Expression And Mechanism of KLF6 And iNOS During Apoptosis of Macrophages Induced By Pseudomonas Aeruginosa Supernatant

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Research Article

Keywords: Pseudomonas aeruginosa, RAW264.7, apoptosis, KLF6, iNOS, SMT

Posted Date: October 25th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-927426/v1

Abstract

Background: Pseudomonas aeruginosa (PA) is one of the most common gram-negative opportunistic pathogens in nosocomial infection. The susceptible population is mainly patients with low immunity, such as pulmonary cystic fibrosis, chronic obstructive pulmonary disease, tumor, bronchiectasis and burn.Macrophages from monocytes are the main innate immune cells recruited to the site of inflammation. After infection leads to local inflammation, these macrophages recruited to the inflammatory site play a key role in the occurrence, diffusion and regression of inflammation. The proliferation ability of macrophages is reduced, which weakens the ability of macrophages to phagocytize pathogens, resulting in persistent infection and lung tissue injury.KLF6 plays an important role in the regulation of gene expression in inflammatory response, and it has been reported that there is a functional relationship between KLF6 and inducible nitric oxide synthase (iNOS). Our previous studies found that PA can induce the expression of KLF6 in lung tissue cells at the animal level, and may mediate apoptosis of lung tissue cells by regulating iNOS. In this study, the effect of PA supernatant on RAW264.7 cells apoptosis and the expression of KLF6 and iNOS were observed. In order to further study the role of iNOS in the apoptosis of RAW264.7 cells induced by PA supernatant, S-methylisothiourea sulfate (SMT) was added to the infected RAW264.7 cells to detect the expression level of iNOS, the change of NO content and the apoptosis of RAW264.7 cells.

Methods: The MTT assay was used to detect the cell proliferation rate. Considering the different volume concentrations(the volume ratio of PA supernatant to complete medium) of PA supernatant, the experiment groups were divided into control group(add same volume of complete medium), 15% PA group, 30% PA group, 45% PA group, the cells in each group were treated for 12h and 24h respectively. In addition, SMT(+) group(SMT was the iNOS blocker) and SMT(-) group(without SMT) were designed for the experiment, the concentration was selected as 6μM according to the manual and preliminary results. According to MTT results, 30% PA supernatant treating for 24h was screened as the processing condition.Flow cytometry was used to detect the apoptosis rate. Hoechst 33342 staining was used to observe the nuclear morphological changes. Western blot was used to detect the expression of KLF6 and iNOS protein. The expression levels of KLF6 mRNA and iNOS mRNA were detected by real-time PCR. The NO content detection kit was used to detect the content of NO. Finally, the data analysis was conducted by using SPSS16.0 software.

Results: When the PA supernatant at different concentrations treated the cells for the same time interval, the proliferation rate increased in a concentration-dependant manner, indicating that the PA supernatant inhibited the proliferation of RAW264.7 cells in a time-concentration-dependent manner. Meanwhile,the same results were achieved in flow cytometry and staining with Hoechst 33342 detected the same conclusion. Furthermore, the expression of KLF6 and iNOS protein, KLF6 mRNA and iNOS mRNA increased significantly after treatment, and NO content was statistically determined after treatment in a concentration-dependent manner and a time-dependent manner. Finally, compared with the SMT(-) group, the apoptosis rate was significantly decreased, and the NO content decreased after treatment with PA supernatant,.

Conclusions: Our findings demonstrste tthat PA supernatant can inhibit the proliferation of RAW264.7 cells, and its inhibitory effect is concentration-time dependent. The mechanism by which PA induces apoptosis of RAW264.7 cells may be caused by the production of excess NO by KLF6 and iNOS. The reduction of iNOS expression has a protective effect on the cytotoxicity caused by PA supernatant infectied RAW264.7 cells.

1. Introduction

Pseudomonas aeruginosa (PA) is one of the most common gram-negative opportunistic pathogens in nosocomial infection. The susceptible population is mainly patients with low immunity, such as pulmonary cystic fibrosis [1], chronic obstructive pulmonary disease [2], tumor [3], bronchiectasis [4] and burn [5]. In recent years, PA has become one of the most common pathogenic factors with high prevalence and mortality of nosocomial infection, and the drug resistance rate is increasing year by year [6]. In this study, PA was selected as the research object to explore its mechanism of action on immune cells, so as to find a new direction for the treatment of its infection.

Macrophages from monocytes are the main innate immune cells recruited to the site of inflammation. After infection leads to local inflammation, these macrophages recruited to the inflammatory site play a key role in the occurrence, diffusion and regression of inflammation. The proliferation ability of macrophages is reduced, which weakens the ability of macrophages to phagocytize pathogens, resulting in persistent infection and lung tissue injury [7]. The proliferation and apoptosis of macrophages play an important role in the process of infection. Therefore, to explore the effect of PA supernatant on the proliferation inhibition and apoptosis of macrophages is the key to control PA infection.

