Novel H₂S donor proglumide-ADT-OH protects HUVECs from ox-LDL-induced injury through NF-κB and JAK/SATA pathway

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Short Running Title: P-A protects HUVECs from ox-LDL-induced injury

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Hydroxyphenyl)-3H-1, 2-dithiole-3-thione) (P-A)
Abstract

Introduction: As a gaseous medilator, hydrogen sulfide (H$_2$S) has many physiological effects and pathological effects in atherosclerosis. In recent years, many exogenous H$_2$S donors have been synthesized to study atherosclerosis diseases.

Methods: Proglumide-(5-(4-Hydroxyphenyl)-3H-1, 2-dithiole-3-thione) (P-A) was synthesized as a H$_2$S donor. The protective effect and mechanism of P-A on HUVEC that injured by ox-LDL was detected.

Results: The HUEVCs was affected by 100μmol/L P-A for 24 hours, the release of H$_2$S was the largest. After 100μmol/L P-A acted on HUVEC damage model for 12h, the cell proliferation activity was the best. The results showed that P-A can down regulate the expression of p-NF-κBp65 protein and reduce the amount of TNF-α and IL-6 and promote the formation of IL-10 by inhibiting the NF-κB pathway, and also induce the expression of superoxide dismutase (SOD) to protect HUVEC from ox-LDL injury. P-A can also regulate JAK/STAT pathway to reduce the expression of p-JAK2 protein and reduce the production of IL-6 and TNF-α.

Conclusion: P-A has protective effect on HUVEC injured by ox-LDL, and the protective mechanism is related to the regulation of JAK/STAT pathway and NF-κB pathway.
**Introduction**

Atherosclerosis is a common cardiovascular disease caused by the interaction of environmental factors and genetic factors. The main manifestations of atherosclerosis include the lipid deposition of the intima, the infiltration of monocytes and macrophages, the formation of foam cells and fat veins, and the formation of fibrous plates that caused by the migration and proliferation of vascular smooth muscle cells (VSMCs), which causes the hardening of the vascular wall and the stenosis of the functional cavity and the formation of thrombus[1].

H$_2$S is a novel gas transmitter and has important physiological functions in atherosclerotic lesions[2]. The deficiency of H$_2$S in vivo may be related to the early development of atherosclerotic lesions. On the contrary, an appropriate amount of hydrogen sulfide is helpful to delay atherosclerosis[3]. Thus far, more and more exogenous H$_2$S donors have been created, including 5-(4-Hydroxyphenyl)-3H-1, 2-dithiole-3-thione (ADT-OH)[4]. ADT-OH is one of the most widely studied slow-releasing H$_2$S donors.

The NF-κB plays an important role in inflammatory response, immune response and cell growth and development[5]. H$_2$S could decrease the production of TNF-α and IL-1β as well as leukocyte adhesion to the endothelium by inhibiting the activation of NF-κB[6]. Meanwhile, in the early atherosclerosis development process, TNF-α, IL-1β, IL-6 and IL-10 is closely related to the activation of JAK/STAT signaling pathway which is a signal transduction pathway that can be stimulated by cytokine and participates in the signal transduction and regulation process of various inflammatory and anti-
Proglumide could reduce the release of cytokines and inflammatory mediators by inhibiting the activation of NF-κB pathway in acute pancreatitis. Considering that one of the pathogenesis of atherosclerosis disease is related to the inflammation and the anti-inflammatory effect of proglumide, we combined proglumide with ADT-OH to create proglumide-(5-(4-Hydroxyphenyl)-3H-1, 2-dithiole-3-thione) (P-A). In this study, we proved that P-A is a novel slow-releasing H2S donor and shows anti-atherosclerotic effect on the HUVECs injured model by inhibiting the activation of JAK/STAT pathway and NF-κB pathway.

**Materials and Methods**

**Synthesis of P-A**

5-(4-methoxyphenyl)-1, 2-dithiole-3-thione (ADT) (Reference Number: 150127002, Yansheng Biotechnology, Shanghai, China). Proglumide (Reference Number: 6620-60-6, Ziqi Biotechnology, Shanghai, China). Anhydrous pyridine hydrochloride (sigma, USA).

Step1: synthesis of ADT-OH (Supplementary Figure S1, Supplementary Table S1).

