Moderate Concentration of H_2O_2 Induced $[Ca^{2+}]_i$ Oscillation in Human Bronchial Epithelial Cells

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Abstract Reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) can damage cells by inducing changes of cytosolic Ca^{2+} ($[Ca^{2+}]_i$). However, different concentration of H_2O_2 induced different changes in $[Ca^{2+}]_i$ which activated different signaling pathways. One type of $[Ca^{2+}]_i$ changing is $[Ca^{2+}]_i$ oscillation which can regulate the activity of NF-κB. The aim of this study is to get the concentrations of H_2O_2 which can induce $[Ca^{2+}]_i$ oscillation in the bronchial epithelial cells. Human bronchial epithelial cells were cultured, and treated without or with different concentration of H_2O_2 (0~1 000 μmol/L). Cytosolic Ca^{2+} concentration was detected by fluorescent Ca^{2+} indicator Fura-2. The results revealed that H_2O_2 concentrations <50 μmol/L just induced $[Ca^{2+}]_i$ spark, 50~500 μmol/L induced $[Ca^{2+}]_i$ oscillation, and 1 000 μmol/L induced constantly high level of $[Ca^{2+}]_i$. Moreover, the findings confirmed that 150 μmol/L H_2O_2 induced $[Ca^{2+}]_i$ oscillation, and the $[Ca^{2+}]_i$ oscillation induced increases of NF-κB activity. In conclusion, these data suggested that moderate concentration (50~500 μmol/L) of H_2O_2 can induce $[Ca^{2+}]_i$ oscillation and NF-κB activation in bronchial epithelial cells, which should be involved in ROS induced damage in chronic airway inflammation.

Key words reactive oxygen species (ROS); H_2O_2; $[Ca^{2+}]_i$ oscillation; bronchial epithelial cells

适当浓度的过氧化氢诱导人支气管上皮细胞发生钙振荡

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摘要 包括过氧化氢(H_2O_2)在内的活性氧通过引起细胞内钙的变化而造成细胞损伤。然而, 不同浓度的H_2O_2可以导致细胞内不同的钙变化, 并激活不同的信号通路。细胞内钙振荡是其中的一种钙信号变化形式, 钙振荡可以调控转录因子NF-κB的活性。该研究探讨可以诱导支气管上皮细胞内钙振荡发生的H_2O_2浓度。体外培养人支气管上皮细胞, 采取钙离子荧光探针Fura-2标记细胞, 并使用离子成像系统, 观测不同浓度的H_2O_2(0~1 000 μmol/L)作用下细胞内钙浓度的变化。结果发

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现, 低于50 μmol/L的H_2O_2仅仅引起“钙火花”; 50~500 μmol/L的H_2O_2导致细胞内钙振荡的发生; 而1 000 μmol/L的H_2O_2引起细胞内持续的高钙; 同时也证实150 μmol/L的H_2O_2诱发明显的钙振荡, 而钙振荡随后引起了NF-κB活性的升高。该研究提示, 适当浓度的H_2O_2可以诱发支气管上皮细胞内钙振荡的发生, 推测可能是活性氧导致慢性气道炎症损伤的一个机制。

关键词 活性氧; 过氧化氢; 钙振荡; 支气管上皮细胞

Studies have demonstrated that reactive oxygen species (ROS) can influence cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in the cells[1-2]. Hydrogen peroxide (H_2O_2), an oxidizing agent and a member of ROS, is commonly used in experimental models to induce oxidative stress[1-3]. H_2O_2 induces $[Ca^{2+}]_i$ rise through phospholipase C (PLC) pathway[1]. Interestingly, H_2O_2 also can induce regular changes of $[Ca^{2+}]_i$. One type of regular changes of $[Ca^{2+}]_i$ is oscillation in the concentration of $[Ca^{2+}]_i$, which has been named $[Ca^{2+}]_i$ oscillation. As a significant event of calcium signaling, $[Ca^{2+}]_i$ oscillation can increase the efficiency and specificity of target gene expression[4-5]. Previous studies reported that different frequency of $[Ca^{2+}]_i$ oscillation regulated the activity of transcriptional factor, such as NF-κB[6].

