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Original article

Heat stress-induced PPAR-β enhances HUVEC resistance to oxidant injury

Bo Zhou[a], Xiaoyuan Huang[a], Pengfei Liang[a], Licheng Ren[a], Bimei Jiang[b], Pengju Fan[a], Xu huang[a], Chunhua Liao[a], Chenchen Zuo[a]

[a]Department of Burns and Plastic Surgery, Xiangya Hospital, Central South University, Changsha, Hunan 410008, [b]Department of Pathophysiology, Xiangya School of Medicine, Central South University, Changsha, Hunan 410078, China

Background: Mild heat stress provides protection to cells, which is known as thermal tolerance. Various kinds of heat shock proteins have been proven to play important roles in this process. Another antiapoptotic factor induced by mild heat stress, peroxisome proliferator activated receptor beta (PPAR-β), provides a protective effect to cells against subsequent oxidant injury.

Objective: To understand the expression of PPAR-β and other proteins in human umbilical vein endothelial cells (HUVECs) during mild heat stress. H_2O_2-induced apoptosis of cells with mild heat pretreatment were also investigated to elucidate cell resistance to oxidant injury.

Methods: HUVECs were chosen in the current study because vascular endothelial cells in burn wounds, especially in the zone of stasis, suffer sequentially from heat stimulus and oxidant injury.

Results: The cells were subjected to 43□C for 25 minutes and allowed to recover for different times (from 1 to 72 hours). The PPAR-β expression was found to be upregulated in the later recovery stage. BCL-2 also showed a similar trend, but P53 showed otherwise. Heat pretreated HUVECs were exposed to 400 μmol/L of H_2O_2 for 12 hours, and apoptosis rate was assessed. H_2O_2-induced apoptosis was attenuated by heat pretreatment and by the PPAR-β agonist GW0742 ($p < 0.01$ and $p < 0.05$ versus control group); HUVECs transfected with PPAR-β shRNA seemed much more susceptible to oxidation damage ($p < 0.05$ versus the control group). Mild heat stress also upregulated the BCL-2 expression relative to PPAR-β.

Conclusion: Heat-induced PPAR-β may be partly responsible for this process, which may also be one of the possible explanations of the antiapoptotic function of PPAR-β, although the specific mechanism needs further examination.

Keywords: Heat stress, PPAR-β, HUVEC, oxidant injury

Heat stress has long been studied by scholars in different cell lines and at a wide temperature range, which has yielded inconsistent results. Higher temperatures and prolonged exposures to heat stimuli tend to induce cell apoptosis [1-3]. By contrast, much milder heat-stress conditions may induce a protective effect in cells through the stimulation and activation of various kinds of stress-related proteins or cytokines, which can protect cells from much severe, even lethal, stimuli that cause apoptosis [4-6]. This protective effect may be explained by thermal tolerance [7], and the upregulation of HSP may be one of the most important mechanisms. Several other factors, upregulated by mild heat stimuli, may also be at least partly responsible for the antiapoptotic characteristic of heat-pretreated cells.

Recently, the nuclear transcription factor PPAR-β has attracted attention because of its antiapoptotic functions in different cells [8, 9]. Mild stimulus may upregulate PPAR-β to protect human umbilical vein endothelial cells (HUVECs) from H_2O_2-induced apoptosis [10]. In the present study, we aimed to understand whether mild heat stress could upregulate PPAR-β expression and to examine the protective effects on HUVECs and its mechanisms.

Correspondence to: Xiaoyuan Huang, Department of Burns and Plastic Surgery, Xiangya Hospital, Central South University, Changsha Hunan 410008, China. E-mail: xiaoyuanhuangcn@163.com

Materials and methods

Cell culture

HUVECs were isolated from fresh, term umbilical cords digested with 0.1% collagenase type

I (Sigma, St. Louis, MO, USA). Cells were maintained at 37°C in a 5% CO_2-humidified atmosphere in DMEM (Hyclone, Logan, UT, USA) supplemented with 20% fetal calf serum (Gibco, Grand Island, NY, USA) and with 2% low serum growth supplement (Invitrogen, Carlsbad, CA, USA). The cells were identified as endothelial cells by morphology and Factor VIII staining as previously described [11], and between 3 and 10 endothelial cell passages were used. As a PPAR-β agonist, we used GW0742 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted in dimethyl sulfoxide until the final concentration in the medium was 1 μM [12, 13]. HUVECs were then cultured in this medium for 24 hours.

Heat stimulation

Culture flasks containing cells to be heat-stimulated were sealed and immersed in a 43°C water bath for 25 minutes as previously described [14]. Then, the flasks were unsealed and incubated at 37°C for different periods. Cells were harvested at 1, 3, 6, 12, 24, 48, and 72 hours after heating.

Reactive oxygen species treatment

H_2O_2 (30%) (Sigma) was diluted and incubated in PBS, followed by further dilution in culture medium. The final H_2O_2 concentration in the medium was 0.4 mM. Cells were cultured in this medium for 12 hours as previously described [9].

Staining of nuclear DNA in apoptotic cells

We used fluorescent DNA-binding dyes to define nuclear chromatin morphological features as a quantitative index of apoptosis within the cell culture system [15]. Cells to be analyzed for apoptosis were stained with Hoechst 33258 and viewed under a fluorescence microscope as previously described [16]. Briefly, after treating cells as described in the cell treatment protocol, cells were incubated with Hoechst 33258 (10 μg/mL in PBS) for 20 minutes at 37°C for DNA staining. Individual nuclei were visualized at 200× to distinguish the normal uniform nuclear pattern from the characteristic condensed coalesced chromatin pattern of apoptotic cells. Apoptosis was quantified by an observer blinded to the treatment groups who analyzed 400 nuclei from random microscopy fields. The total number of apoptotic cells in each section was summed and expressed as a percentage of the total cell number. At least 10 individual fields of view were evaluated per slide.

Flow cytometry

Apoptosis was also examined by analysis of DNA fragmentation using flow cytometry. HUVECs were collected using trypsin and washed once with PBS. Cell pellets were resuspended in 50% cold ethanol and fixed at -20°C. After fixation, cells were washed once with cold PBS and incubated in 0.5 ml of PBS containing 100 μg/mL RNase A for 20 minutes at 37°C. HUVECs were then pelleted by centrifugation, and 250 μL of PBS containing 50 μg/mL propidium iodide was added to the pellet. Flow cytometric analysis was performed after 30 minutes using a Beckman Coulter Elite Esp Cell Sorter in the Flow Cytometry Core Facility at the Cancer Research Institute of Central South University. Cells with DNA content less than that of the untreated cells in G0/G1 were considered apoptotic.

Vector construction

Three kinds of PPAR-RNAi-plasmid were constructed by Genechem (Shanghai, China) according to *Homo sapiens* PPAR-δ mRNA (cDNA clone MGC: 3931 IMAGE: 3630487). The three siRNAs targeting different nucleotide sites were as follows: plasmid-1: 5′-CCGCAAACCCTTCAGTGATAT-3′ (1218–1238); plasmid-2: 5′-CCTATTCATTGCGGC CATCAT-3′ (1305–1325); plasmid-3: 5′-GACCACAG CATGCACTTCCTT-3′ (353–373). The shRNA-encoding gene was ligated with the plasmid expressing vector pGCsi-U6/Neo/GFP/shRNA (Invitrogen).

