Role of PPARβ in fibroblast response to heat injury

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Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor family of ligand-inducible transcription factors. Our previous study has shown that in human umbilical vein endothelial cells PPARβ initiates a protective mechanism that limits the extent of damage due to H2O2-induced injury. Although fibroblasts are one of the main cell types involved in wound repair, the role of PPARβ in the fibroblast response to heat injury has not been investigated. Thus, in this study, we examined possible protective role of PPARβ in fibroblasts from heat injury. We developed a novel dermal fibroblast heat injury model to characterize the mechanisms of the heat injury healing response that involved PPARβ. The specific PPARβ ligand GW0742, a PPARβ activator and a short hairpin RNA (shRNA) plasmid against PPARβ were used to reveal the action mechanism of PPARβ in heat injury-induced fibroblast changes in morphology and increased proliferation. In response to heat injury (52°C for 30 s), fibroblast activation of PPARβ increased 1.56-fold. Administration of GW0742 significantly induced a protective effect on heat injury-induced fibroblasts by minimizing the structural damage and increasing the cell proliferation response. Likewise, inhibition of PPARβ using shRNA exacerbated the damage by inhibiting the de novo synthesis of PPARβ. These results indicated that heat injury enhanced PPARβ expression and PPARβ protected fibroblast structure and proliferation.

Keywords: PPARβ, Fibroblast, Heat injury, In vitro wound healing model

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor family of ligand-inducible transcription factors. The three isotypes of PPAR that have been identified to date are encoded by separate genes (PPARα or NR1C1; PPARβ, also known as PPARδ or NR1C2, and PPARγ or NR1C3). All three have been identified in amphibians, rodents and humans1. PPARα and PPARγ play important roles in the regulation of intermediary metabolism, in particular energy homeostasis, lipid catabolism and glucose homeostasis2-5. However, little is known about the function of PPARβ, although it has been implicated in inflammation, cell proliferation and apoptosis. Recently, it has been shown that mice lacking the full level of PPARβ demonstrate a wound healing defect6,7. PPARβ heterozygous mice show defects in maintaining the cutaneous barrier and in these mice wound repair is delayed by 2–3 days8. In addition, PPARβ selectively inhibits cell proliferation to prevent scar formation9,10. Thus, the combined evidence suggests that PPARβ may regulate several parameters of wound healing.

Our recent studies have indicated that DNA-binding and transcriptional regulation activities and expression of PPARβ are upregulated by tumor necrosis factor (TNF)-α11,12. Enhancement of PPARβ activity by repetitive low-grade H2O2 stress protects human umbilical vein endothelial cells from subsequent high-grade oxidative stress-induced apoptosis13. These studies suggest that PPARβ is induced in response to cell stresses as a protective mechanism. However, whether PPARβ plays a similar role in heat injury, including the injury response has not been shown. In this study, we have investigated the possible protective role of PPARβ in fibroblasts from heat injury. We demonstrate that PPARβ has a protective role in the survival of heat-stressed dermal fibroblasts.

Materials and Methods
Primary cultures of mouse dermal fibroblasts
All animal procedures were approved by the Ethical Committee of the XiangYa Hospital. Primary cultures of mouse dermal fibroblasts were obtained from 1 to 3-day-old wild-type Kunming mice

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(Experimental Animal Center of Xiang Ya Hospital, Central South University), isolated from dermis after sacrifice. Dermis was separated from the epidermis following overnight incubation at 4°C in 2.5 U/mL of dispase. Dermis extracts were incubated for 30-40 min at 37°C in cell culture-grade type I collagenase (Dingguo Biological Technology, Beijing, China). Following filtration and centrifugation, cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO) supplemented with 100 U/mL penicillin/streptomycin and 10% fetal calf serum (FCS; Dingguo Biological Technology, Beijing, China). Fibroblasts were used at passages 3 to 5

A total of 6 isolations were used, with ≥3 isolations used per experiment.

Chemicals and plasmids
GW0742, a PPARβ activator (purity, 98%, from Cayman Chemical, Boston, Massachusetts) was dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration did not exceed 0.5% v/v, and this concentration was used in the control (untreated) wells. The PPARβ shRNA plasmid and control shRNA plasmid were purchased from Santa Cruz Biotechnology, CA, USA. The sequence of shRNA for PPARβ was:

5’-CCTTCTCTAAAGCAGAATCTATTTCAAGAGTAGATGTGCTTGAGAAGGTGTTTTT-3’, 5’-CCATCTTCACACATTGTCCTTTCAAGAGAGACAAATGTGAGGACTGTTGTTTTTT-3’, and 5’-CCTCTCTATCCCTTCAAAATTTCAAGAGTTGAGGATT-3’ (mRNA accession: NM_011145).