After the product were cooled to 25°C, 1mol/L HCL solution was added to dissolve the product and then the solution was filtered. After the filtrate is removed, the product is washed to neutral by distilled water. And then vacuum filtration, the residue was retained. The ADT-OH was obtained by recrystallization of anhydrous ethanol. The productivity of ADT-OH was 86.32%, with a total of 9.1382 g.
Step 2: synthesis of P-A (Figure 2A, Supplementary Table S2). Dichloromethane (DCM), N, N-Dimethyl-4-pyridinamine (DMAP), sodium hydroxide (NaOH), N, N'-dicyclohexylcarbodiimide (DCC) are from sigma in USA. After the reaction was stopped, a small amount of NaOH solution (1mol/L) was added to the reaction termination system until the color of the pH test paper displayed 7. After vacuum filtration for 2-3 times, filter the residue and filtrate. The filtrate is added to water in the separation funnel and layered, the water layer was removed and the dichloromethane layer was retained. The residual water in the dichloromethane layer was removed by adding anhydrous sodium sulfate. After vacuum filtration 2-3 times, the residue was filtered and the filtrate was left. The products were obtained after drying with rotary evaporator at 50℃. The product is dissolved in anhydrous ethanol. The product was recrystallized in the refrigerator at -20℃ for one night. After vacuum filtration for 2-3 times the product was retained. The oil pump is evacuated to remove the product water and organic solvent to obtain P-A.

**Detection of H$_2$S releasing**

Human umbilical vein endothelial cells (HUVECs) were incubated in 12-well plates, and four microporous filtering films of 0.22µm were adhered to the inner side of the 12-well plates of each hole to set up the filter membrane adsorption device. After 500µL 1% (g/100mL) zinc acetate (Kelong, Chengdu, China) solution was added to each filter membrane, the P-A solutions were added in HUVECs. The filter membranes were collected after P-A acted on HUVEC for 1, 3, 6, 12, 24, 48h, respectively. Then the filter membrane was soaked in 2.5mL ultrapure water. The release of H$_2$S was detected
by methylene blue spectrophotometry at 670 nm, and the Na$_2$S standard curve was
drawn according to the OD.

**Establishment of ox-LDL induced HUVECs injured model**

HUVECs were damaged by 80μg/mL ox-LDL for 24h. The oil red O staining method
was used to judge whether the HUVEC had been damaged.

**CCK-8 assay**

HUVECs were seeded in 96-well plates and cultured for overnight at 37°C. HUVECs
were induced by 80μg/mL ox-LDL for 24h, then the P-A were acted on HUVECs. Then
Cell proliferation was detected by CCK8 kits (Boster Biotechnology, Chengdu, China)
according to the manufacturer’s protocol. Absorbance was determined at the 450nm by
enzyme-linked immunosorbent assay reader.

**ELISA assay of IL-6, IL-10 and TNF-α**

After the HUVECs were injured for 24h by 80μg/mL ox-LDL in 12-well plates, the
HUVECs were treated with 100μmol/L P-A for 12h. The supernatant from each well
was collected and used to detect the secretion of IL-6, IL-10 and TNF-α by ELISA
assay with commercial ELISA kits of IL-6, IL-10 and TNF-α (Boster Biotechnology,
Chengdu, China) according to the manufacturer’s protocol. In the AG490and PDTC
pre-treated assay, AG490 and PDTC were added in HUVECs respectively for 1h before
80μg/mL ox-LDL induced HUVEC for 24h. All other methods were the same as
described above.

**Determination of intracellular SOD**

**After injured by 80μg/mL ox-LDL for 24h, HUVECs were treated with 100μmol/L P-**
A for 12h. Then HUVECs were collected to lysis at 4℃ in RIPA buffer. The lysate was clarified by centrifugation at 12000 rpm for 15 min at 4℃. Protein concentration of HUVEC lysate was determined by BCA assay kits (Yiyuan Biotechnology, Guangzhou, China). The activity of intracellular SOD was determined by SOD assay kits (Yiyuan Biotechnology, Guangzhou, China) according to the manufacturer’s protocol.

**Protein expression by western blotting**

p-NF-κBp65, p-JAK2, p-STAT3, NF-κBp65, JAK2, STAT3 antibody and goat anti-rabbit IgG were from Cell Signaling Technology, China. The total protein was extracted from HUVECs according to the standard procedures. Protein samples (40μg) were separated by 10% SDS-PAGE and then transferred into PVDF membranes. The membrane was blocked with 5% nonfat dry milk solutions. After washing the PVDF membranes with TBST, the PVDF membranes were incubated overnight at 4℃ with the above antibody, respectively. It was followed by secondary antibody for 2h with goat anti-rabbit IgG. After washing, the membrane was developed with ECL kit and detected with VILBER Fusion FX5 system.