Bronchial epithelial cells play a pivotal role in bronchial diseases, such as bronchial asthma, and chronic bronchitis[7-8]. Airway inflammation is the key point of bronchial diseases, and induces abundant of ROS generation. Overproduction of ROS induced by inflammation is considered as a direct cause of airway injury[9]. However, during the inflammatory status of bronchial diseases, the effect of ROS on bronchial epithelial cells was poorly understood. Constantly high level of $[Ca^{2+}]_i$ leads to cell death, $[Ca^{2+}]_i$ oscillation should have more chronic effect because $[Ca^{2+}]_i$ oscillation regulated the activity of NF-κB. So, the concentration of ROS or H_2O_2 which can induce $[Ca^{2+}]_i$ oscillation needs to be clarified.

Thus, the aim of this study is to get the concentrations of H_2O_2 which can induce $[Ca^{2+}]_i$ oscillation in the bronchial epithelial cells.

1 Materials and Methods

1.1 Materials and reagents

DMEM medium and fetal calf serum were obtained from Hyclone (Logan, UT, USA). 30% H_2O_2 was purchased from Tianjin Chemicals Co. Ltd (Tianjin, China). Fura-2 AM was obtained from Molecular Probes (Eugene, OR, USA). Tetrandrine (Tet) was purchased from Zhejiang Jinhua Pharmaceutical Factory (Jinhua, Zhejiang, China).

1.2 Cell culture

Human bronchial epithelial cells (HBE) were cultured in DMEM supplemented with 10% fetal bovine serum, 20 mmol/L HEPES, 2.2 g/L $NaHCO_3$ and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin) at 37 °C in 5% CO_2 and 95% air. HBE were 1:2 subcultured when cells grown to confluence, and cultured medium was changed every 2~3 days. Before experimentation, HBE were subcultured on glass coverslips (diameter, 24 mm) placed in the wells of 6-wells culture plate. 24 hours later, cells would be ready for experimentation with 40% confluence.

1.3 Cytosolic Ca^{2+} measurement

Cytosolic Ca^{2+} measurement was performed as described previously[10]. Briefly, HBE on glass coverslips were incubated with 1 μmol/L fluorescent Ca^{2+} indicator of Fura-2 at 37 ºC, 5% CO_2 and 95% air for 30 min. The coverslips were then gently washed 3 times with indicator-free Hepes buffered saline (HBS) containing: NaCl 140 mmol/L, KCl 4.5 mmol/L, $CaCl_2$ 1.5 mmol/L, $MgSO_4$ 1.0 mmol/L, D-glucose 10 mmol/L, and HEPES 21 mmol/L, pH7.4, at room temperature to allow deesterification of the indicator. Glass coverslips were gently transferred to a perfusion chamber mounted on the stage of an inverted epifluorescence microscope (IX-70; Olympus, Tokyo, Japan). Monolayers were exposed to HBS for equilibrium and inspecting $[Ca^{2+}]_i$ changing. In some procedure, Tet was added to a final concentration of 15 μmol/L in HBS, and H_2O_2 was added to a final concentration of 0~1 000 μmol/L in HBS.

For all experiments, the way of H_2O_2 delivery was bolus addition. Fura-2 fluorescence was alternatively excited at 340 nm and 380 nm using a polychrome (FV300; Olympus, Tokyo, Japan) corresponding to the Ca^{2+}-bound and -free forms of the indicator, respectively. Emitted fluorescence through bandpass interference filters with selected wavelength bands at 410 nm and 510 nm was captured by computer coupled device and transferred to IPA software. Autofluorescence from unloaded HBE was subtracted from Fura-2 fluorescence recordings before the calculation of the ratio of the emitted fluorescence intensity excited at 340 nm and 380 nm, respectively. The ratio of F340 and F380 (F340/F380) was used as a relative indicator of $[Ca^{2+}]_i$.

1.4 NF-κB activity analysis

An ELISA-based analysis was used to measure the NF-κB activity as described previously[10]. In brief, after HBE treated with H_2O_2 and/or Tet for 1 h, cells were rinsed twice with cold PBS, detached with trypsin, and centrifuged for 10 min at 1 000 r/min. The pellet was then resuspended in 100 μL lysis buffer (20 mmol/L HEPES, pH7.5, 0.35 mol/L NaCl, 20% glycerol, 1% NP-40, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, and 0.1 mmol/L EGTA) containing a protease inhibitor cocktail. After incubation on ice for 10 min, the lysate was centrifuged for 20 min at 14 000 r/min. The supernatant constituted the total protein extract. After being quantified with BCA reagent, the cell extract was kept frozen at –80 °C until NF-κB activity measurement. Cell extracts were incubated in a 96-well plate coated with the oligonucleotide containing the NF-κB consensus-binding site (5′-GGG ACT TTC C-3′). Activated transcription factors from extracts specifically bound to the respective immobilized oligonucleotide. NF-κB activity was then detected with the primary antibody to NF-κB p65 and secondary antibody conjugated to horseradish peroxidase. NF-κB activity was finally determined as absorbance values measured with a microplate reader at a wavelength of 450 nm.