Induction of nucleic acid into cells and treatment
In a 6-well tissue culture plate, cells were grown to 50% confluency in antibiotic-free normal growth medium DMEM (GIBCO). Transfections were performed at 37°C in a 5% CO₂ incubator for 6 h using 2 µg PPARβ shRNA plasmid or 2 µg control shRNA plasmid and 3 µL shRNA plasmid transfection reagent (Santa Cruz Biotechnology, CA, USA). Transfection media was then removed, the wells were washed with phosphate buffered saline (PBS), the media was replaced with Dulbecco’s modified Eagle medium (DMEM) and the cells were allowed to recover for 48 h. Cells were reseeded into 96-well plates (Corning) and after adhering to the well bottom, they were then selected with puromycin at 2 µg/mL (Santa Cruz). Every 2-3 d, the media was aspirated and replaced with freshly prepared media.

Fibroblasts were serum-starved for 2 h before GW0742 (10 µM) treatment. After the 2 h treatment period, cells were maintained in DMEM with GW0742 or control (DMSO) for 24 h. The concentration of GW0742 used has been shown to specifically activate PPARβ.

Experimental groups and heat injury model
Cells were randomly allocated to the following groups: (Group I, normal): cells were cultured normally; (Group II, shRNA): cells were transfected with the PPARβ shRNA plasmid prior to heat injury; (Group III, shRNA control): cells were transfected with the control shRNA plasmid prior to heat injury; (Group IV, GW0742): cells were treated with GW0742 (10 µM) prior to heat injury; and (Group V, DMSO control): cells were treated with only DMSO prior to heat injury.

All cells described above were subjected to heat injury by incubating the resuspended cells in a 52°C thermostatic water-bath for 30 s (second-degree burn). The cells were immediately seeded in a 6-well plate and incubated at 37°C in the presence of 5% CO₂ under a humidified atmosphere.

Reverse transcription polymerase chain reaction (RT-PCR)
To verify expression of PPARβ mRNA in cultured fibroblasts, the cells were cultured as described above and treated with heat injury after 24 h. Total RNA was extracted from fibroblasts using TRIzol reagent (Invitrogen, Life Technologies Corporation, USA). For the RT-PCR amplification of PPARβ transcripts, 1-2 µg of total RNA was reverse transcribed with M-MLV reverse transcriptase according to the manufacturer’s instructions (Invitrogen, Life Technologies Corporation, USA). The RT-PCR amplification was performed by adding template cDNA, 20 mM Tris–HCl (pH 8.3), 100 mM KCl, 3 mM MgCl₂, 500 µM of each dNTP, 1 mM of each primer, and 0.1 U Taq DNA polymerase in 25-µL reaction volumes. The PCR cycle conditions included 28 cycles of denaturation (95°C for 30 s), annealing (63°C for 40 s), elongation (72°C for 50 s) and one final elongation cycle (72°C for 10 min).

All RT-PCR products were characterized by electrophoresis through 2% agarose gels and visualized by ethidium bromide staining under UV light. Images were acquired using the electrophoresis image analysis system (Bio-PRO).
The following primers were used: PPARβ forward: 5’-CCAGAAGACCGCAACA-3’, and reverse: 5’-GTCACCTGTCATTGAGGAAGA-3’; β-actin forward: 5’-GAGACCTTCAACACCCACGC-3’ and reverse: 5’-ATGTCACGCACGATTTCCC-3’.

Electron microscopy
For fibroblast ultra-structural analysis, cells were fixed in 2.5% glutaraldehyde for 24 h, post-fixed with 1% osmium tetroxide for 2 h and then dehydrated by a graded acetone series (50%, 70%, 90%, 100%, each 10 min × 3). Samples were soaked in a 1:1 epoxy resin:pure acetone mixture for 24 h in 37°C and embedded in epoxiaequivalentgewicht 145-160 (Epon812), dodecenylsuccinic anhydride (DDSA), methyl nadic anhydride (MNA) and dimethylaminomethyl phenol (DMP-30). Ultrathin (500 Å) sections cut with an LKB III ultramicrotome (LKB, Switzerland) were contrasted with uranyl acetate and lead nitrate and imaged with a JEOL-1230 electron microscope fitted with a camera (Nikon, Japan).

Methyl-thiazolyl-tetrazolium (MTT) reduction assay
Mitochondrial reduction capacities were determined by MTT assay, i.e., quantification of the reduction of methyl-thiazolyl-tetrazolium bromide (Sigma) to formazan dye crystals. Fibroblasts were stimulated by heat injury (52°C for 30 s) and plated in quadruplicate in 96-well plates at a density of 2 × 10^4 cells/well. MTT solution (final conc. 0.5 mg/mL PBS per well) was added to the cultures, which were then incubated for 4 h. The cells were washed gently with PBS and 100 µL of DMSO was added to the wells, followed by mild shaking to dissolve the MTT precipitate. Absorbance was measured for each well using a Bio-Tek Powerwave X microplate reader (BioTek Instruments, USA) at a wavelength of 490 nm. MTT reduction was expressed as a percent of controls. All experiments represented ≥ 3 independent replications performed in quadruplicate.