Kruppel like factor 6 (KLF6) is a member of the Kruppel like C2H2 zinc finger transcription factor family, which is involved in a variety of cell proliferation, apoptosis and other processes [8]. KLF6 plays an important role in the regulation of gene expression in inflammatory response [9], and it has been reported that there is a functional relationship between KLF6 and inducible nitric oxide synthase (iNOS) [10]. INOS is generally not expressed in cells. In macrophages, it is usually stimulated by some pathogens or cytokines to induce iNOS synthesis and produce nitric oxide (NO) [11], which can destroy cell stability, play a cytotoxic role, promote apoptosis, and also cause damage to surrounding tissues [12, 13].

Domestic and foreign studies have found that the overexpression of KLF6-specific protein 1(SP1) in endothelial cells can reduce the expression of activin receptor like kinase 1 (ALK1) and reduce the formation of vascular intima [14]. KLF6 interacts with the related transcription factor SP1 to bind to the endoglycoprotein promoter. Endothelial glycoprotein has been confirmed to be involved in leukocyte transport during vascular inflammation. Therefore, KLF6 in endothelial cells can promote the infiltration of immune cells during vascular injury [15]. However, the role of KLF6 and iNOS in PA supernatant induced macrophage apoptosis is rarely studied.

Our previous studies found that PA can induce the expression of KLF6 in lung tissue cells at the animal level, and may mediate apoptosis of lung tissue cells by regulating iNOS [16]. In this study, the effect of

PA supernatant on RAW264.7 cells apoptosis and the expression of KLF6 and iNOS were observed. In order to further study the role of iNOS in the apoptosis of RAW264.7 cells induced by PA supernatant, S-methylisothiourea sulfate (SMT) was added to the infected RAW264.7 cells to detect the expression level of iNOS, the change of no content and the apoptosis of RAW264.7 cells.

2. Material And Methods

2.1 Cell culture, bacterial strains and incubation

Raw 264.7 macrophages were derived from mouse monocyte macrophages, which were donated by Institute of Cardiovascular Disease, North Theater General Hospital. They were infected by the supernatant of PA which was the standard strain ATCC27853, which came from the microbiology laboratory of Jinzhou Medical University.

2.2 MTT assay

RAW264.7 cells were seeded into 96 well plates according to 13000 cells per well and cultured overnight. The cells were divided into control group, 15% PA group, 30% PA group and 45% PA group, with 6 multiple wells in each group. The cells were cultured in 5% CO_2 cell incubator at 37℃ for 6h, 12h, 24h and 48h, respectively. After that, add 20μL in each hole. The cells were incubated for 4 hours in the incubator. Suck out the supernatant and add 150μL in each hole. Until dimethyl sulfoxide(DMSO) was completely dissolved. The absorbance of each well was measured at 560nm by full wave microplate reader, and the experiment was repeated three times. The cell proliferation inhibition rate (%) was calculated as: 1-OD value of experimental group/OD value of negative control group×100%.

2.3 Groups

RAW264.7 cells were divided into control group (added with equal volume of complete medium), 15% PA group, 30% PA group and 45% PA group (volume ratio of 15%, 30% and 45% PA supernatant and medium mixture).

In addition, S-methylisothiourea sulfate（Biyuntian Biotechnology Co., Ltd, China）SMT（-) group (without SMT) and SMT (+) group (as the blocker of iNOS, the concentration of SMT was 6 μM). According to the results of MTT, 30% PA supernatant was used to treat the cells for 24 hours.

2.4 Annexin V-FITC / PI double staining flow cytometry

The cells in logarithmic growth phase were inoculated on 6-well plate(2×10^5 cells / well) and cultured overnight at 37℃ in 5% CO_2. PA supernatant was added according to the concentration gradient. The control group was the normal cultured cells, and 24h was the detection point. Flow cytometry(BD Biosciences, USA) was used within 1 hour.

2.5 Hoechst 33342 staining

The cells were treated with 1.8% ethanol and were inoculated in 12 well plates and cultured in 5% CO_2 incubator at 37℃ for 24 hours. The cells were divided into control group and PA supernatant group (15%, 30%, 45%), with 24h as the detection point. The culture medium was sucked up 300 μL fixative was added for 15 min. Then, the fixed solution was removed washed with PBS twice for 5 min each time, and the liquid was sucked up. Next, 300 μL Hoechst 33342 was added, incubated at room temperature for 15 min and washed withe PBS wash 3 times for 5 min each time. Then the morphological changes of apoptotic nuclei were observed by the cystation3 cell imaging multifunctional detector(BIO-Tek, USA).

