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Inhibitory effects of prostaglandin A1 on apoptosis of rat cardiac microvascular endothelial cells was mediated by NF-κB[1]

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KEY WORDS prostaglandins A; vascular endothelium; cultured cells; NF-kappa B; apoptosis; *bcl*-2 genes; *in situ* nick-end labeling

ABSTRACT

AIM: To study the effects of prostaglandin A1 (PGA1) on rat cardiac microvascular endothelial cells. **METHODS**: Isolated rat cardiac microvascular endothelial cells were cultured in hypoxia and reoxygen conditions, respectively. Endothelial cell apoptosis was detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining. The activity of NF-κB was detected by electrophoretic mobility shift assay. Bcl-2 and Bax protein expression were examined by Western blot and *bcl*-2 mRNA expression was examined by Northern blot. **RESULTS**: PGA1 reduced endothelial cell apoptosis markedly, inhibited activity of NF-κB, and increased expression of Bcl-2 protein and *bcl*-2 mRNA. However, PGA1 did not alter Bax protein expression resulting in an increase in the ratio of Bcl-2 to Bax. **CONCLUSION**: PGA1 can inhibit rat cardiac microvascular endothelial cell apoptosis by inhibiting activity of NF-κB.

INTRODUCTION

Recently, endothelium and cardiac ischemia reperfusion injury have been widely studied. The effects of endothelium in the occurence and development of scleroderma is attached with more and more importance. Scleroderma, systemic sclerosis, is an autoimmune disease. Besides the characteristic skin thickening, symptoms include vascular involvement of the heart. NF-κB plays an important role in the transcriptional regulation of many cytokines and adhesive molecules[1,2]. Though there are some reports to manifest the effects of NF-κB on apoptosis, it is not understood completely whether NF-κB is involved in the mediation of the endothelial apoptosis.

Former researches on the conservation of endothelium focused on such remedy as antioxidant, calcium antagonist, protease and lipase inhibitor, and some traditional Chinese medicines[3]. Prostaglandin A1 (PGA1) is a kind of circulus with twenty carbon fatty acids which has lots of biological activities. The knowledge about PGA1 was limited before, but it has decompression, diuresis, and anti-cancer effects. Recently there are reports abroad, that PGA1 is a potent antagonist of NF-κB[4]. NF-κB has an intimate relationship with apoptosis, it can stimulate the expression of IL-1β, c-*myc*, and TNF-α and subsequently cause the apoptosis of the cells. There are also many proofs indicating that NF-κB has an anti-apoptosis effect, which may be due to different cell types and signal transduction. Here, we tried to investigate the influence of PGA1 on the apoptosis of rat cardiac microvascular endothelial cells and the activity of NF-κB.

MATERIALS AND METHODS

Reagents PGA1 was purchased from Sigma (USA). Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining (TUNEL) kits were purchased from Nanjing Jiancheng Bioengineering Institute; NF-κB gel shift assay system kits were provided by Promega; [γ-^{32}P]ATP was provided by Beijing Yahui Biomedical Engineering Company Ltd. RPMI-1640 was from Sigma; Bcl-2 antibody was purchased from Wuhan Boster Biological Engineering Company Ltd; *bcl*-2 probe was generously provided by Dr KONG Xiang-Rong.

Cell culture Six Wistar rats (7.8 g ± 2.1 g, Grade Ⅱ, Certificate № 98018) were provided by Experimental Animal Center of Medical College of

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Soochow University. Rat cardiac microvascular endothelial cells (RCMEC) were isolated as described by Nishida[5] with some modifications. The cells were sifted and treated with glutin, endothelium growth factor, and RPMI-1640, and then identified with ABC immunohistochemistry method. Cultures of RCMEC between the 5th and 10th passage were used for the experiments[6].

Hypoxia-reoxygenation (H/R) model[7] Cultured RCMEC were replicated in six-well culture plate (medium bubbled with 100 % oxygen for 45 min). Cells 6×10^6 were placed in each well and then placed in an airproof container filled with 95 % nitrogen and 5 % CO_2 at 37 ℃ (gas rate = 1 L/min), p_{O_2} was maintained at 0.5 kPa (the instrument provided by 304 Hospital of Chinese People's Liberation Army). After 15-h hypoxia, cells were put back into an incubator for reoxygenation.

Experimental protocal The cells were divided into four groups: normal oxygen (containing 5 % CO_2) group (normoxia), hypoxic group (hypoxia), hypoxia/reoxygenation action group (H/R), and H/R + PGA1 40 μmol/L group (PGA1 was dissolved in 100 % ethanol ane was diluted to a final concentration of 40 % with saline, which has no hasm to endothelial cells).