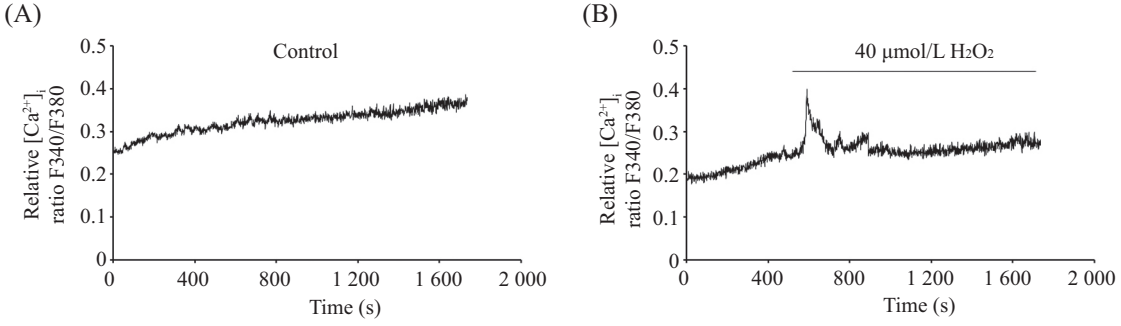
1.5 Statistical analysis

In each experiment, experimental and control HBE were matched for age, seeding density and number of passages to avoid variation in culture factors. Results were shown as the mean±S.E.. Student's *t*-test was used to determine the significant difference between the means of different groups. Results with $P<0.05$ were considered statistically significant.

2 Results

2.1 Low concentration of H_2O_2 (<50 μmol/L) induced $[Ca^{2+}]_i$ spark in HBE

According to the method, HBE on the glass coverslips were incubated with Fura-2 for 30 min, and transferred to the HBS perfusion chamber which was mounted on the stage of an inverted epifluorescence microscope. Then, H_2O_2 was added into the HBS, and the final concentration of H_2O_2 was 30, or 40 μmol/L. At the same time, $[Ca^{2+}]_i$ changes in HBE were detected. As showing in Fig.1, <50 μmol/L H_2O_2 induced a $[Ca^{2+}]_i$ spark, the $[Ca^{2+}]_i$ returned to the basal level very



Cultured HBE were loaded with Fura-2, H_2O_2 was added into the Hepes buffered saline, and the final concentration of H_2O_2 was 30 μmol/L or 40 μmol/L, and then $[Ca^{2+}]_i$ was monitored on a fluorescent microscope. A: a representative tracing shown that the level of $[Ca^{2+}]_i$ is stable in HBE in the absence of H_2O_2 (n=15); B: a representative tracing shown that 40 μmol/L H_2O_2 induced a $[Ca^{2+}]_i$ spark (n=15).