2.6 Western blot

Total proteins were extracted from whole-cell lysates, and then subjected to western blot as previously described. Membranes were incubated with primary antibodies against KLF6 and iNOS, then the first antibodies are added, marked and shaked overnight at 4℃(16-18h). Gray value analysis was performed by analysis software, and the gray value of the target protein band was compared with the internal reference β, the ratio of gray value of actin protein bands represented the relative surface level of each protein.

2.7 Fluorescence quantitative PCR analysis

Total RNA was isolated from whole cell, and cDNA was synthesized using the Reverse Transcriptase kit (TaKaRa, Japan). SYBR Green real-time PCR (TaKaRa, Japan) was performed in duplicate for each sample to determine relative gene expression using GAPDH as a housekeeping control with the $2^{-\Delta\Delta Ct}$ method.

Primers used in reverse transcription-quantitative polymerase chain reactions. Primer sequence in qPCR as Table 1.

2.8 Determination of nitric oxide (NO) content

The cells were cultured and groups were subjected to Western blot as previously described.The measurement steps were followed the instructions. After the operation, A_{550} was measured after the mixture standing at roon temperature for 15min.

No content calculation

No content definition: at 25 ℃, 1μmoL NO_2^- equivalent to 1μmoL NO is generated every 15 minutes of sample per litre.

No content（μmoL /L）=（A_{550}+0.0103）÷0.016×$V_{\text{inverse total}}$÷V_{sample}140×（A_{550} +0.0103）.

2.3.8 Effect of PA supernatant on apoptosis of RAW264.7 after blocking iNOS

On the basis of the results of previous experiments, 30% PA supernatant was added to SMT (-) group and SMT (+) group for 24 hours respectively. The expression of iNOS was detected by Western blot. The content of NO was determined by the same method as before. The apoptosis of cells was detected by flow cytometry, the same as before.

2.3.9 Statistical analysis

All the data analyses were performed using SPSS 16.0 software.Results were expressed as ($\bar{x}\pm s$). T-test was used to compare the two groups' mean, one-way ANOVA was adopted to compare the multi groups' mean, LSD (least significant difference) test was used for further pairwise comparison between groups, and Dunnett's T3 test was applied for uneven variance. Data with a *P* value of <0.05 were considered statistically significant.

3. Results

3.1 Inhibitory effect of PA supernatant on proliferation of RAW264.7 cells

Compared with the control group, the inhibition rate of cell proliferation increased significantly in a concentration-dependent manner from 12 h ($P < 0.05$). Therefore, the apoptosis of RAW264.7 cells induced by PA supernatant for 12 h may be the beginning. And with the increase of time, the inhibition rate of cell proliferation increased gradually. When the 15% PA group was treated for 48 h, the inhibition rate of cell proliferation was 44.88%, which indicated that the 15% PA group had strong effect damage to RAW264.7 cells after 48 h treatment, leading to more cell necrosis. Therefore, the PA supernatant with the concentration of 15%, 30%, 45% was selected as the concentration, and the detection time points were 12 h and 24 h.

When the cells were treated with the same PA supernatant at the same concentration for 12h and 24h, respectively,the cell proliferation rate increased in a time-dependent manner, and the difference was statistically significant ($P < 0.05$). When treated by PA supernatant of different concentrations, the cell proliferation rate increased with the increase of concentrations, and the difference was statistically significant ($P<0.05$). The results showed that PA supernatant could inhibit the proliferation of RAW264.7 cells in a time-concentration-dependent manner. See table 2-1, 2-2 and Fig 1(a) for details.

3.2 Flow cytometry

The results of flow cytometry showed that the apoptotic rate of RAW264.7 cells treated with PA supernatant of different concentrations of PA supernatant for 24 hours gradually increased with the increase of PA supernatant concentrations, and the difference was statistically significant compared with the control group ($P<0.05$). Therefore, PA supernatant can induce apoptosis of RAW264.7 cells in a concentration-dependent manner, as shown in Table 3 and Fig. 1(b c).

3.3 Morphological changes

After Hoechst 33342 staining, the nuclei of RAW264.7 cells in the control group were blue round or oval, and the chromosomes were uniform; When RAW264.7 cells were treated with PA supernatant of 15%, 30% and 45% for 24 hours, the nuclei of RAW264.7 cells were found pyknosis and became hyperchromatic, and dense massive granules could be seen in the nuclei. The morphological changes of these nuclei were the expression of apoptosis, especially in the 45% PA group, as shown in Fig. 1d.