Cell counting Trypan-blue (0.5 %) stain was used to count cell number. Endothelial cells were observed and the number was counted under a microscope (×200). Cell viability [vital cell number/(vital cell number + dead cell number)] was calculated.

Detection of DNA fragmentation Cells were collected and broken in breaking buffer. After the suspension was centrifuged, DNA fragmentation in the sediment and solution were determined[8] and then DNA fragmentation ratio (DNA amount in the solution/DNA amount in both of the sediment and solution) was calculated.

Determination of apoptosis using TUNEL Apoptosis of endothelial cells were determined using colorimetric TUNEL[9]. The ratio of apoptosis (apoptotic cell number/total cell number) was counted.

Electrophoretic mobility shift assay (EMSA) of NF-κB activity Nuclear extracts were isolated according to the methods of Schreiber[10]. Folin-phenol reagent was applied to quantify the protein. The extracted protein 10 μg was used to react with [γ-^{32}P] ATP (185 GBq/mol) labeled NF-κB oligonucleotide at 37 ℃ for 20 min and then the reactions were subjected to electrophoresis on 6 % acrylamide (w/v) gel. Autoradiography was done after gel was dried. The developed film was scaned by an IBAS-2000 graphic analyzer.

Western blot Western blot was performed as previously described[11]. In brief, cells were lysed in 1 × SDS sample buffer, then protein was collected and stored at −80 ℃ for later use. Total protein 10 μg was subjected to 12 % SDS-PAGE and electrotransfered at 4 ℃ for 2 h onto Nitrocellulose membrane. Membrane was incubated with anti-Bcl-2 antibody (1 : 100, Sino-America Biotech) at 37 ℃ for 2 h. Membrane was subsequently incubated with HRP-conjugated second antibody (1:2000, Vector) at room temperature for 2 h. Finally, membrane was developed with diaminobenzidine (Amersico) and H_2O_2. Photoes were taken to note the results.

Northern blot RNA was extrated by acid guanidinium thiocyanate-phenol-chloroform single-step method[12]. RNA sample (30 μg) was separated by electrophoresis through 1 % agarose-formaldehyde gel and transferred onto positively charged nylon membrane. Hybridization was made with *bcl*-2 probe labeled by [γ-^{32}P]-ATP. β-Actin was used as an internal control. Optical density was scaned with IBAS-2000 image analyzer.

Statistics Data were expressed as $\bar{x} \pm s$ and analyzed by t test. Comparison of multi-groups were made by one-way ANOVA.

RESULTS

Effects of PGA1 on cell number after hypoxia/reoxygenation After 15 h hypoxia, some cells started to become round and eventually detached from the plate and floated in the medium, leaving many holes in the confluent cells. Cell number under hypoxic conditions was decreased greatly. After 12 − 24 h reoxygenation, the declination of cell number was even more obvious ($P < 0.01$). The group treated with PGA1 had obviously more cells than H/R group ($P < 0.01$) (Fig 1).

Effects of PGA1 on DNA fragmentation ratio after hypoxia/reoxygenation DNA fragmentation ratio of hypoxia group had an obvious increase compared with control, and that of H/R group was even higher ($P < 0.01$); but the ratio in PGA1 group was decreased markedly compared with hypoxia and H/R group respectively ($P < 0.01$), which indicated that PGA1 could relieve the injury of endothelial cells (Fig 2).

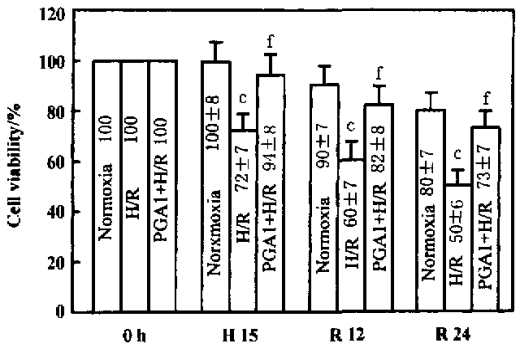

Fig 1. Effects of PGA1 40 μmol/L on cell number. H15: 15 h after hypoxia; R12: 12 h after reoxygenation; R24: 24 h after reoxygenation. $n = 8$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normoxia. $^fP < 0.01$ *vs* H/R.