Fig.1 Effect of low concentration of H_2O_2 (40 μmol/L) on $[Ca^{2+}]_i$ in HBE

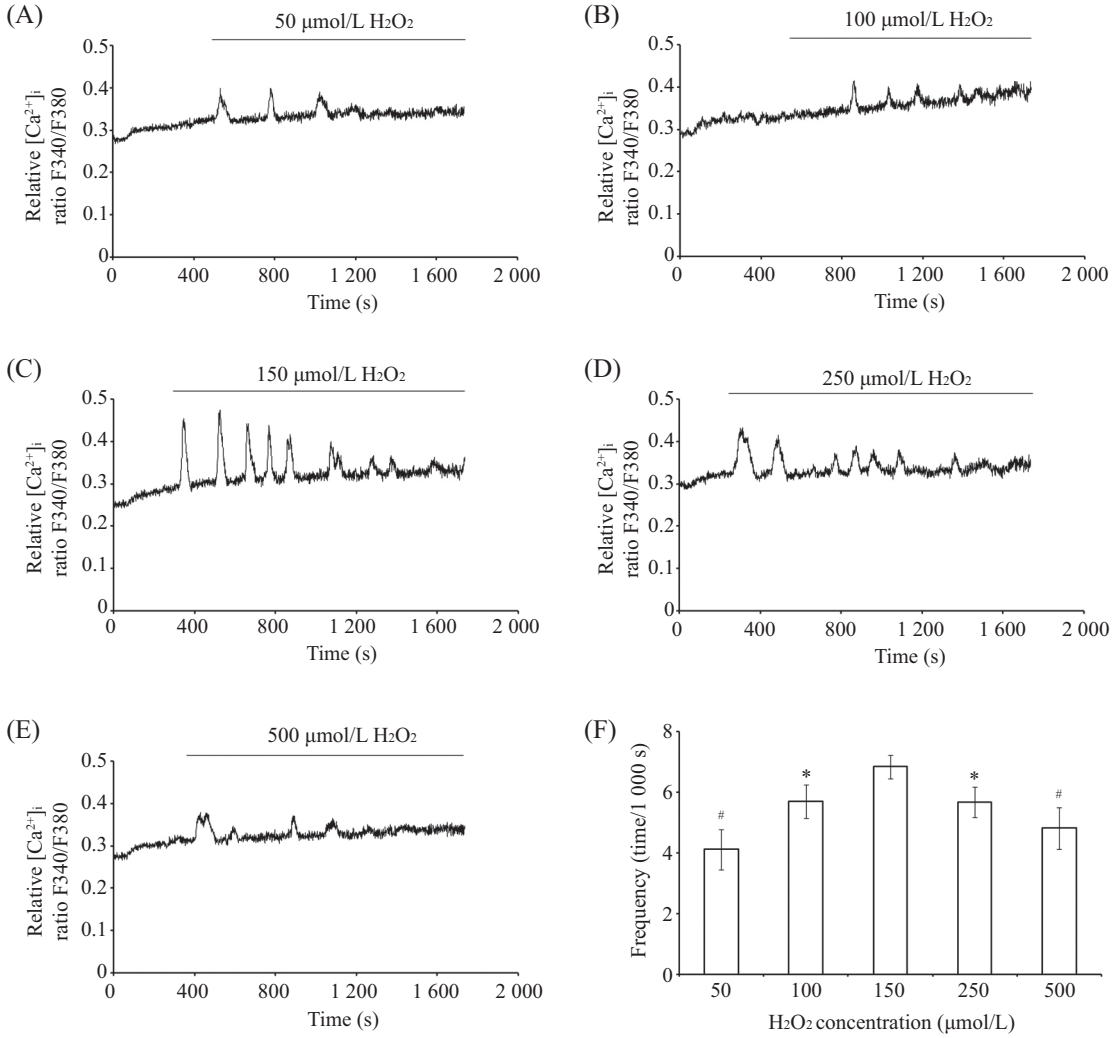
quickly.

2.2 Moderate concentration of H_2O_2 (≥50 μmol/L, ≤500 μmol/L) induced $[Ca^{2+}]_i$ oscillation in HBE

Different concentrations of H_2O_2 were added into the HBS, and the final concentration of H_2O_2 was 50, 100, 150, 250, 500 μmol/L respectively, then $[Ca^{2+}]_i$ in the HBE was detected. As showing in Fig.2, 50 μmol/L H_2O_2 induced $[Ca^{2+}]_i$ oscillation, but there were only a couple of times $[Ca^{2+}]_i$ oscillation existed. 100 μmol/L H_2O_2 induced more times of $[Ca^{2+}]_i$ oscillation than that of 50 μmol/L H_2O_2. 150 μmol/L H_2O_2 induced classical $[Ca^{2+}]_i$ oscillation, and the oscillation frequency was about 7 times per 1 000 seconds. When concentration of H_2O_2 was 250 μmol/L or 500 μmol/L, the frequency of $[Ca^{2+}]_i$ oscillation was decreased. Fig.2F showed different concentrations of H_2O_2 and the corresponding frequency of $[Ca^{2+}]_i$ oscillation.