Cell transfection

HUVECs for transfection were seeded in six-well plates at a density of 1×10^5 cells/well to 2×10^5 cells/well in 2 mL complete medium and grown for 1 to 3 days until 80% to 95% confluence. One day prior to transfection, cells were cultured in complete growth medium without antibiotics. Lipofectamine 2000 (Invitrogen) was used in transfection. For each well, 4 μg of plasmid diluted in a serum-free medium optimized minimal essential medium (OMEM; Invitrogen) was complexed with 10 μL of the reagent and incubated for 20 minutes at room temperature. The transfection mix was added to the cells growing in the OMEM medium, and the cells were incubated at 37°C in a CO_2 incubator. The medium was changed after 6 hours. Cells were determined according to whether the cell could emit green fluorescence under a fluorescence microscope. Transfection efficiency was calculated as the ratio of transfected cells in one

hundred HUVECs. Transfected cells were passaged 1:10 into fresh growth medium 24 hours later to select and allow proliferation of positive cell clones. Then, 200 μg/mL G-418 (Sigma) was added to the medium in the following 14 days. The PPAR-β expression was detected by RT-PCR and western blot assay.

RT-PCR

Total RNA was extracted from cultured HUVECs using the TRIzol Reagent (Invitrogen). For the RT-PCR amplification of PPAR-β, BCL-2, P53, and β-actin transcripts, 1 μg total RNA were reverse-transcribed using a SuperScriptIII First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. RT-PCR amplification was then achieved in reactions by 2× Taq PCR MasterMix (Tiangen Biotech, Beijing, China) mixed with template cDNA (corresponding to 1 μg total RNA) in reaction volumes of 25 μL. The primer used for PCR was listed as follows: PPARδ: sense 5′-ACTGAGTTCGCCAAGAGCATC-3′, antisense 5′-TTAGTACATGTCCTTGTAGATCT-3′ (products: 546 bp); BCL-2: sense 5-TATCCAATCCTGTGCT GCTATC-3′, antisense 5′-ACTCTGTGAATCCCG TTTGAA-3′ (product: 174 kb); P53: sense 5′-TCTGA CTGTACCACCATCCACTA-3′, antisense 5′-CAA ACACGCACCTCAAAGC-3′ (product: 146 bp); and β-actin: sense 5′-TCCTTCCTGGGCATGGAGTC-3′, antisense 5′-GTAACGCAACTAAGTCATAGTC-3′ (product: 361 bp). All RT-PCR products were characterized by electrophoresis through 1.5% agarose gels and visualized by ethidium bromide staining under UV light.

Western blot analysis

PPAR-β, BCL-2, BAX, and GAPDH were detected and quantitated using gel electrophoresis and western blotting. At 1, 3, 6, 12, 24, 48, and 72 hours after heating, cells in heated and unheated flasks were lysed in a solution containing 1% SDS, 50 mM Tris-HCl, and 0.1 mM phenylmethyl sulfonyl fluoride, 10 mM NaCl, and 1 mM PMSF. Protein concentration was measured using an enhanced BCA protein assay kit (Beyotime, Hai Men, China). All proteins (30 μg) were denatured (95°C, 5 minutes), chilled on ice (5 minutes), and applied to electrophoresed (8% to 15% SDS-PAGE). Proteins were transferred onto PVDF membranes, washed three times with PBS, blocked with 5% skimmed milk in PBS (room temperature, 1 hour), and incubated at 4°C overnight with one of the primary antibodies (PPAR-β: catalog number S2709; BCL-2: catalog number 1017-1; BAX: catalog number 1063-1 (Epitomics, Burlingame, CA, USA); and GAPDH: catalog number PR-M 001; GoodHere Technology, Hangzhou, China). Unbound antibodies were washed and membranes were incubated with HRP-labeled goat anti-rabbit IgG secondary antibodies (catalog number A0208, Beyotime) for 1 hour (room temperature). After washing (3 × 15 minutes), the membranes with blocking buffer-bound antibody signals were detected by ECL substrate (Pierce, Rockford, IL, USA) and analyzed using an autoradiography system (Bio-Rad, Richmond, CA).

Statistical analysis

Data are presented as mean values ± SE. Differences at the 95% confidence level were considered significant ($p < 0.05$). The experimental groups were compared using an ANOVA with a Bonferroni–Dunn post hoc test.

Results

PPAR-β and BCL-2 expression

In the current experiment, PPAR-β mRNA levels were gradually upregulated after heat stress remained elevated from 6 to 48 hours and decreased subsequently **(Figure 1A)**. Interestingly, BCL-2 mRNA levels showed little change during the early stage of the recovery period post-heat stress. Until cells were recovered for 12 hours, BCL-2 was also upregulated, reaching the maximum level of expression 48 hours post-heat treatment, before decreasing subsequently **(Figure 1B)**. By contrast, P53 levels showed little change post-heat stress **(Figure 1C)**.

The PPAR-β protein expression was detected by western blotting. The PPAR-β protein levels were upregulated post-heat stress **(Figure 2A** and **B)**. The PPAR-β expression also reached maximum levels 48 hours post-heat treatment. Similar to the mRNA expression, BCL-2 proteins were downregulated post-heat stress at the beginning of the recovery period. However, this protein was soon upregulated 12 hours post-heat stress and reached maximum expression 48 hours post-heat treatment **(Figure 2C** and **D)**. The most interesting finding was the expression trend of BAX, which showed slight upregulation, changed 1 to 6 hours post-heat treatment. However, 24 and 48 hours post-heat treatment, this molecule was downregulated dramatically **(Figure 2E)**.

The BCL-2/BAX ratio initially showed effects of downregulation, but eventually showed upregulation, especially 48 hours post-heat stress, whereon it reached its maximum value. However, the rates subsequently returned to normal **(Figure 2F)**.

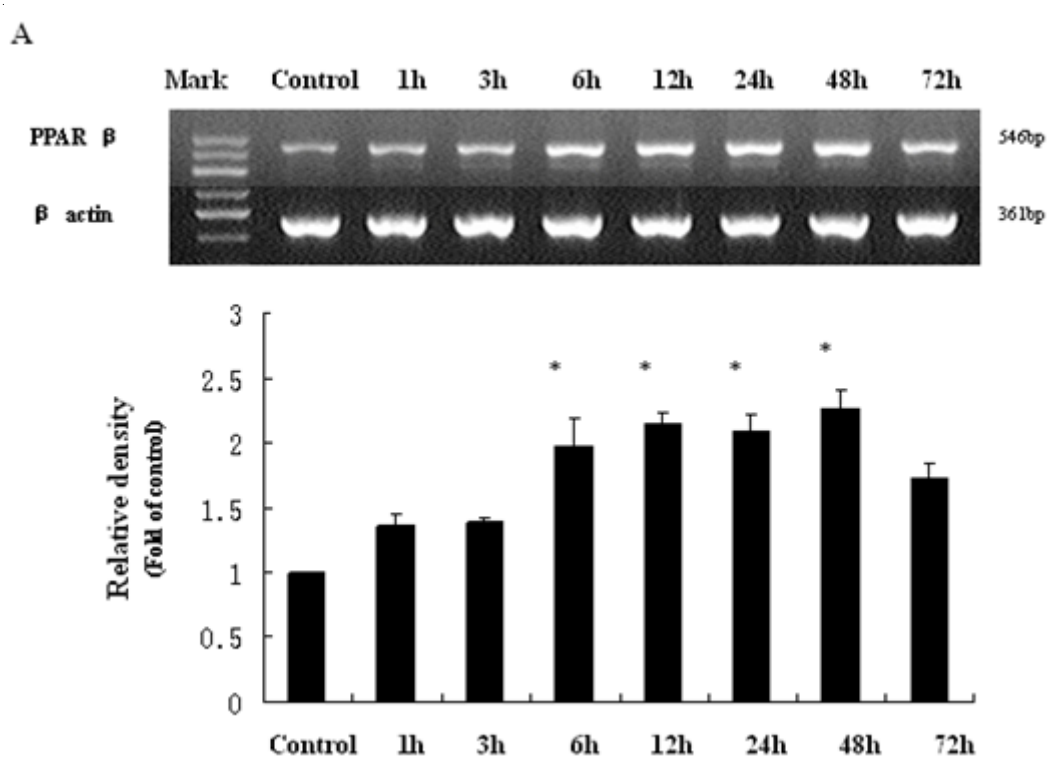
All of these data indicate that heat stress could induce the PPAR-β and BCL-2 expression. Previous research has shown that a mild stimulus such as topical application of tetradecanoylphorbol acetate (TPA) on skin or hair plucking could markedly upregulate PPAR-β expression in the epidermis [17]. Previous research has indicated that repeated exposure to oxidant stimuli could upregulate PPAR-β levels in HUVECs [10]. Through microarray analysis, Tzika et al. found that the PPAR-β expression is upregulated remarkably in the skeletal muscle of burn patients [18].