3.4 PA supernatant promotes the expression of KLF6 and iNOS in RAW264.7 cells

Western blot results showed that the expression levels of KLF6 and iNOS protein in RAW264.7 cells infected with PA supernatant at different concentrations for 24 hours were higher than those in the control group, and the difference was statistically significant ($P < 0.05$). The expression levels of KLF6 and iNOS in RAW264.7 cells treated with 30% PA for 12 h and 24 h were significantly increased in a time-dependent manner ($P < 0.05$). It is suggested that KLF6 and iNOS proteins were expressed in the process of apoptosis of RAW264.7 cells induced by PA supernatant, which may be involved in the process of apoptosis of RAW264.7 cells induced by PA supernatant. See Fig. 1(e. f) and Fig. 1(g. h).

3.5 PA supernatant promotes the expression of KLF6 mRNA and iNOS mRNA in RAW264.7 cells

The results of fluorescence quantitative PCR showed that the expression levels of KLF6 mRNA and iNOS mRNA in RAW264.7 cells treated with different concentrations of PA supernatant for 24 hours were higher than those in the control group, and the difference was statistically significant ($P<0.05$). The expression levels of KLF6 mRNA and iNOS mRNA in RAW264.7 cells treated with 30% PA for 12 h and 24 h were significantly higher than those in the control group ($P< 0.05$). It was suggested that PA supernatant could promote the expression of KLF6 mRNA and iNOS mRNA in RAW264.7 cells. KLF6 mRNA and iNOS mRNA played an important role in the process of apoptosis Fig. 1(i.j).

3.6 Effect of PA supernatant on NO content in RAW264.7 cells

The results of NO content detection showed that after RAW264.7 cells were infected with different concentrations of PA supernatant for 24 h, the content increased with the increase of PA supernatant concentration, and the difference was statistically significant ($P < 0.05$). The content of NO in RAW264.7 cells infected with 30% PA for 12h and 24h increased in a time-dependent manner ($P < 0.01$). The results suggested that KLF6 and iNOS may activate the release of NO, exert cytotoxicity and lead to apoptosis in the process of apoptosis of RAW264.7 cells induced by PA supernatant. See Table 4, Fig. 1(k), Table 5 and Fig. 1 (L) for details.

3.7 Effect of iNOS inhibitor SMT on apoptosis of RAW264.7 cells induced by PA supernatant

After RAW264.7 cells were infected with 30% PA supernatant for 24 hours, the expression level of iNOS protein in SMT (+) group was lower than that in SMT (-) group ($P < 0.01$), as shown in Fig. 2(a. b).

Western blot results showed that SMT blocked iNOS successfully, and the NO content in the supernatant of cells treated with PA supernatant was detected. Compared with SMT (-) group, the NO content in SMT

(+) group was significantly decreased, and the difference was statistically significant (P < 0.05), as shown in Table 6.

In order to verify the possibility of the decrease of apoptosis rate induced by PA supernatant after SMT blocking iNOS, the apoptosis rate of cells after SMT intervention in iNOS was detected by flow cytometry. Compared with SMT (-) group, the apoptosis rate of cells treated with PA supernatant decreased significantly (P < 0.01), as shown in Table 7 and Fig.2(c.d).

The results further showed that iNOS could induce apoptosis by releasing NO in the process of PA supernatant induced apoptosis of RAW264.7 cells, and the decrease of iNOS expression could protect the inflammatory response induced by PA.

4. Discussion

4.1 Effect of PA on proliferation of RAW264.7 cells

PA is one of the main pathogens causing high mortality of nosocomial infection. The virulence factor of PA can counteract host defense, cause direct damage to host tissue or improve the competitiveness of bacteria, induce apoptosis of a variety of immune cells, and lead to acute and chronic infection [17]. In recent years, PA is resistant to more and more antibiotics. Therefore, it is important to study the mechanism of PA on immune cells for the treatment of PA infection. The results showed that PA supernatant could significantly inhibit the proliferation of RAW264.7 cells in a time-concentration-dependent manner. It is suggested that the pathogenic mechanism of PA may be through inhibiting the immune cells of the body, thus inhibiting the immune response of the body, leading to persistent or local inflammatory reaction, therefore, long-term application of antibiotics leads to the drug resistance of PA.

4.2 Effect of PA on KLF6 and iNOS expression in RAW264.7 cells

So far, the molecular mechanism of PA induced apoptosis has been widely studied. Fas ligand (FasL), Bax and Bcl-2 are the three most studied apoptosis inducing genes. Among them, FasL is a ligand that can bind with FAS, the death receptor, to form an apoptotic complex, transmit the signal to the cell interior, start the apoptotic process, and mediate apoptosis [18-19]. Bcl-2 can inhibit apoptosis, however, Bcl-2 can also induce apoptosis through mitochondrial pathway. Bax inhibits the activity of Bcl-2 and antagonizes its anti-apoptotic effect. The ratio of Bax and Bax can start the "switch" of apoptosis [20]. KLF6, a new transcription factor, has been found in oncology related studies, the expression of KLF6 promotes the apoptosis of tumor cells and reduces the metastasis of tumor cells. KLF6 plays an important role in the occurrence and development of cancer [21, 22]. When researchers find that KLF6 plays an important role in the occurrence and development of diseases, KLF6 is studied more widely.