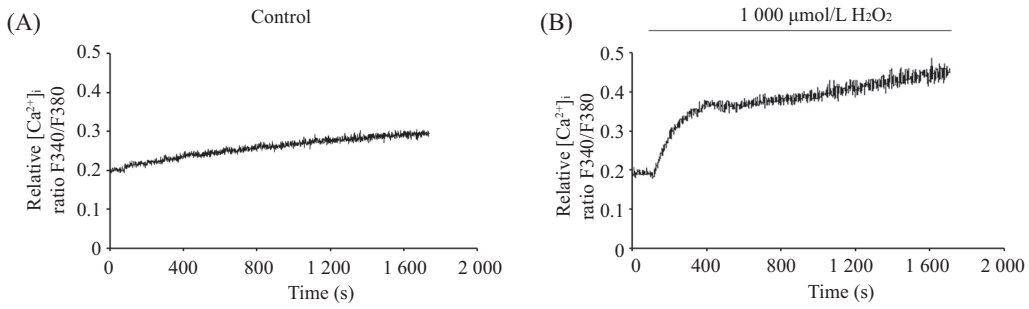
2.3 High concentration of H_2O_2 (1 000 μmol/L) induced constantly high level of $[Ca^{2+}]_i$ in HBE

Low concentration of H_2O_2 (30, 40 μmol/L) only induced a $[Ca^{2+}]_i$ spark in HBE, and moderate concentration of H_2O_2 (≥50 μmol/L, ≤500 μmol/L) induced $[Ca^{2+}]_i$ oscillation. At last, HBE were treated with 1 000 μmol/L H_2O_2. As showing in Fig.3, 1 000 μmol/L H_2O_2 induced constantly high level of $[Ca^{2+}]_i$ instead of $[Ca^{2+}]_i$ oscillation.



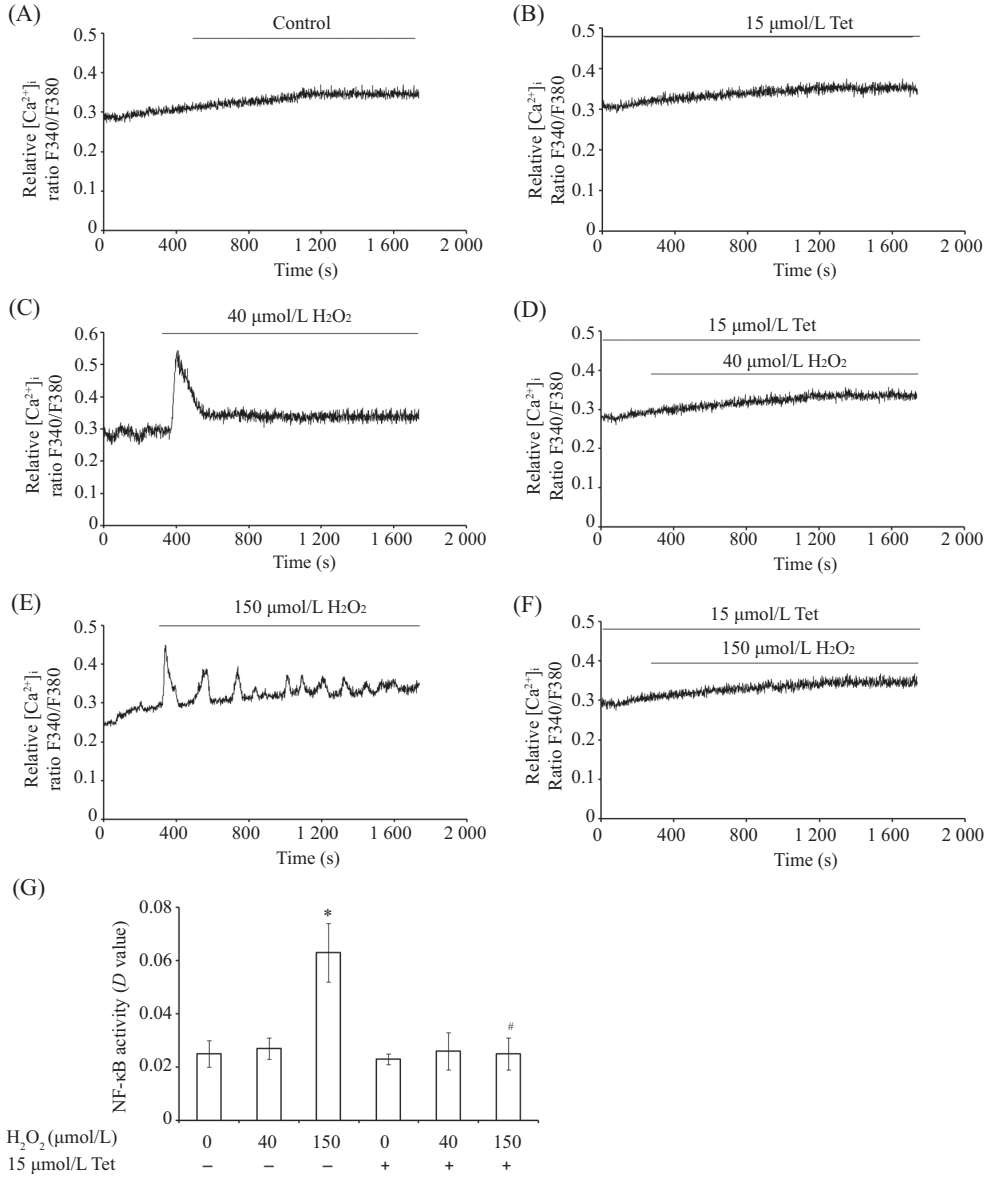
Cultured HBE were loaded with Fura-2, H_2O_2 was added into the Hepes buffered saline, and the final concentration of H_2O_2 was 50, 100, 150, 250, 500 μmol/L respectively, and then $[Ca^{2+}]_i$ was monitored on a fluorescent microscope. A~E: representative tracing shown that series of concentration of H_2O_2 induced $[Ca^{2+}]_i$ oscillation in HBE. A: concentration of H_2O_2 is 50 μmol/L (n=15); B: concentration of H_2O_2 is 100 μmol/L (n=15); C: concentration of H_2O_2 is 150 μmol/L (n=15); D: concentration of H_2O_2 is 250 μmol/L (n=15); E: concentration of H_2O_2 is 500 μmol/L (n=15); F: shown different concentrations of H_2O_2 and the corresponding frequency of $[Ca^{2+}]_i$ oscillation (*P<0.05, $^{\#}P$<0.01 *vs*. 150 μmol/L H_2O_2).

