaction solution at 37°C for 1 hour using the apoptosis detection kits (Roche, China). After washing with PBS, then incubating with 4', 6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA) for 5 min, the sections were analyzed and photographed

with a microscope (SP12-AOBS Leica) at 400× magnification. Both TUNEL-positive cells and total cells in 5 sections of each group were counted respectively. The apoptosis rate was calculated as following: TUNEL-positive cell number/total cell number× 100%.

Statistical analysis

GraphPad Prism software 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was conducted to analyze the data. All data are presented as mean ± standard deviation and compared using an analysis of variance (ANOVA) followed by Fisher's exact test. A *p* value of less than 0.05 indicated statistical significance.

Results

Body weight, tumor volume, and tumor weight

After 5 weeks of the experiment, none mouse died in each group, no severe TSA-related adverse events occurred. Mice under the IH condition gained less body weight at 2th to 5th week (*p*<0.05); when compared with the CTL group, both tumor volume (at 5th week) (*p*<0.05) and tumor weight (*p*<0.001) were significantly higher in the IH group. Treatment with TSA can suppress tumor volume (*p*<0.01) and tumor weight (*p*<0.05) under the IH condition (Figure 1).

Effect of TSA on tumor oxidative stress

From the data in Figure 2, it is apparent that compared with the CTL group, mice in the IH group had significantly higher MDA levels and lower SOD levels; after treated with TSA, SOD levels were increased, while MDA levels were decreased under IH condition.

Effect of TSA on tumor apoptosis

As Figure 3 depicts that mice exposed to the IH had significantly lower Bax and Cleaved Caspase-3 levels; after treatment with TSA, Bax and Cleaved Caspase-3 were increased under the IH condition (Figure 3 A and B). In contrast to the CTL group, mice in the IH group had a lower percentage of TUNEL positive cell; while the percentage of TUNEL positive cell was increased in mice receiving TSA treatment under IH exposure (*p*<0.001) (Figure 3 C and D).

Effect of TSA on Nrf2 and NF-kB expression

When compared with the CTL group, the expression of Nrf2 protein was downregulated in the IH group (*p*<0.01); and the levels could be neutralized by TSA under the IH condition (*p*<0.01). The mean NF-kB level was significantly higher in the IH group than that of the CTL group (*p*<0.001); and this level can decrease after receiving TSA treatment under the IH condition (*p*<0.001). The differences of Nrf2 and NF-kB between groups are highlighted in Figure 4 and Figure 5.

Discussion

The present study confirmed that IH can accelerate tumor growth. The oxidative stress levels of tumor tissue were significantly high under IH exposure. TSA treatment can attenuate oxidative stress under the IH condition. The low tumor apoptotic rate in IH-induced mice can be recovered after receiving TSA administration.

TSA is the Chinese herb *Salvia miltiorrhiza* extracting compound. Its anti-inflammation and antioxidant activity was widely used in clinical practice for treating a large number of cardiovascular or chronic organ diseases [3, 28]. More recently, experimental studies have illustrated that TSA had an anti-cancer effect through antioxidant activity and apoptosis regulation [6, 7].

Evidence demonstrated oxidative stress appears to be a common phenomenon in tumor tissue. There are progressively data elucidated that overproduction of ROS can accelerate sustained proliferation, angiogenesis, invasiveness, and metastasis in the tumor[29]. Numerous anti-cancer therapies including chemotherapy and radiotherapy can induce oxidative stress which further aggravates tumor progression. Antioxidant therapy can suppress ROS-driven tumor progression and metastasis[30]. As a novel hallmark of OSA, IH induces oxidative stress, systemic inflammation [13]. Tumor progression was also found in subjects exposed to the IH in recent studies [15, 16, 18, 20], and our previous study also found that IH aggravates the tumor growth [23, 31]. It is unclear what the potential molecular mechanism of IH accelerating tumor and the antioxidant effects on tumor-bearing mice exposed to the IH. The present study accessed the tumor oxidative stress levels in the IH mice mimicking OSA, and determined the antioxidant effects of TSA. The results demonstrated that high oxidative stress levels were observed in the IH exposed tumor-bearing mice, and TSA can attenuate these levels, especially under the IH condition. This phenomenon may partly attribute to Nrf2, one of the antioxidant genes[32, 33].