In recent years, there are many studies on the role of KLF6 in the regulation of inflammation. When the body encounters physical stimulation such as heat shock protein and hypoxia, KLF6 is produced by cells to induce iNOS expression, which catalyzes the synthesis of NO and regulates various physiological

functions of the body [23,24]. When the body is stimulated by inflammation, the high expression of NO in the body can make the cells produce a series of pathophysiological changes through its cytotoxic effect, and eventually cause different degrees of cell damage, inflammatory reaction and apoptosis [25]. For example, KLF6 can directly form KLF6-DNA complex with iNOS promoter, regulate the expression of iNOS, release NO [26], destroy the stability of cells, play a cytotoxic role, and participate in the inflammatory response induced by virus infection. After down regulating the expression of KLF6 in epithelial cells, the expression of iNOS decreases, the activity of NO decreases, alleviates airway hyperresponsiveness, and delays the development of inflammation [27]. However, the role of KLF6 and iNOS in PA infected macrophages is rarely reported. The results showed that KLF6 and iNOS protein expression were detected by Western blot in the process of PA induced RAW264.7 cells apoptosis, and the expression level increased in a time-concentration-dependent manner, which may be involved in the process of PA supernatant induced RAW264.7 apoptosis. Subsequently, the expression of KLF6-mRNA and iNOS-mRNA were detected by fluorescence quantitative PCR. The expression of KLF6-mRNA and iNOS-mRNA was detected in the process of RAW264.7 apoptosis induced by PA supernatant, which played an important role in the process of apoptosis. The results showed that with the increase of PA supernatant concentration, the content of NO increased gradually. After treated with PA supernatant for 12h and 24h, the NO content in the cells increased gradually. It is speculated that KLF6 expression is increased in PA induced macrophage apoptosis, and the up-regulation of iNOS expression may be induced by KLF6. INOS catalyzes the production of NO and participates in the process of apoptosis.

4.3 Effect of iNOS inhibitor SMT on apoptosis of RAW264.7 induced by PA supernatant

As early as 1994, Kamijo et al. [28] have confirmed that macrophages can activate the release of NO by increasing the expression of iNOS. Mgbemena et al. And Wang Chunbo et al. [27,29] showed that iNOS could transcribe NO and promote cell apoptosis. Although iNOS and NO have always been considered as an important factors in the occurrence of diseases, the role of iNOS and NO in the process of macrophage apoptosis induced by PA infection has rarely been reported. In this study, in order to explore the role of iNOS in the process of apoptosis of RAW264.7 cells induced by PA supernatant, SMT was given to interfere with the production of iNOS in RAW264.7 cells, and the content of NO in the process of apoptosis was decreased correspondingly, and the apoptosis rate of RAW264.7 cells was decreased. The results further indicate that iNOS catalyzes the synthesis of NO in the process of apoptosis of RAW264.7 cells induced by PA supernatant, which forms the basis of monocyte macrophage growth inhibition and cytotoxicity, promotes macrophage apoptosis, and the decrease of iNOS expression plays a protective role in PA induced inflammation.

In conclusion, PA supernatant can inhibit the proliferation of RAW264.7 cells in a time-concentration-dependent manner, and induce apoptosis of RAW264.7 cells, which may be due to the increase of KLF6 expression stimulated by PA supernatant, KLF6 regulates the release of iNOS activated NO and promotes apoptosis. iNOS is involved in the apoptosis of RAW264.7 cells induced by PA supernatant. However, whether the expression of iNOS depends on the induction of KLF6, and whether KLF6 plays a decisive role in PA supernatant induced apoptosis of RAW264.7 cells remains to be further studied. This study

provides a new theoretical basis and research direction for reducing the inflammatory response caused by PA infection.

Declarations

Availability of data and materials

The dataset supporting the conclusions of this article is included within the article.

Author contributions

Meng-ru Liu performed the experiments and analyzed the data;Ya-qi Wang drafter the manuscript; Wen-Shu Chai designed the project and finalized the manuscript.

Funding

This study was supported by the Key Project of Natural Science Foundation of Liaoning Province （No.20170540390）

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study does not involve the use of human, animal and plant data or tissue.

Competing interests

All authors declare that they have no competing interests.