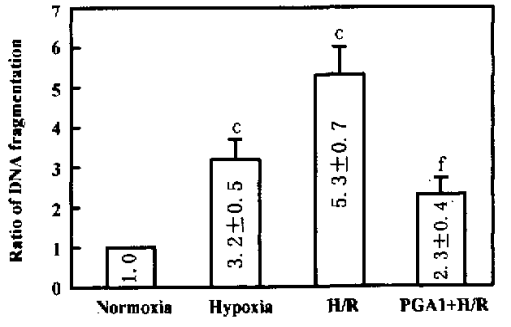

Fig 2. Effects of PGA1 40 μmol/L on ratio of DNA fragmentation. H/R, hypoxia/reoxygenation. $n = 8$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normoxia. $^fP < 0.01$ *vs* H/R.

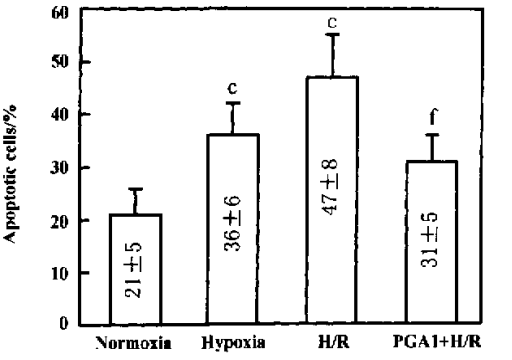

Fig 3. Effects of PGA1 40 μmol/L on cell apoptosis. $n = 8$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normoxia. $^fP < 0.01$ *vs* H/R.

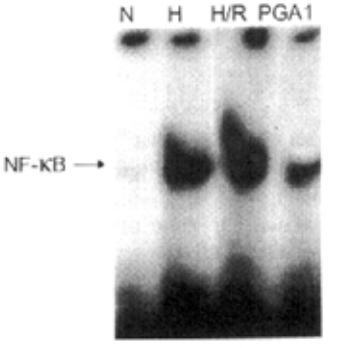

Fig 4. Gel mobility shift assay for NF-κB activity. N, normoxia group; H, hypoxia group; H/R: hypoxia/reoxygenation group; PGA1: PGA1 40 μmol/L + H/R group.

Effects of PGA1 on apoptosis after H/R RCMEC after H/R are liable to apoptosis. The nuclei of apoptosis cells dyed with TUNEL have a blue-purple color. The number of apoptosis cells in H/R group had a great increase compared with those cultured under normal oxygen pressure ($P < 0.01$); and apoptosis cell number in PAG1 group was fewer than that in the H/R group ($P < 0.01$), suggesting that PGA1 can antagonize the apoptosis induced by H/R in RCMEC (Fig 3).

Effects of PGA1 on NF-κB activity after H/R NF-κB activity in RCMEC was increased obviously after 15-h hypoxia, and there was still an uprise after 24-h reoxygenation. While the endothelial cells were treated with PGA1, NF-κB activity was decreased ($P < 0.01$ *vs* H/R), indicating that PGA1 can effectively reduce the NF-κB activity in RCMEC (Fig 4).

Effects of PGA1 on the expression of Bcl-2 and Bax protein in RCMEC Endothelial cells cultured in normal oxygen had a high Bcl-2 expression, while Bcl-2 protein level was much lower in the hypoxia and H/R group ($P < 0.01$). In PGA1 treated group the expression of Bcl-2 protein was increased compared with hypoxia and H/R group ($P < 0.01$). However, there was no significant difference in the expression of Bax protein between PGA1 group, hypoxia group, and H/R group (Fig 5).

Effects of PGA1 on the expression of *bcl*-2 mRNA Endothelial cells cultured in normal condition had a high *bcl*-2 mRNA expression, while it was much lower in the hypoxia and H/R group ($P < 0.01$), and PGA1 increased *bcl*-2 mRNA expression compared with the hypoxia and H/R group respectively ($P < 0.01$, Fig 6).

DISCUSSION

Development of scleroderma, hypoxia-reoxygenation

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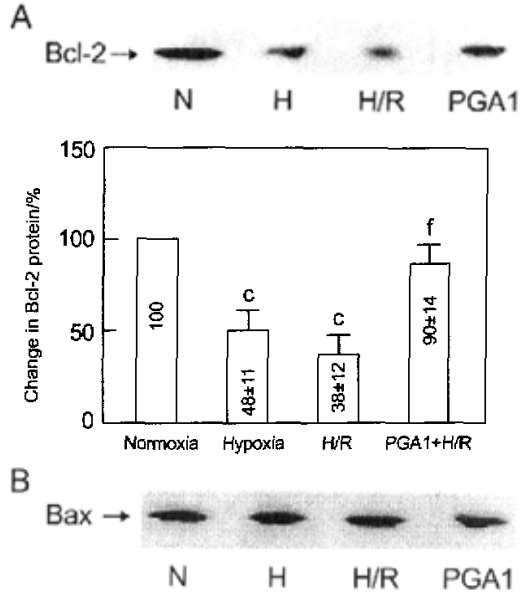