Apoptosis is a natural way of removing aged cells from the body. In cancer, however, the activation of anti-apoptotic systems leads to the reduction of apoptotic cells, and further the uncontrolled proliferation of cancer cells [34]. Previous studies have elucidated that TSA can induce apoptosis in several cancer cells, and can be considered a potential therapeutic medicine for cancer patients. The present experimental study illustrated that the reduced apoptotic levels were detected in tumor-bearing mice exposed to the IH environment. The apoptotic levels can be improved by TSA under the IH condition in which NF- κ B signaling pathway may be partly involved [35, 36].

It is widely accepted that Nrf2 has a pivotal function in the regulation of antioxidant genes[37]. As a transcription factor, Nrf2 is downregulated under oxidative stress environment. Numerous antioxidant therapies have the function to activate Nrf2-related signaling pathways[38-40]. Evidence has shown the lower expression of Nrf2 in OSA patients or the IH animal models[41, 42]. These results are in agreement with previous studies indicating that the Nrf2 expression was downregulated in tumor-bearing mice exposed to IH condition. TSA treatment can upregulate its expression under IH condition. The present study offers valuable insight into the significant role of Nrf2 in IH-induced oxidative stress. NF- κ B is also showed a crucial role in inflammation, oxidative stress, and apoptosis processes[43, 44]. Prior studies have noted the importance of NF- κ B in the OSA or IH investigation. Israel et.al[45] illustrated that NF- κ B is

activated in children with OSA. Another study by Ryan and coworkers[46] pointed out that NF-kB-dependent genes were elevated in OSA patients. Experimental studies elucidated that NF-kB was activated after mice or cell exposing to the IH condition[47, 48]. TSA may protect mice from immune-mediated liver injury via NF-kB signaling pathway[36]. Without doubt, our study confirmed the activated NF-kB in tumor-bearing mice exposing to IH. TSA administrated can attenuate the NF-kB expression in IH-induced mice. There is a large number of researches have proved the correlation between Nrf2 and NF-kB[49-51], but the exact function and underlying molecular mechanism of Nrf2 and NF-kB in the IH-induced oxidative stress and apoptosis are yet from the conclusion.

The generalisability of these results is subject to certain limitations. Firstly, similar to previous studies[24-27], only one dose (10mg/kg) rather than several doses of TSA was administrated to the mice, the dose-effect relationship was hard to be concluded from our study. Secondly, only tumor oxidative stress and apoptotic levels were measured for evaluating the effect of the IH and the therapeutic efficacy of TSA. The proliferation, migration, and invasion of the tumor cell should be assessed in the future study. Thirdly, we only observed that the antioxidant and apoptotic regulated effects of TSA was significant in mice under the IH exposure and the discrepant expressions of Nrf2 and NF-kB between groups; More studies with RNA interference were required to elucidate the role and underlying mechanism of the Nrf2/NF-kB[51] in the effect of TSA on cancer under IH condition.

Conclusions

Taken together, these findings support the idea that IH can contribute to high oxidative stress and low apoptosis in tumor-bearing mice. We infer that TSA appeared to improve IH-induced oxidative stress and apoptosis via Nrf2/NF-kB signaling pathway. From this experimental study we indicate that with the antioxidant and apoptotic regulated effects, TSA can be recommended as an adjunctive therapeutic medicine for OSA patients with cancer.

Abbreviations

TSA: sodium Tanshinone IIA sulfonate

ROS: reactive oxygen species

OSA: obstructive sleep apnea

IH: intermittent hypoxia

CTL: control group

LLC: Lewis lung carcinoma

MDA: malondialdehyde

SOD: superoxide dismutase

PVDF: polyvinylidene fluoride

TUNEL: terminal deoxynucleotidyl transferase dUTP nick-end labeling

Declarations

Notes

X-B Zhang X-Y Chen and X-M Su contributed equally to this works.