The current RT-PCR results show that BCL-2 mRNA levels show little change during the early stage of the recovery period post-heat stress, but were soon upregulated. BCL-2 protein level and the level of expression of another important apoptotic relevant gene, BAX, were then investigated.

Similar to the RT-PCR results, BCL-2 protein levels showed an early relatively stable trend before upregulation with the passage of time during the recovery stage post-heat stress treatment. The BAX expression level contradicted that of BCL-2. During the early stage of recovery period, BAX levels were upregulated and maintained at a relatively high level compared with the control group. As time passed, a gradually decreasing trend was observed. The ratio of BCL-2/BAX initially obtained in the early stage of recovery time was low, which rose to higher levels during the later period.

Protection from oxidation damage

Given that the maximum expression levels of PPAR-β and BCL-2 almost overlapped 48 hours post-heat stress, the cells recovered from heat stress after 48 hours were considered as the heat-pretreated model. Considering that mild heat stress can also generate a considerable number of apoptotic cells in the early stage of recovery, the cell culture medium was changed after HUVECs recovered for 48 hours. The flow treatment was applied afterward to exclude interference factors.



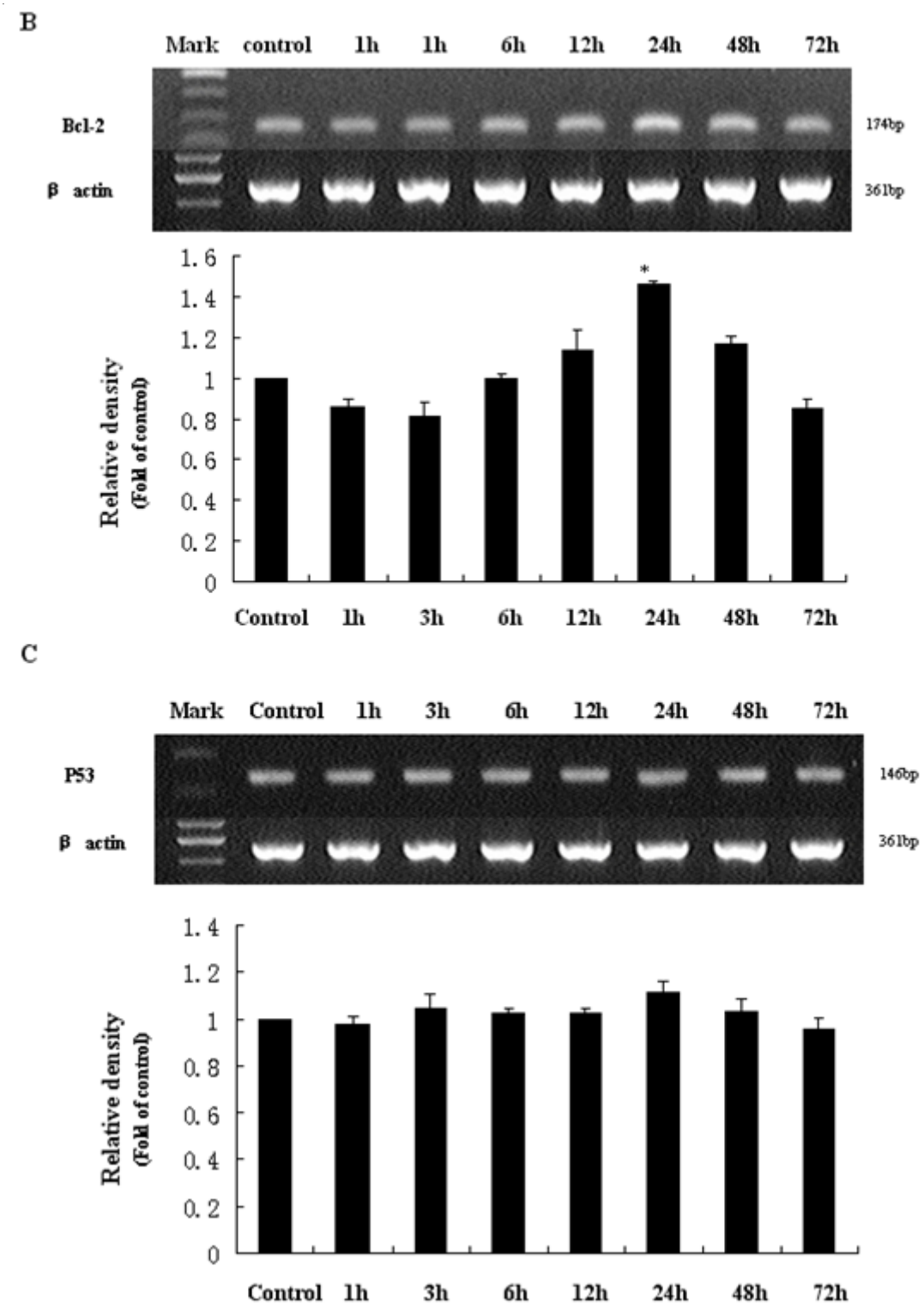
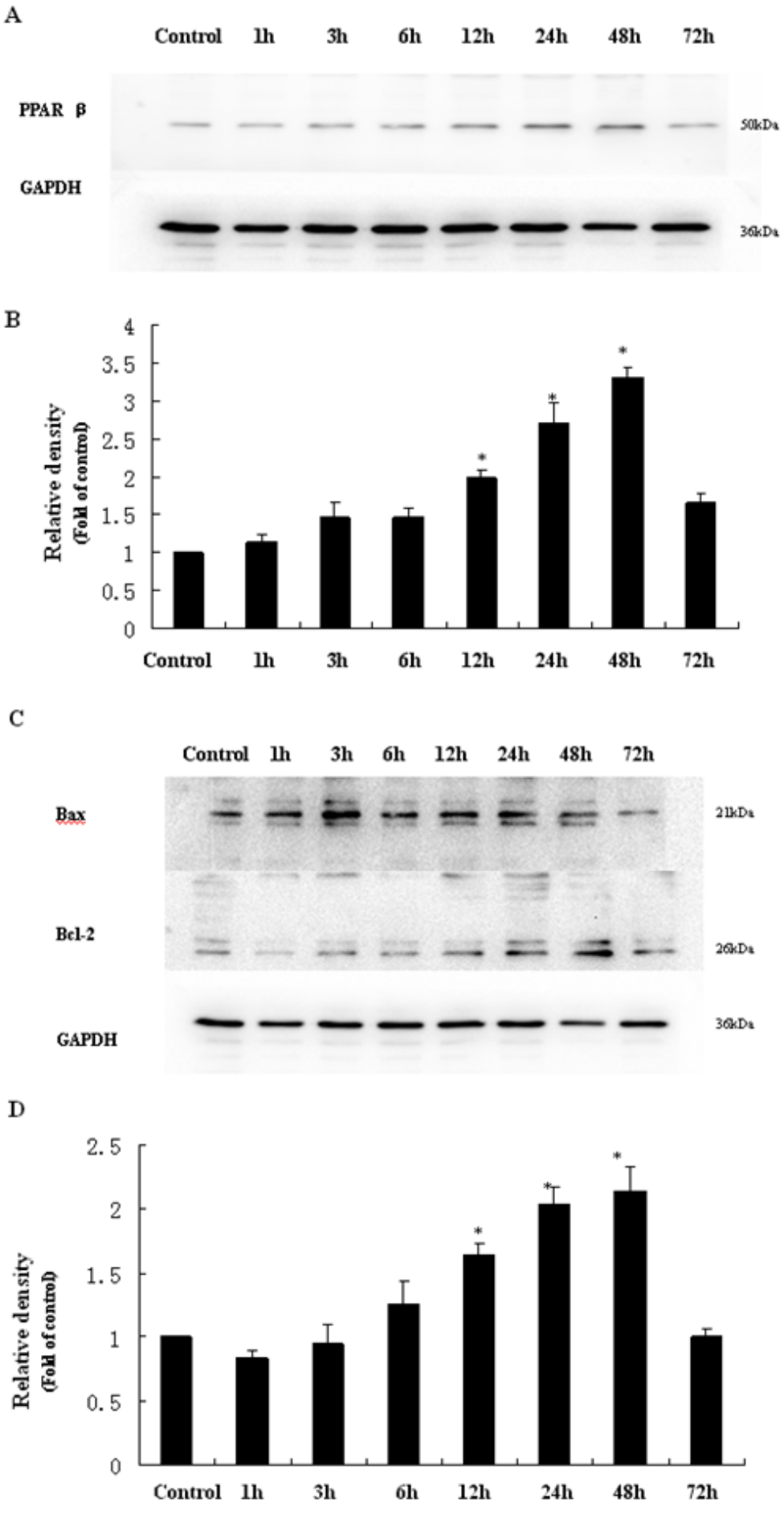