Fig.2 Effect of moderate concentration of H_2O_2 (≥50 μmol/L, ≤500 μmol/L) on $[Ca^{2+}]_i$ in HBE



Cultured HBE were loaded with Fura-2, H_2O_2 was added into the Hepes buffered saline, and the final concentration of H_2O_2 was 1 000 μmol/L, and then $[Ca^{2+}]_i$ was monitored on a fluorescent microscope. A: a representative tracing shown that the level of $[Ca^{2+}]_i$ is stable in HBE in the absence of H_2O_2 (n=15); B: a representative tracing shown that 1 000 μmol/L H_2O_2 induced constantly high level of $[Ca^{2+}]_i$ in HBE (n=15).

Fig.3 Effect of H_2O_2 (1 000 μmol/L) on $[Ca^{2+}]_i$ in HBE



Cultured HBE were loaded with Fura-2. H_2O_2 was added into the Hepes buffered saline, and the final concentration of H_2O_2 was 0, 40, 150 μmol/L respectively (A,C,E). In tetrandrine (Tet) treated groups (B,D,F), Tet was added into the Hepes buffered saline before H_2O_2 added, and the final concentration of Tet was 15 μmol/L. Then, $[Ca^{2+}]_i$ was monitored on a fluorescent microscope in HBE of all groups. A~F: representative tracing shown that H_2O_2 and/or Tet induced $[Ca^{2+}]_i$ changes in HBE (n=15); G: bar graph shown NF-κB activity in HBEs treated with H_2O_2 and/or Tet for 1 h (*P<0.01 *vs*. control, #P<0.01 *vs*. 150 μmol/L H_2O_2, n=5).

Fig.4 Effect of H_2O_2 on $[Ca^{2+}]_i$ oscillation and NF-κB activity in HBE

2.4 $[Ca^{2+}]_i$ oscillation induced by H_2O_2 (150 μmol/L) increased NF-κB activity in HBE