Fig 5. Western blot of Bcl-2 (A) and Bax (B) protein. H/R: hypoxia/reoxygenation group; PGA1 + H/R: PGA1 40 μmol/L + H/R group. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normoxia. $^fP < 0.01$ *vs* H/R.

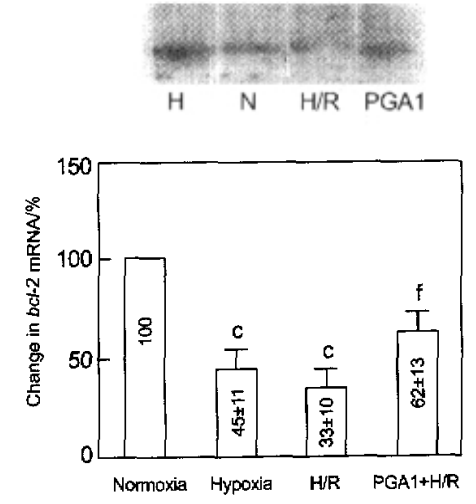

Fig 6. Northern blot analysis of *bcl*-2 mRNA in rat cardiac microvascular endothelial cells. H/R: hypoxia/reoxygenation group; PGA1 + H/R: PGA1 40 μmol/L + H/R group. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* normoxia. $^fP < 0.01$ *vs* H/R.

process of cardiac muscle, and injured endothelial cells would has apoptosis besides putrescence. Many factors such as leukocyte, active oxygen, and inflammative factors could do harm to and cause the dysfunction of the endothelial cells.

In this study, we found that the number of normal cells was decreased but that of apoptosis cells was increased remarkably after H/R, and the activity of NF-κB was reduced greatly in PGA1 group compared with that in H/R group. The inhibition of the NF-κB activity may be achieved by upregulation of I-κBα, the inhibitory gene of NF-κB, caused by PGA1[13]. NF-κB played an important role in the activation of endothelial cells and the expression of many genes[14].

The studies revealed a marked decrease in Bcl-2 protein, an antiapoptotic factor, and no change in Bax protein, a proapoptotic factor, after H/R treatment. So the apoptosis induced by H/R may be due to the unbalance between antiapoptotic factor and proapoptotic factor. These findings are further supported by the fact that overexpression of Bcl-2 protein attenuated endothelial cell death and apoptosis under H/R conditions. PGA1 upregulates the expression of *bcl*-2 mRNA and Bcl-2 protein, but did not influence the level of Bax protein. Unfortunately, the present study cannot explain how NF-κB decreased *bcl*-2 mRNA expression. But NF-κB has been believed to inhibit apoptosis in the caspase-dependent pathways[15,16].

In conclusion, PGA1 obviously inhibit the apoptosis of RCMEC mediated by NF-κB, which may attribute to new approaches to conservation of endothelial cells and the therapy of scleroderma and cardiac H/R injury.

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前列腺素 A1 通过 NF-κB 介导抑制大鼠心脏微血管内皮细胞凋亡[1]

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关键词 前列腺素 A; 血管内皮; 培养的细胞; NF-κB; 细胞凋亡; *bcl*-2 基因; 原位切口末端标记

目的: 研究前列腺素 A1 (PGA1)对心脏微血管内皮细胞凋亡的影响. **方法**: 培养分离大鼠心脏微血管内皮细胞, 建立缺氧再给氧模型, 通过原位缺口末端标记观察 PGA1 对内皮细胞凋亡的作用; 通过凝胶电泳迁移率测定 NF-κB 的活性; 用 Western blot 法测定 Bcl-2 和 Bax 蛋白表达; 用 Northern blot 法测定 *bcl*-2 mRNA 的表达. **结果**: PGA1 能明显减少缺氧再给氧内皮细胞的凋亡, 抑制 NF-κB 的活性, 升高 Bcl-2 蛋白及 *bcl*-2 mRNA 的表达, 不改变 Bax 蛋白的表达, 导致 Bcl-2/Bax 增加. **结论**: PGA1 通过 NF-κB 介导抑制大鼠心脏微血管内皮细胞的凋亡.

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