Figure 1. Heat stress induced PPAR-β and BCL-2 mRNA expression, but not P53. HUVECs were exposed to 43°C in a water bath for 25 minutes, and then recovered to 37.0°C for different times, control group cells were exposed to 37.0°C in a water bath and allowed to recover for 72 hours. PPAR-β (**A**), bcl-2 (**B**), and p53 (**C**) expression were analyzed by RT-PCR; equal loading was confirmed by β-actin mRNA density using ethidium bromide staining. Results are shown as mean ± SEM and representative of three independent experiments. *$p < 0.01$, compared with the control group.


A
Control 1h 3h 6h 12h 24h 48h 72h
PPAR β
GAPDH
50kDa
36kDa
B
Relative density (Fold of control)
4
3.5
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2.5
2
1.5
1
0.5
0
Control 1h 3h 6h 12h 24h 48h 72h
C
Control 1h 3h 6h 12h 24h 48h 72h
Bax
Bcl-2
GAPDH
21kDa
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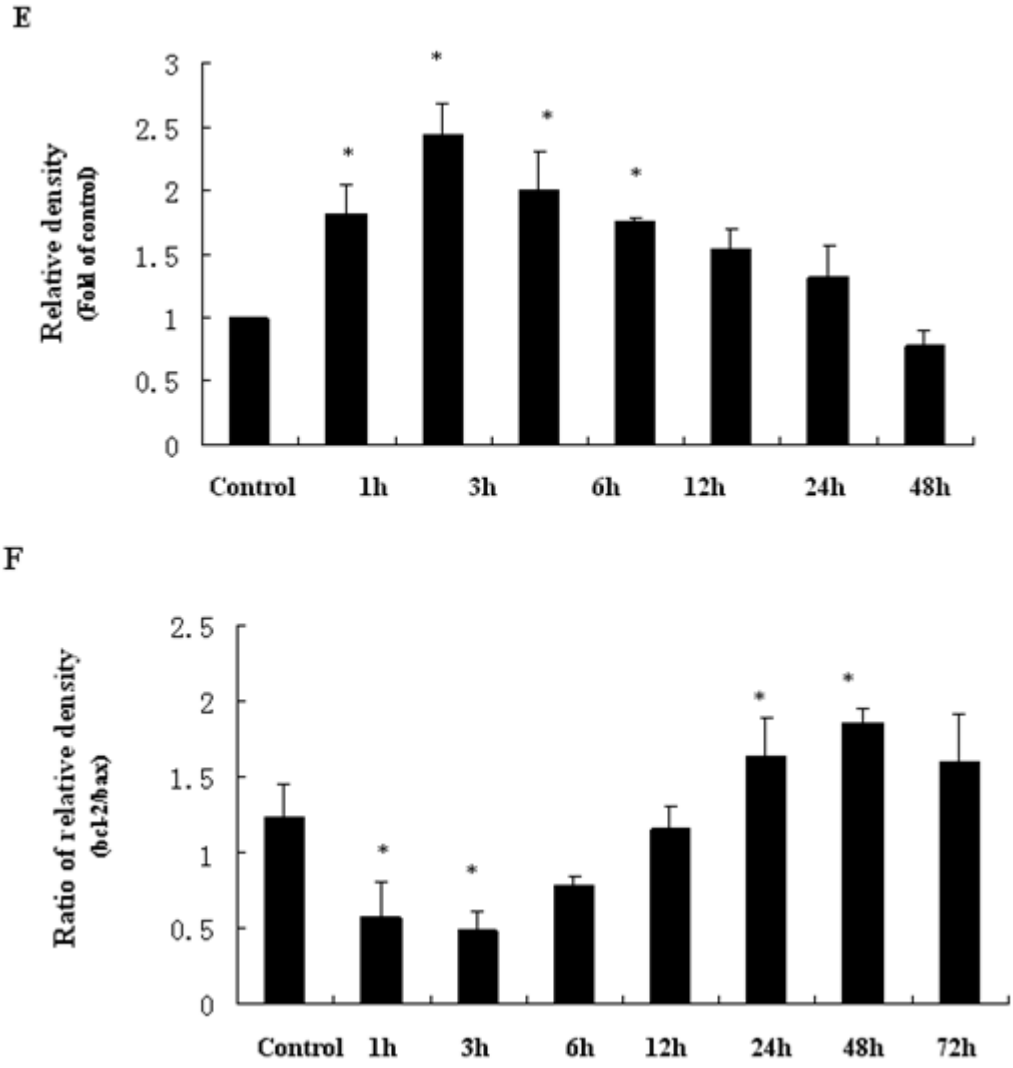

Figure 2. Effect of heat stress on PPAR-β and BCL-2/BAX expression levels. **A** and **B:** show heat stress upregulated the expression of PPAR- at the recovery period after heat treatment, from 6 to 48 hours after treatment, PPAR- was maintained a high level. **C** and **D:** The level of bcl-2 was initially downregulated after heat stress, but became much higher later. **E:** by contrast the level of BAX was initially upregulated during recovery after heat stress and maintained a high level, but show a gradually decreased trend. **F:** the ratio of BCL-2/BAX was low at first but became higher later. The data are presented as mean ± SEM and representative of three independent experiments.

Four kinds of shRNA plasmid were transfected into HUVECs. Positive cells were selectively isolated by G418, and the effects of shRNA were investigated using RT-PCR and western blotting. PPAR-β shRNA plasmid-2 showed the most effective inhibition of PPAR-β mRNA. Thus, in subsequent experiments, we chose plasmid-2 as the PPAR-β shRNA as can be seen in **Figure 3**.