Ackowlegedgement

Not applicable.

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Tables

Table 1 Primer sequence in qPCR

Genes		Primer sequences（5’-3’）
GADPH	Forward primer	5’-ATTGTCAGCAATGCATCCTG-3’
KLF6	Reverse Primer	5’-ATGGACTGTGGTCATGAGCC-3’
iNOS	Forward primer	5’-CGGACGCACACAGGAGAAAA-3’
	Reverse Primer	5’-CGGTGTGCTTTCGGAAGTG-3’
	Forward primer	5’-CTGCAGCACTTGGATCAGGAACCTG-3’
	Reverse Primer	5’-GGGAGTAGCCTGTGTGCACCTGGAA-3’

Table 2-1 Effect of PA supernatant of the same concentration on the proliferation rate of RAW264.7 cells at different time（$\bar{x}\pm s$, n=3）

Groups	Control	15%PA group	30% PA group	45% PA group
6h	0	3.17±1.89	3.6±0.53	7.02±0.6
12h	0	12.97±0.63$^{\&}$	27.47±2.13$^{\&}$	49.17±3.92$^{\&}$
24h	0	32.19±3.23$^{\&}$●	48.14±3.85$^{\&}$●	63.79±3.73$^{\&}$●
48h	0	44.88±7.67$^{\&}$●●	58±4.58$^{\&}$●●	79.73±6.27$^{\&}$●●
F		39.261	114.454	79.929
P		0.000	0.000	0.000

$^{\&}$ *P*<0.05 vs PA supernatant of the same concentration for 6h group.

●*P*<0.05 vs the same concentration of PA supernatant 12h group.

●*P*<0.05 vs the same concentration of PA supernatant 24h group.

Table2-2 Effects PA supernatant of different concentrations on the proliferation rate of RAW264.7 cells （$\bar{x}\pm s$, n=3）

Groups	6h	12h	24h	48h
Control	0	0	0	0
15%PA group	3.17±1.89	12.97±0.63**	32.19±3.23**	44.88±7.67**
30% PA group	3.6±0.53*	27.47±2.13**#	48.14±3.85**#	58±4.58**#
45% PA group	7.02±0.6*▲	49.17±3.92**#▲	63.79±3.73**#▲	79.73±6.27**#▲
F	21.251	175.212	152.058	76.105
P	0.000	0.000	0.000	0.000

*$P<0.05$ **$P<0.01$ vs control group.

#$P<0.05$ vs 15% PA group.

▲$P<0.05$ vs 30% PA group.

Table 3 Effects of PA supernatant on apoptosis of RAW264.7 cells（$\bar{x}\pm s$, n=3）

Groups	Apoptosis rate（%）
Control	9.48±0.91
15%PA group	12.24±0.63*
30% PA group	15.92±1.41*ΔΔ
45%PA group	39.15±0.75*ΔΔ▲▲
F	390.442
P	0.000

*$P<0.05$ vs control group; ΔΔ$P<0.01$ vs 15% PA group; ▲▲$P<0.01$ vs 30% PA group.

Table 4 Effect of PA supernatant on NO content in RAW264.7 cells（$\bar{x}\pm s$, n=3）

Groups	NO content（μmoL/L）
Control	4.55±0.54
15%PA group	7.87±0.57*
30%PA group	10.42±1.26**#
45%PA group	13.45±1.83**##&
F	20.570
P	0.000

*P<0.05,** P<0.01 vs control group;

#P<0.05，##P<0.01 vs 15% PA group;

&P<0.05 vs 30% PA group.

Table 5 Changes of NO content in RAW264.7 cells treated with PA supernatant for in different time

Groups	NO content（μmoL/L）
Control	7.22±1.2
12h	15.31±0.98**
24h	21.59±1.02**##
F	89.909
P	0.000

**P<0.01 vs control group;

##P<0.01 VS 12h group

Table 6 Effect of iNOS inhibitor SMT on NO content in RAW264.7 cells apoptosis induced by PA supernatant（$\bar{x}$±s, n=3）

Groups	NO content（μmoL/L）
SMT（-）group	11.14±1.7
SMT（+）group	1.66±0.61*
t	13.129
P	0.001

**P<0.01 vs SMT-group.

Table 7 effect of iNOS inhibitor SMT on apoptosis of RAW264.7 cells induced by PA supernatant

Groups	Apoptosis rate(%)
SMT-group	36.58±2.62
SMT+group	13.58±1.34**
t	11.055
P	0.002