To further confirm the role of H_2O_2 in $[Ca^{2+}]_i$ oscillation and NF-κB activity, a calcium entry blocker, Tet was used in the experiments. Tet (final concentration is 15 μmol/L) was added into HBS before HBE exposed to H_2O_2. As shown in Fig.4C and 4E, 40 μmol/L H_2O_2 induced a $[Ca^{2+}]_i$ spark, 150 μmol/L H_2O_2 induced $[Ca^{2+}]_i$ oscillation in the absence of Tet. Neither 40 μmol/L H_2O_2 nor 150 μmol/L H_2O_2 made changes in $[Ca^{2+}]_i$ in the presence of Tet (Fig.4D and 4F). On the same time, HBEs were treated with H_2O_2 and/or Tet for 1 h, NF-κB activity was detected. As shown in Fig.4G, NF-κB activity was only increased in HBEs treated with 150 μmol/L H_2O_2 which induced $[Ca^{2+}]_i$ oscillation. There was no changes in NF-κB activity when HBEs were treated with 40 μmol/L H_2O_2 or Tet plus 150 μmol/L H_2O_2 which did not induce $[Ca^{2+}]_i$ oscillation. These data suggested that 150 μmol/L H_2O_2 induced $[Ca^{2+}]_i$ oscillation, and $[Ca^{2+}]_i$ oscillation induced increases of NF-κB activity.

3 Discussion

Bronchial epithelial cells are the barrier of the airway, and play a pivotal role in the respiratory diseases especially in bronchial inflammatory diseases. In bronchial asthma, inflammatory substances that are produced in airway inflammation damage the bronchial epithelial cells and lead to a subsequent asthma attack[11]. In chronic bronchitis, chronic inflammation affects the integrity of bronchial epithelial cells and induces airway remodeling. ROS, primarily generated during respiration, has been widely acknowledged that they can evoke cell damage[12]. It has been confirmed that ROS are overproduced during inflammation with the respiratory diseases. As the barrier of airway, bronchial epithelial cells damaged by ROS should be the early event in the respiratory diseases.

However, studies confirmed that different concentration of H_2O_2 induced different signaling pathway, and had different effects. For example, researchers determined that Pap1 signaling pathway responded to moderate increases in H_2O_2, whereas the complex Sty1 pathway was activated by higher levels of the oxidant in the yeast *Schizosaccharomyces pombe*[13-14]. In human embryonic kidney cells (HEK-293), Sobotta *et al*[15] reported that high or low concentration of H_2O_2 induced different effects. They also suggested that exogenous H_2O_2 concentrations (>25~50 μmol/L) temporarily overwhelmed the catalase filter and therefore led to nonphysiological effects.

In the current study, we used 0~1 000 μmol/L H_2O_2 to treat human bronchial epithelial cells and detected $[Ca^{2+}]_i$. We found that H_2O_2 concentrations <50 μmol/L just induced $[Ca^{2+}]_i$ spark, 50~500 μmol/L induced $[Ca^{2+}]_i$ oscillation, and 1 000 μmol/L induced constantly high level of $[Ca^{2+}]_i$. To our knowledge, this is the first time to report the relationship between different concentration of H_2O_2 and $[Ca^{2+}]_i$ in human bronchial epithelial cells. High concentration of H_2O_2 are majorly produced in acute serious inflammation, so in acute serious inflammation, high concentration of H_2O_2 induced high level $[Ca^{2+}]_i$ should be one main cause of cell death. But in mild chronic inflammation, moderate concentration H_2O_2 occurred which should induce $[Ca^{2+}]_i$ oscillation according our current finding. Previous studies revealed that $[Ca^{2+}]_i$ oscillation could induce activity of NF-κB[6,10]. To further confirm the relationship between $[Ca^{2+}]_i$ oscillation and NF-κB activity in HBE, we detected NF-κB activity. The results revealed that 150 μmol/L H_2O_2 induced $[Ca^{2+}]_i$ oscillation and increases of NF-κB activity. With the molecular formula $C_{38}H_{42}O_6N_2$, Tet is a blocker of Ca^{2+} channels[16]. Studies confirmed that Tet could abolish $[Ca^{2+}]_i$ oscillation in the cells[17]. So we used Tet to abolish $[Ca^{2+}]_i$ oscillation induced by 150 μmol/L H_2O_2. Not surprisingly, there were no increases of NF-κB activity without $[Ca^{2+}]_i$ oscillation happening. Thus, it confirmed that 150 μmol/L H_2O_2 induced $[Ca^{2+}]_i$ oscillation, and $[Ca^{2+}]_i$ oscillation induced increases of NF-κB activity in HBEs. Taken together, moderate-H_2O_2/$[Ca^{2+}]_i$-oscillation/NF-κB should be a novel signaling pathway in the chronic inflammation.

In conclusion, moderate concentration (50~500 μmol/L) of H_2O_2 can induce $[Ca^{2+}]_i$ oscillation and

NF-κB activation in bronchial epithelial cells, which should be involved in ROS induced damage in chronic airway inflammation.

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