H_2O_2 was added into the cell culture medium at a final concentration of 400 μmol/L to study the effects of heat stress on oxidant injury. Different groups of cells were cultured in this medium for 12 hours for oxidant injury. HUVECs were randomly divided into the following groups: normal control (control); H_2O_2 (H_2O_2); heat pretreated recovered 48 hours prior to H_2O_2 treatment (heat + H_2O_2); GW0742 added before H_2O_2 treatment (GW+H_2O_2); and PPAR-β shRNA H_2O_2 (shRNA+H_2O_2). In the heat+H_2O_2 group, cells were preincubated in a water bath at 43°C for 25 min and were recovered under normal cell culture conditions for 48 hours. Cell culture medium was changed, and then H_2O_2 was added. In the GW+H_2O_2

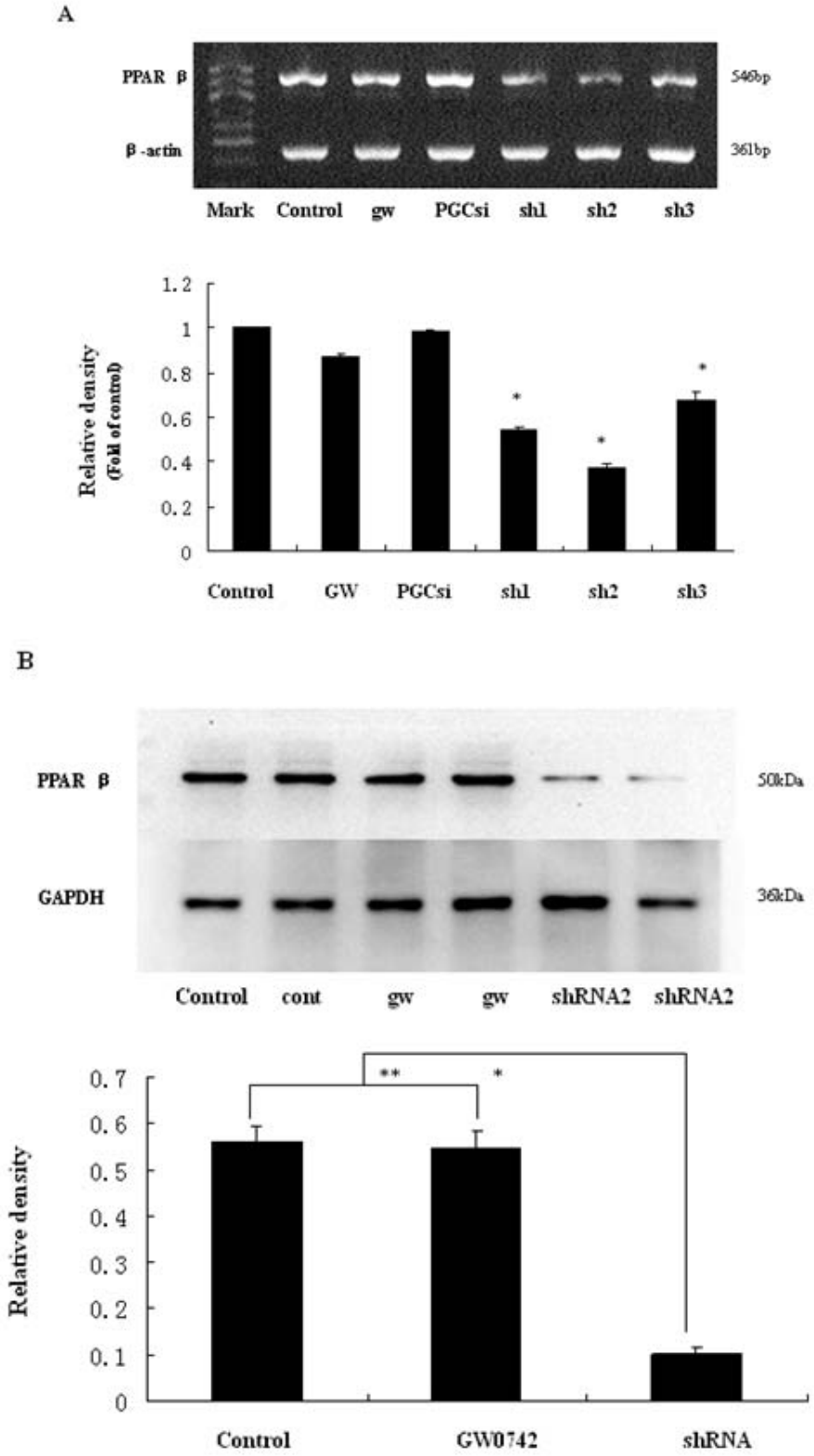

Figure 3. **A:** The efficiency of GW0742 and four kinds of shRNA plasmid on PPAR-β mRNA 4 kinds of plasmid were transfected into HUVECs, the level of PPAR-β expression was detected by RT-PCR, compared with PGCsi all three plasmid types decreased the expression of PPAR-β, and plasmid 2 show the most effect on interfering mRNA of PPAR-β, GW0742 shows little influence on the expression of PPAR-β mRNA. **B:** shRNA plasmid 2 decreased expression of PPAR-β, but GW0742 showed little effect on PPAR-β HUVECs either transfected with shRNA or cultured in the medium that contained 1 μM GW0742 for 24 hours, total protein was extracted and investigated by western blotting with antibody specific for PPAR-β, compared with the control group, shRNA apparently decreased PPAR-β, in the GW0742 group, the level of PPAR-β showed little change. Data are mean ± SEM. n = 3 independent experiments. *$p < 0.01$, **$p > 0.05$.

group, cells were cultured under normal conditions for 24 hours with medium containing 1 μM GW0742. After which, the medium was changed and H_2O_2 was added.

Both heat pretreated and GW0742-supplemented media remarkably improved the morphology of shrunken and deformed endothelial cells induced by H_2O_2. **Figure 4** shows a representative set of micrographs for all five groups of HUVECs. The control group of HUVECs showed normal cobblestone morphology. HUVECs treated with H_2O_2 show a deformed and shrunken appearance. Oxidant injury caused increasing cell aggregation and floatation. Cell adherence ability apparently declined. Interestingly, deformed and shrunken HUVECs were far less extensive in the heat+H_2O_2 group, whereas the GW+H_2O_2 group demonstrated similar conditions to the heat pretreated group. In contrast, the shRNA+ H_2O_2 group showed much more severe oxidant injury than the other H_2O_2 groups.