**P<0.01 vs SMT-group.

Figures

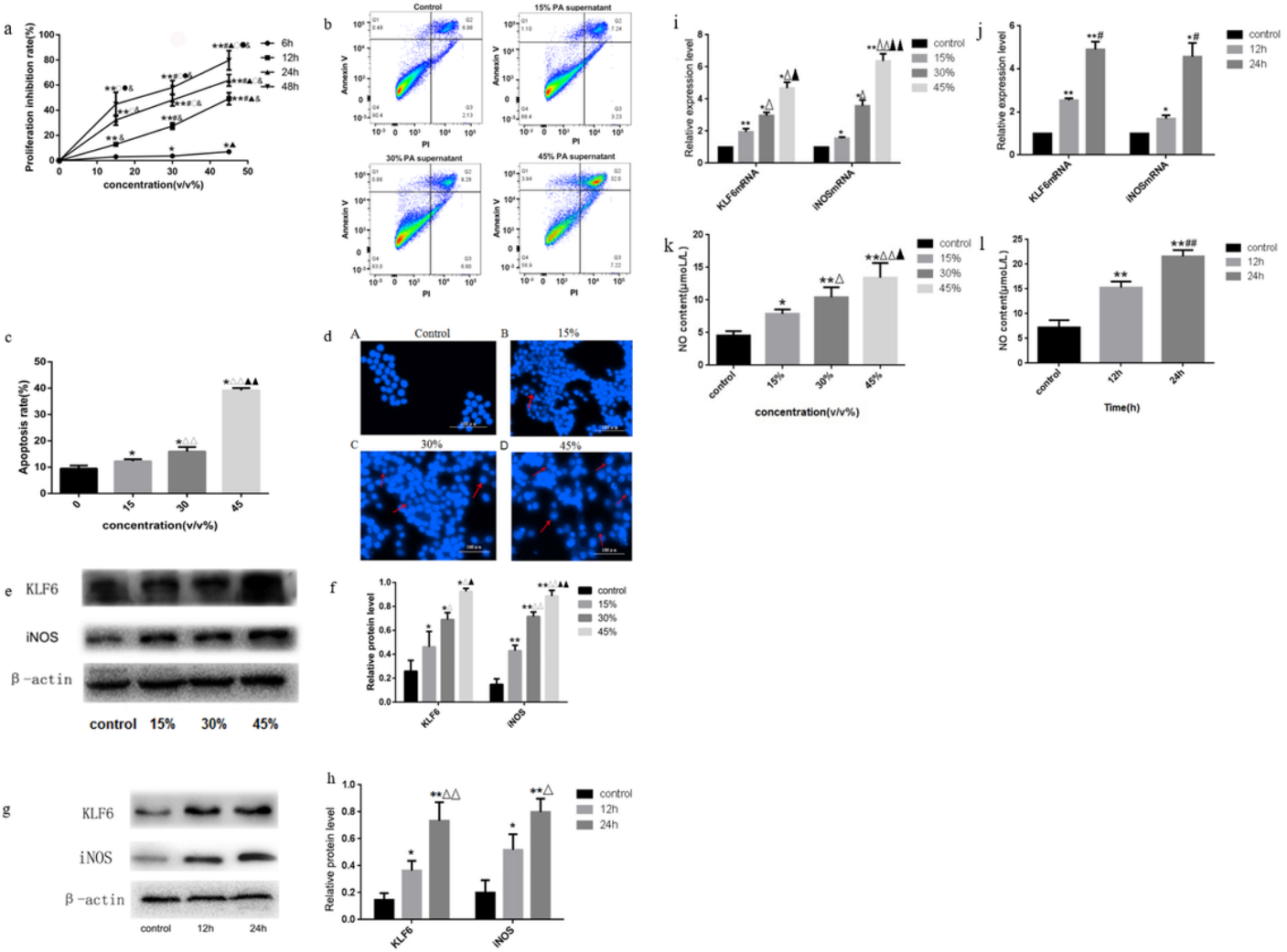

Figure 1

PA supernatant has an obvious proliferation inhibition on RAW264.7 cell.(a) Detection of proliferation inhibition rate of RAW264.7 cells in MTT. (b. c) Effect of PA supernatant on apoptosis of RAW264.7 cells. Apoptotic rate (%) = early apoptotic rate + late apoptotic rate, namely Q1 + Q2. (d)The morphological changes were observed by cystation3 cell imaging system.(e. f) The detection of KLF6 and iNOS protein expression in RAW264.7 cells treated with PA supernatant in different concentration. (f. h)Expression of KLF6 and iNOS in RAW264.7 cells treated with PA supernatant in different time. (i)Effect of PA supernatant on KLF6 mRNA and iNOSmRNA expression in RAW264.7. (j)Expression of KLF6 mRNA and iNOS mRNA in RAW264.7 cells treated with PA supernatant for different time. (k)Effect of PA supernatant on NO content in RAW264.7 cells. (l)Changes of NO content in RAW264.7 cells treated with PA supernatant for different time.*P<0.05□**P<0.01 vs control group; ΔP<0.05□ΔΔP<0.01 vs 15% PA group; ▲P<0.05□▲▲P<0.01 vs 30% PA group□#P<0.05□##P<0.01 vs 12h group.