**Statistical analysis**

All data were analyzed with Graph Pad Prism5 and were presented as the mean ± SD. For all tests, P<0.05 was considered statistically significant.

**Results**

**P-A was synthesized successfully**

AS proved by ¹H-NMR (Table 1), MS (Figure 1B) and HPLC (Figure 1C), P-A was
successfully synthesized and used for subsequent experiments.

**P-A is a slow-releasing H$_2$S donor**

The H$_2$S productivity of P-A was analyzed in HUEVCs, and we found that the release of H$_2$S from P-A increased in a time and concentration dependent manner, generally. However, the release rate decreased after the incubation time reached 24h or the concentration reached 100μmol/L. (Table 2, Figure 2).

**P-A reliefs ox-LDL induced HUVECs injury**

To test the protective effect of P-A on vein endothelial cells, we established the ox-LDL injured HUVECs in vitro model. After the HUVECs were induced by 80μg/mL ox-LDL for 24h, oil red O staining showed that a large number of red dye particles appeared in the cells (Figure 3A & B). This phenomenon indicated that HUVECs had formed damage which causing the oil red O enter into the cell and dissolve in the lipid. Then we treated the ox-LDL injured HUVECs with P-A, as shown in figure 3 C & D, the cell proliferation activity increased with time in a concentration dependent manner in 24h. When the concentration of P-A reached to 200 mol/L, the declined cell viability indicated that P-A produces cytotoxicity at very high concentration above 100mol/L. (Figure 3C). After 100μmol/L P-A acted on HUVECs damage model for 12h, the cell proliferation activity was the best (Figure 3D).

**P-A regulates the expression of IL-6, IL-10, TNF-α and SOD**

Compared with normal cell control group, the amount of IL-6, TNF-α, IL-10 in the HUVEC injury model group increased significantly (P<0.01). As shown in Table 3, compared with the HUVEC damage model group, the secretion of IL-6 and TNF-α
reduced significantly after treated with P-A as well as positive control NaHS and ADT-OH (P<0.01), while the secretion of IL-10 increased significantly (P<0.01).

SOD can regulate the level of superoxide anion in the vascular wall, and alleviate the oxidative damage of oxygen free radicals to endothelial cells, as well as protect endothelial cells from atherosclerosis[8], therefore we analyzed the SOD level after P-A treatment. As shown in Figure 4, compared with normal cell control group, the activity of SOD in the HUVEC injury model group decreased significantly (P<0.01).

Compared with the HUVEC damage model group, the activity of SOD in the NaHS group, P-A group and proglumide group increased significantly (P<0.01).

**P-A regulates expression of IL-6, IL-10 and TNF-α through NF-κB and JAK/SATA pathway**

Compared with P-A group, the expression of p-NF-κBp65 protein increased in the PDTC+P-A group (Figure 5A, P<0.05); the expression of p-NF-κBp65 protein reduced and the expression of p-JAK2 protein as well as the expression of p-STAT3 protein increased in NaHS group (Figure 5A-C); the expression of p-JAK2 protein increased in the AG490+P-A group(Figure 5B, P<0.01). Compared with NaHS group, the expression of p-NF-κBp65 protein increased in the PDTC+ NaHS group (Figure 5A, P<0.05); the expression of p-JAK2 protein increased in the AG490+P-A group (Figure 5B, P<0.01).

As shown in Table 4, compared with NaHS group, the amount of IL-6 and TNF-α increased significantly as well as the amount of IL-10 reduced significantly in the PDTC+ NaHS group in the PDTC+NaHS group (P<0.05). Compared with P-A group,
the amount of IL-6 increased significantly and the amount of IL-10 reduced significantly in the PDTC+P-A group and AG490+P-A group (P<0.05); the amount of TNF-α increased significantly in the PDTC+P-A group (P<0.05). Compared with Proglumide group, the amount of IL-10 reduced significantly in the PDTC+Proglumide group (P<0.05). Compared with ADT-OH group, the amount of IL-6 increased significantly (P<0.05).