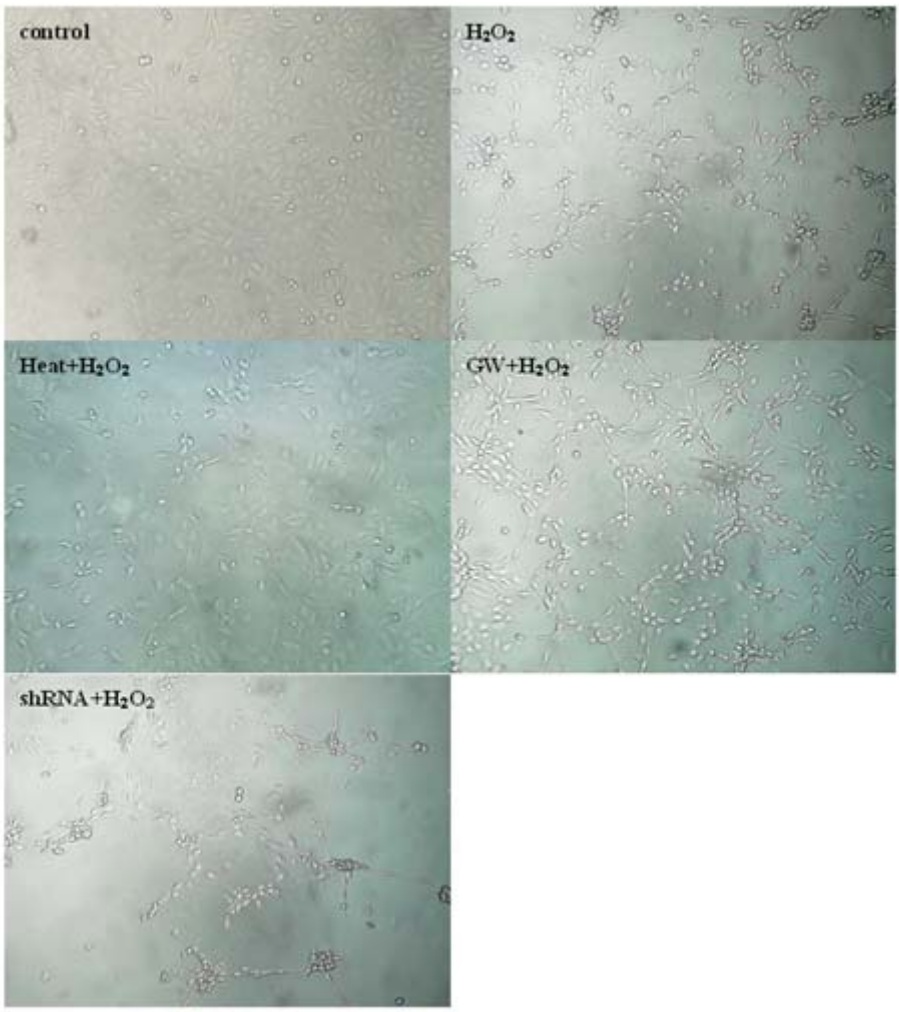

Figure 4. Observation of morphological changes (×100). Control: untreated HUVECs demonstrating normal cobblestone morphology. H_2O_2: exposure to 400 μM H_2O_2 showing HUVEC shrinkage, resulting in increased cell aggregation and floatation and a roughened cell profile. Heat+H_2O_2 and GW+H_2O_2 attenuated HUVEC shrinkage and the HUVECs show an improved appearance in the micrographs compared with the H_2O_2 group. More severe oxidant damage is seen in HUVECs of the PPAR-β shRNA H_2O_2 group compared with the H_2O_2 group.

Nuclear morphology and apoptotic rate

Almost no signs of morphological nuclear damage or condensation were found in the control group. However, nuclear condensation and fragmentation and the appearance of apoptotic body-like structures were frequently observed in the H_2O_2 groups. There were far less apoptotic cells in the heat+H_2O_2 and GW+H_2O_2 groups than that in the H_2O_2 group. By contrast, HUVECs transfected with PPAR-β shRNA seemed much more sensitive to oxidant injury, after which the number of apoptotic cells significantly increased, as shown in shRNA+ H_2O_2 group **(Figure 5A** and **B)**.

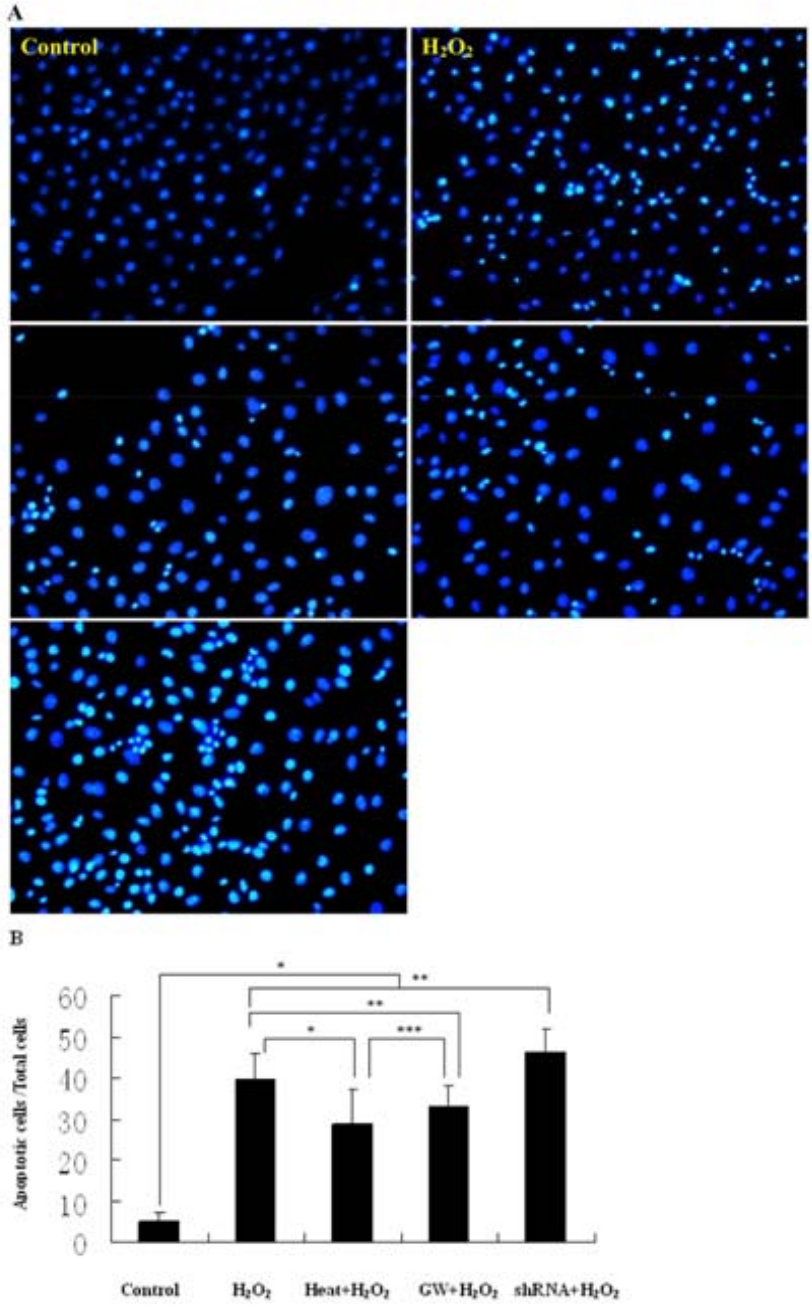

Figure 5. A: typical effects of heat stress and GW0742 or PPAR-β shRNA on nuclear morphology of cells stained with Hoechst 33258 (×200). Control: untreated HUVECs demonstrating normal nuclear morphology. H_2O_2: exposure to 400 μM H_2O_2 caused nuclear margination, condensation and the appearance of apoptotic-like bodies. Heat+H_2O_2: pretreated with heat stress shows attenuated cell nuclear damage. GW+H_2O_2: HUVECs pretreated with GW0742 also shows a protect effect against oxidant injury. shRNA+H_2O_2: HUVECs transfected with PPAR-β shRNA show much more apoptotic-like bodies than other groups. Representative of 3 independent slides. **B**: effects of heat stress or GW0742 or transfection with PPAR-β shRNA on apoptotic index of cells injured by H_2O_2. Data are shown as mean □ SEM. n = 7–9 independent experiments. *$p < 0.01$. **$p < 0.05$. ***$p > 0.05$

Figure 6 shows the normal appearance represented by the control group, including normal G1 and G2-M DNA peaks and a small proportion of apoptotic cells (0.7 ± 0.27%). H_2O_2-treatment produced a significant increase in the proportion of apoptotic cells (15.97 ± 1.77%). Heat-pretreatment produced attenuated apoptosis compared with the H_2O_2-treated group (12.55 ± 1.43%, $p < 0.01$). Cells pretreated with GW0742 showed similarly attenuated apoptosis compared with the H_2O_2 group (13.70 ± 1.49%, $p < 0.05$). The statistical difference between the heat pretreated and GW0742 groups was not significant ($p > 0.05$). By contrast, HUVECs transfected with PPAR-β shRNA seemed much more sensitive to oxidant injury (19.72 ± 3.05 compared with the H_2O_2-treated group $p < 0.01$).