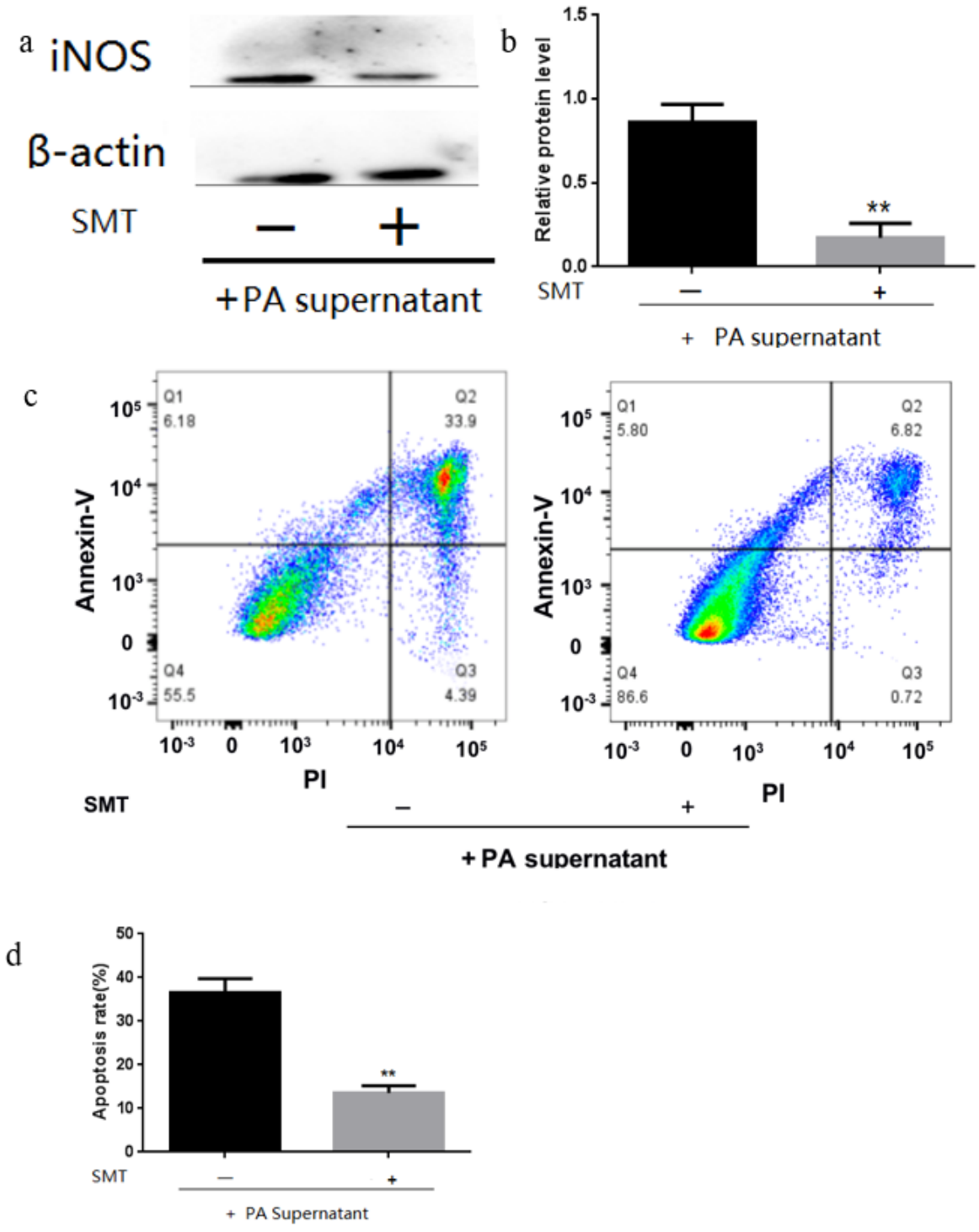

Figure 2

Effect of iNOS inhibitor SMT on apoptosis of RAW264.7 cells induced by PA supernatant.a. bEffect of iNOS inhibitor SMT on iNOS expression in RAW264.7 cells apoptosis induced by PA supernatant.(c. d)Effect of iNOS inhibitor SMT on apoptosis of RAW264.7 cells induced by PA supernatant.Apoptotic rate (%) = early apoptotic rate + late apoptotic rate, namely Q1 + Q2. **P<0.01 vs SMT-group.