Declarations

Ethics approval and consent to participate: The study was approved by the Ethics Committee of Zhongshan Hospital, Xiamen University.

Consent for publication: no applicable.

Availability of data and material: All data generated or analyzed during this study are included in this published article.

Competing interests: The authors declare that they have no conflict of interest.

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Authors' contributions: Conception and design: XBZ, XYC, and HQZ. Collection and assembly of data: XMS and MW. Data analysis and interpretation: XBZ, XMS, XBL, and HQZ. Manuscript writing: All authors. Final approval of manuscript: All authors.

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Figures

Figure 1

Body weight, tumor volume, and tumor weight in each group. A: Mice exposed to the IH gained less body weight than those exposed to normoxia (CTL group) at 2-5 week ($p < 0.05$). B: Tumor volume of mice in the IH group was higher than that in the CTL group ($p < 0.05$); and when compared with the IH group, the tumor volume was decreased in the IH+TSA group ($p < 0.01$). C: There was increased tumor weight in the IH group than that in the CTL group ($p < 0.01$); the tumor weight was lower in the IH+TSA group than that of the IH group ($p < 0.05$). D: Picture showed the tumor size in different groups. Abbreviations: IH, intermittent hypoxia, CTL: control group, CTL+TSA, control + sodium tanshinone IIA sulfonate group, IH+TSA: intermittent hypoxia+ sodium tanshinone IIA sulfonate.

Figure 2

Oxidative stress levels in each group. A: Compared with the CTL group, IH group had an increased MDA levels ($p < 0.05$); after treatment with TSA, MDAs were decreased under the IH condition ($p < 0.001$); B: The SOD levels in the IH group were lower than that of the CTL group ($p < 0.01$); after treatment with TSA, the SOD levels were increased under IH condition ($p < 0.01$). Abbreviations: IH, intermittent hypoxia, CTL, control group, CTL+TSA, control + sodium tanshinone IIA sulfonate group, IH+TSA, intermittent hypoxia+ sodium tanshinone IIA sulfonate.

Figure 3

Apoptosis levels in each group. A and B: Bax and Cleaved Caspase-3 levels in the IH group were lower than that of the CTL group ($p < 0.001$); TSA can increase both proteins under the IH condition ($p < 0.001$ under IH condition). C and D: The percentage of TUNEL positive cell in the IH group was markedly lower than that of the CTL group ($p < 0.05$). After receiving TSA treatment, the percentage of TUNEL positive cell was increased under the IH condition ($p < 0.001$). Abbreviations: IH, intermittent hypoxia, CTL, control group, CTL+TSA, control + sodium tanshinone IIA sulfonate group, IH+TSA, intermittent hypoxia+ sodium tanshinone IIA sulfonate.

Figure 4

Protein expression of Nrf2 between groups The Nrf2 expression in the IH group was lower than that of the CTL group ($p < 0.01$); while its levels were upregulated after treatment with TSA under the IH condition ($p < 0.01$ when compared with the IH group); the similar founding was failed to be observed under normoxia condition. Abbreviations: IH, intermittent hypoxia, CTL, control group, CTL+TSA, control + sodium tanshinone IIA sulfonate group, IH+TSA, intermittent hypoxia+ sodium tanshinone IIA sulfonate.

Figure 5

Protein expression of NF-κB between groups The NF-κB expression in the IH group was higher than that of the CTL group ($p < 0.001$); while its expressive levels were suppressed after treatment with TSA under the IH condition ($p < 0.001$ when compared with the IH group); the similar founding was not observed under normoxia condition. Abbreviations: IH, intermittent hypoxia, CTL, control group, CTL+TSA, control + sodium tanshinone IIA sulfonate group, IH+TSA, intermittent hypoxia+ sodium tanshinone IIA sulfonate.

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