In the current research, in the early stage of heat-pretreatment stage, both BCL-2 mRNA and protein levels showed little change, but those of BAX were upregulated. Subsequently, the ratio of BCL-2/BAX was downregulated. BCL-2 and BAX levels were downregulated, upregulated, or showed no significant change after heat stress. The results actually depend on the temperature and the time the cells were treated [3, 24, 25]. Different cell lines also showed different responses to heat stress. During the early stage of recovery period post-heat stress (1 to 24 hours), an increase in apoptotic HUVEC numbers was observed with time. In the later stage of recovery (48 to 72 hours), no further increase in the numbers of apoptotic cells was observed, even after the addition of H_2O_2. Thus, we propose that heat stress provides some kind of delayed protective effect against oxidant injury, which may be relevant to the upregulation of BCL-2.

HUVECs were randomly divided into the following groups to investigate the relationship between BCL-2 and PPAR-β: HUVECs transfected with PPAR-β shRNA (shRNA); HUVECs treated by heat stress and recovered for 48 hours (HT); HUVECs treated with GW0742 (GW); and control group. (Control) Total mRNA was extracted and analyzed by RT-PCR. Results showed that compared with levels in the control group, BCL-2 mRNA levels in the GW and shRNA groups showed little change **(Figure 7A)**. This finding suggests that PPAR-β does not regulate BCL-2 expression directly at the level of transcription.

HUVECs were divided into 6 groups randomly **(Figure 7B)** to understand whether PPAR- affects BCL-2 protein levels and to investigate whether heat stress-induced BCL-2 upregulation is PPAR-β-dependent. BCL-2 expression was detected by western blotting. The PPAR-β agonist GW0742, as well as heat stress apparently induces BCL-2 upregulation. Furthermore, heat stress apparently enhances the effect of GW0742, which may be more or less relevant to the heat stress-induced PPAR-β expression. The effects of both GW0742 and heat-induced BCL-2 can be abolished by transfection with PPAR-β shRNA.

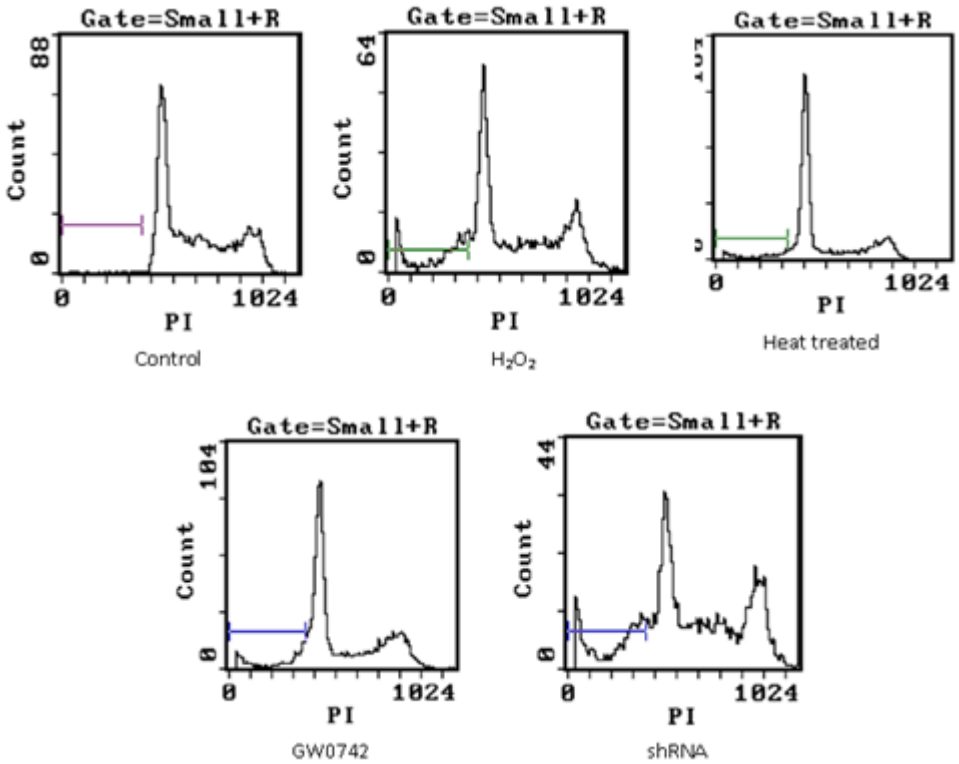

Figure 6. Effect of heat stress and activation or blockage of PPAR-β on the apoptotic ratio of HUVECs measured by flow cytometry with PI staining. HUVECs cells were either untreated (control) or treated with H_2O_2 (H_2O_2) at 400 μM, or pretreated with heat stress or GW0742, or transfected with PPAR-β shRNA followed by H_2O_2 at 400 μM. n = 7 independent experiments.