Discussion

The current research on H$_2$S presents the trend of cross disciplinary research in pharmacology, physiology, chemistry, biology, materials science and so on[9]. In addition to the endogenous H$_2$S and the traditional hydrogen sulfide donor NaHS, more and more exogenous hydrogen sulfide donor[10], and some sulfur compounds extracted from natural plants have also been widely studied[11]. In this study, a novel hydrogen sulfide donor P-A was successfully synthesized as proved by $^1$H-NMR and MS. The demethylation reaction of ADT is the key in the whole synthesis reaction. The purity of the P-A will be affected by the purity of the ADT-OH. The addition of DCC in this system can activate carboxyl. After the reaction was stopped, adding NaOH solutions to the reaction system to pH 7 can wash out some acidic by-products, and can also adjust the reaction system pH to the neutral to avoid the degradation of the products.

The chemical synthesis method in this experiment is simple and the reaction conditions are mild, while the post-processing is also simple. The product is easy to be purified through recrystallization. The study shows that the synthetic method in this experiment
can be used to obtain the target product P-A, which also provides valuable reference for the synthesis of other H$_2$S donors in the future.

With the increase amount of P-A used for treating HUVECs, the release of hydrogen sulfide and the cell proliferation gradually increased. However, excessive P-A has cytotoxic effect on cells, the proliferation and release of hydrogen sulfide was inhibited. Vascular endothelial cells, smooth muscle cells and macrophages can secrete interleukin at different stages of inflammation[12]. In our study, we found that intracellular triglyceride and cholesterol metabolism disorder cause lipid aggregation to damage endothelial cells after the HUVEC was injured by ox-LDL, and the inflammatory reaction started at the same time, as well as the secretion of inflammatory factors TNF-α, IL-6 and anti-inflammatory factor IL-10 increased. IL-6 can cause chronic inflammation and magnify acute inflammatory response to some extent, and promote the release of some chemokines and reactive oxygen species to participate in and further aggravate the atherosclerosis process[13]. TNF-α is present in atherosclerotic plaques, which stimulates the production of inflammatory factors and directly promotes the development of inflammation. IL-10 has the functions of anti-inflammatory for the atherosclerosis disease[14].

The release of TNF-α, IL-6 and IL-10 can activate NF-κB to promote the production of inflammatory factors such as IL-6 and IL-8, that further aggravate the inflammatory reaction in the atherosclerosis process[15, 16]. Our study shows that P-A and ADT-OH can significantly reduce the secretion of IL-6 and TNF-α in the HUVEC damage model. It is indicated that P-A reduces the secretion of IL-6 and TNF-α, which is related to the
structure of ADT-OH. P-A and proglumide can significantly increase the secretion of IL-10 in the HUVEC damage model. It is indicated that P-A increase the secretion of IL-10, which is related to the structure of proglumide.

H$_2$S can inhibit the expression of intercellular adhesion molecule-1 mediated by NF-$\kappa$B pathway in HUEVC, and induce the expression of SOD in endothelial cells at the same time[17]. SOD can regulate the level of superoxide anion in the vascular wall, and alleviate the oxidative damage of oxygen free radicals to endothelial cells, as well as protect endothelial cells from atherosclerosis[8]. we found that P-A can significantly increase the activity of SOD in the experimental ox-LDL affected HUVECs, which is related to the structure of proglumide.

As the main transcription factor of the inflammatory response, NF-$\kappa$B can be activated by IL-6, TNF-$\alpha$, CRP, and so on, which participates in the whole process of atherosclerosis[18, 19]. JAK/STAT signal transduction pathway is activated after the JAK2 phosphorylation. Inhibition of JAK2 activity can inhibit STAT3 phosphorylation so that inhibiting the production of IL-6, IL-8, TNF-$\alpha$ and other inflammatory factors. Blocking the JAK/STAT signal pathway can effectively prevent the occurrence and aggravation of atherosclerosis diseases[20]. Our study found that P-A can down regulate the expression of p-NF-$\kappa$Bp65 protein and reduce the production of TNF-$\alpha$ and IL-6 and promote the formation of IL-10 by inhibiting the NF-$\kappa$B pathway, and also induce the expression of SOD in HUVEC damage model to protect HUVEC from ox-LDL. P-A can also regulate JAK/STAT signal transduction pathway to reduce the expression of p-JAK2 protein and reduce the production of TNF-$\alpha$ and IL-6.
However, there is no direct evidence showing that P-A protect HUVEC from ox-LDL damage only through the NF-κB pathway and JAK/STAT signaling pathway. Other associated signaling pathways may also play important roles in protecting HUVECs from ox-LDL damage. Based on the existing basis, the further study of the P-A is needed to find the downstream targets and genes of NF-κB pathway and JAK/STAT signaling pathway protect HUVECs, as well as the receptors of IL-6, IL-10 and TNF-α mediated by NF-κB signaling pathway and JAK/STAT signaling pathway, and other related signaling pathways and indicators.

In conclusion, P-A has the protective effect for experimental ox-LDL affected HUVEC, and the protective mechanism is related to the regulation of JAK/STAT pathway and NF-κB pathway to some extent. What’s more, our study provides direct evidence that JAK/STAT pathway and NF-κB pathway participate in the atherosclerosis process.

Statement of Ethics

There is no human or animal studies were conducted in this research.

Conflict of Interest Statement

The authors declared no potential conflicts of interest with respect to the research, authorship and publication of this article.

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**Author Contributions**

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. XO, SZ, RH, CC, and TX conducted the experiments; CY, CYU and CZ wrote the manuscript.

**Reference**

7. Wang, R., et al., *Protein Inhibitor of Activated STAT3 Suppresses Oxidized LDL-


Figure Legends

Figure 1. P-A was successfully synthesized. (A) The method of synthesizing P-A. (B) MS of P-A. The molecular weight of P-A was 542 by MS. The molecular weight was 543, indicating that the combination of P-A and H. The molecular weight was 565, indicating that the combination of P-A and Na+. The molecular weight was 1107, indicating that the combination of 2 molecule of P-A and Na+. (C) P-A has higher purity and less impurity content, as well as a single peak was showed by HPLC.

Figure 2. HUVECs produce H_{2}S after P-A was added in cells. The release of H_{2}S of different concentrations P-A acted on HUVECs at different time. The release of H_{2}S was the largest after the HUVECs was affected by 100\mu mol/L P-A for 24 hours. (Mean ± SD, n=3).

Figure 3. The cell proliferation of different concentrations P-A acted on HUVEC at different time. (A) representative images of HUVECs. (B) representative images of ox-LDL induced HUVECs. After the HUVECs were induced by 80\mu g/mL ox-LDL for 24h, the cells had been damaged. (C)The cell proliferation activity increased with time in a concentration dependent manner in 0-24h. \( ^-P<0.01 \) vs control; \(^#P<0.05\) vs 25\mu mol/L; \(^\Delta P<0.01\) vs 50\mu mol/L; \(^&P<0.01\) vs 100\mu mol/L. (D) After 100\mu mol/L P-
A acted on HUVEC 12h, the cell proliferation activity was the largest. *P<0.05 vs 1h; 
**P<0.05 vs 3h; ***P<0.001 vs 6h; &P<0.01 vs 12h; ⋆⋆⋆ P<0.001 vs 24h. (Mean ± SD, n=3).

Figure 4. Effect of P-A acted on the activity of SOD in HUVEC damage model.
(Mean ±SD. n=4) **p<0.01 vs cell control; △△ p<0.01 vs model; ♦p<0.05 vs P-A.

Figure 5. P-A regulates expression of IL-6, IL-10 and TNF-α through NF-κB and JAK/SATA pathway. (A) The expression of p-NFκB p65 protein in HUVEC damage model, 1: cell control; 2: model; 3: NaHS; 4: P-A; 5: PDTC+NaHS; 6: PDTC+P-A. *p<0.05 **p<0.01 vs model; ♦p<0.05 vs NaHS. Mean ± SD, n=3. (B)The expression of p-JAK2 protein in the HUVEC damage model 1: cell control; 2: model; 3: NaHS; 4: P-A; 5: AG490+NaHS; 6: AG490+P-A. (C)The expression of p-STAT3 protein in the HUVEC damage model 1: cell control; 2: model; 3: NaHS; 4: P-A; 5: AG490+NaHS; 6: AG490+P-A. **p<0.01 vs model; ⋆⋆ P<0.01 vs P-A; △△ p<0.01 vs NaHS. Mean ± SD, n=3.
Fig. 3

A and B: Images of cell culture under different conditions.

C: Graph showing relative cell viability over time.

D: Graph showing relative cell viability at different concentrations.

Legend:
- control
- 12.5
- 25
- 50
- 100
- 200

Time:
- 1h
- 3h
- 6h
- 12h
- 24h
- 48h

Concentration:
- 100 μmol/L
Fig. 4

The activity of SOD (U/mgprot)

- Cell control
- Model
- NaHS
- P-A
- Proghmide
- ADT-OH
Supplementary materials

Supplementary Figure S1

Supplementary Figure 1. Reaction equation for the synthesis of ADT-OH compounds.

Supplementary Table S1

Supplementary table S1. Synthesis of ADT-OH

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### Supplementary Table S2

#### Supplementary table S2. Synthesis of P-A

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