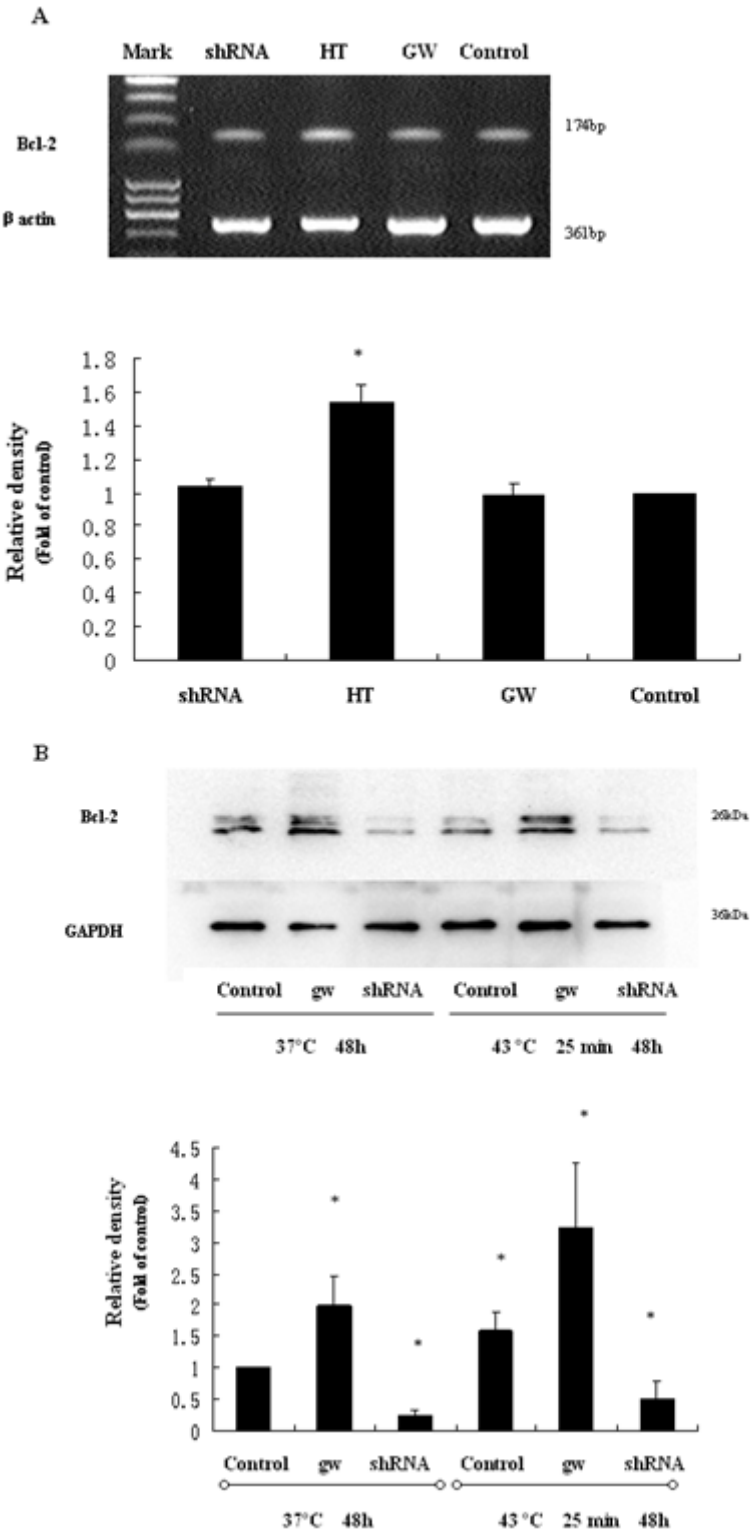

Figure 7. **A:** GW0742 or PPAR-β shRNA have little effect on BCL-2 on level of mRNA in HUVECs. Cells were divided into four groups: a PPAR-β shRNA transfection group, heat pretreated group; GW0742 pretreated group, and a control group. Compared with the control group, all except the heat treated group were upregulated, BCL-2 shows little change in other groups at the level of its mRNA. **B:** Heat induced upregulation of BCL-2 was relevant to PPAR-β. The level of BCL-2 was upregulated in HUVECs pretreated with GW0742 or heat stress, and the effect of GW0742 can be enhanced by heat stress, both of these effects can be abolished by transfection with PPAR-β shRNA. Data are presented as mean ± SEM. n = 3 independent experiments. Compared with the control group $*p < 0.01$.

Discussion

Heat stress can cause severe damage to cells and tissue. A number of studies have indicated that heat shock-induced cell apoptosis is manifested through a variety of mechanisms [1, 2]. However, other findings show that mild thermal preconditioning could prevent cells from subsequent lethal damage caused by adverse factors such as oxidation [21]. This phenomenon has been known as thermo tolerance, which depends on the temperature and the time for which cells are subjected to it. Different cell lines also show different responses to thermal treatments. The mechanism of the protective effect of thermal pretreatment may be associated with some types of HSP, such as HSP72 [4], HSP70 [22], and HSP27 [23]. Downstream mechanisms of the protective effect of HSPs have been proven. However, not all HSPs produce a protective effect [24]. Thus, some other heat-related mechanism must be inducing this protective effect.

PPARs are members of the super family of ligand-inducible transcription factors known as nuclear receptors, which regulate many cellular and metabolic processes. Three isotypes, namely, PPAR-α, PPAR-β/δ, and PPAR-γ [35], are distributed differently in different tissues. Their biological effects and mechanisms vary from one another. PPAR-α and PPAR-γ are important in the regulation of lipid metabolism [26] and inflammatory responses [27], whereas PPAR-β functions to regulate different cells in terms of proliferation, migration, and differentiation [28-30] and play an important role in regulating cell apoptosis. Previous studies have indicated that the upregulation of PPAR-β has an antiapoptotic effect on different cell lines [31, 32]. In a previous study, we found that oxidation causes HUVEC apoptosis, the expression of PPAR-β is downregulated, and L-165041, a PPAR-β agonist, significantly inhibits H_2O_2-induced apoptosis in HUVECs [9]. We have also demonstrated that repetitive mild stress enhances PPAR-β expression and activity, thereby inhibiting subsequent oxidative stress-induced apoptosis [10].

Many factors such as injury, inflammation, and ischemia-reperfusion cause upregulation of PPAR-β as a mechanism of self-protection [33, 34]. Tzika et al. found that the PPAR-β expression in skeletal muscle was apparently upregulated after burn injury [18]. The possibility that mild heat stress may also cause the upregulation of PPAR-β during the recovery time post-heat stress was explored further in the current study. HUVECs were chosen for the heat stress model because vessel endothelial cells play an important role during the recovery toward the stasis zone after burn injury, and endothelial cells are more vulnerable to heat injury than other types of cells in the skin, such as fibroblast and keratinocytes. The recovery of endothelial cells after burn injury is critical for burn wound healing.

Mild heat stress can induce PPAR-β expression in HUVECs. As revealed in other studies, heat stress itself causes considerable cell apoptosis in the early stage of recovery post-heat treatment. An increase in the number of apoptotic cells with time was observed initially. However, after 48 hours or later, no further increase in the numbers of apoptotic cells was seen, even with H_2O_2 treatment. PPAR-β expression was upregulated during the entire recovery period. The protective effect of PPAR-β could be partly mimicked by treating HUVECs with the PPAR-β agonist, GW0742. The protective effect can be diminished by transfecting HUVECs with PPAR-β shRNA. These results suggest that heat-induced PPAR-β may at least be partly responsible for protecting heat-treated HUVECs from severe oxidant injury.

BCL-2 and BAX are very important in regulating cell apoptosis. Previous studies had provided different conclusions for different cells treated with a variety of temperatures for different times. Takasu et al. showed that the expression of the BCL-2 and BAX proteins in the HL-60 human promyelocytic leukemia cell lines are unchanged after heating [19]. Deng et al. demonstrated that after treatment at different high temperatures, BCL-2 and BAX expression are not changed in human colon cancer HCT116 cells [20]. However, Meldrum et al. indicated that heat preconditioning can prevent renal tubular cells from ischemia-induced apoptosis by upregulating BCL-2 expression [4]. Recent research results show that heat pretreatment prevents K562, U937, and HeLa cells from sorbitol-induced apoptosis by upregulating BCL-2 and downregulating BAX [5]. In the current study, we found that BCL-2 expression showed little change in the early stage of recovery after heat stress, both at the mRNA and protein levels. However, much of previous research only investigated the BCL-2 expression at the early stage (<48 hours) of the recovery period. In the current results, the BCL-2/BAX ratio was downregulated at the first stage of recovery period, but upregulated in the later stages.

This finding is consistent with previous research and explains why heat stress initially causes apoptosis in HUVEC, but subsequently provides protection against oxidant injury.

The relationship between PPAR-β and BCL-2 was also investigated in the current research. PPAR-β mRNA levels were found unchanged when HUVECs were treated with GW0742 or transfected with PPAR-β shRNA. As a nuclear transcription factor, PPAR-β regulated the expression of other genes by interacting with specific DNA-response elements (PPRE) located upstream of the responsive genes [35]. No PPRE was found in the upstream promoter of the BCL-2 gene using bioinformatics analysis [36, 37]. Surprisingly, some agonists of PPAR-β, such as GW0742, can upregulate BCL-2 expression relatively [38, 39]. Yin et al. found that another agonist of PPAR-β, GW50156, upregulates BCL-2 through an indirect pathway [40]. In the present study we observed that the BCL-2 protein level was upregulated by heat stress or PPAR-β activation. Both of these factors can be attenuated by transfection with PPAR-β shRNA. These results suggest that PPAR-β may regulate BCL-2 expression through an indirect pathway, such as post-transcriptional regulation.

In conclusion, mild heat stress can provide HUVECs with a delayed protective effect against oxidant injury. Upregulation of PPAR-β may be partly responsible for this protective effect. Active PPAR-β indirectly upregulates BCL-2, which may in part explain its antiapoptotic effect. However, discovery of the specific mechanism needs further study. These results suggest that activation of PPAR-β may be beneficial to endothelial cells in burn wounds.

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