Flow cytometry analysis
For each experimental group, cells (1×10^6) were treated with heat injury for the indicated conditions of temperature and time. Floating and attached cells were harvested, washed with PBS and fixed in 70% ethanol overnight at 4°C. The cells were stained with propidium iodide (PI; 20 mg/mL; Invitrogen), measured by flow cytometry (Coulter, USA) and analyzed by MCYCLE software. The proliferation index was calculated as (S% + G2%) / (G1% + S% + G2%) × 100.

Immunoblot analysis
Cells were washed with cold PBS and suspended in 1×10^6 of cold lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM phenylmethanesulfonyl fluoride, and 1× protease inhibitor cocktail). The cell lysate was incubated on ice for 30 min and centrifuged at 10,000 × g for 10 min at 4°C. Insoluble material was removed by centrifugation and the protein content of the supernatant was determined with a BCA Protein Assay kit (Genstar Biosolution). Equal amounts of total protein (20 μg) were loaded and separated by SDS–PAGE, and the resolved proteins were transferred on to a polyvinylidene fluoride membrane. The blot was blocked with 5% dried skim milk in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) overnight at 4°C and probed with anti-PPARβ, anti-proliferating cell nuclear antigen (PCNA) anti-β-tubulin or anti-GAPDH polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactive bands were visualized with a horseradish peroxidase-conjugated secondary antibody and diaminobenzidene (Boster Biological Technology, Wuhan, China).

Statistical analyses
Data were presented as mean ± SEM. Differences between two groups were analyzed by the unpaired Student’s t-test. Differences among three groups were analyzed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls tests. A P-value < 0.05 was considered significant. Analyses were performed using SPSS13.0 software.

Results
Heat injury significantly alters the fibroblast morphology and cell survival
To investigate the effect of temperature on morphology and cell survival, mouse dermal fibroblasts were treated in a thermostatic water bath at 52°C for 30 s. Significant morphological changes were observed and cell blebbing was apparent in the heat injury group, indicative of lesions (Fig. 1A and B). Cell viability and proliferation were then determined by MTT assay. The survival rate of fibroblasts was 49.6% when cells were treated at 52°C for 30 s and proliferation was inhibited on the first day (Table 1 and Fig. 1C). Our results showed that fibroblasts subjected to heat injury could be defined
as an *in vitro* fibroblast wound healing model, with the most serious cell damage occurring within 24 h.

Heat injury increases PPARβ expression in dermal fibroblasts

In order to determine the role of PPARβ in heat-treated fibroblasts, we first determined whether PPARβ expression existed in mouse dermal fibroblasts before and after heat injury. There were detectable expression levels of PPARβ in normal fibroblasts and PPARβ protein levels increased 1.56-fold in heat-treated fibroblasts compared to the normal group (Fig. 2).

GW0742, a PPARβ agonist protects dermal fibroblasts from heat injury

Electron micrographs revealed that cell structure in cells pre-treated with 10 µM GW0742 was preserved, compared with control (DMSO-treated) cells (Fig. 3A). After 30 s exposure to heat injury followed by a 24 h recovery period, the cell proliferation index was significantly higher in cells pretreated with GW0742 than in the DMSO-treated controls (Table 2). An increased level of proliferating cell nuclear antigen (PCNA) was also observed in...
GW0742-treated cells, compared with the DMSO-treated control cells (Fig. 3B). No toxic effects were observed after GW0742 treated (cell survival data >90%).

**PPARβ shRNA treatment enhances the heat injury response in dermal fibroblasts**

To further characterize the role of PPARβ in fibroblasts after heat injury, PPARβ shRNA plasmids were transfected into the cells. Using the shRNA

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**Table 2—Cell cycle of GW0742-treated fibroblasts**

[Fibroblasts were treated with heat injury for 30 s and recovered for 24 h in the presence of GW0742 or DMSO. A flow cytometry assay showed that cell proliferation index in the cells pretreated with GW0742 was significantly higher than that of the DMSO-control and normal cells. The percentage of cells in G0/G1 phase was significantly lower than in the normal and control groups. The percentage cells in S and G2/M phase was higher than in the normal and control groups. Values expressed as the mean ± SEM of three independent experiments]

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1 (%)</th>
<th>G2/M (%)</th>
<th>S (%)</th>
<th>Proliferation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>50.4 ± 1.4</td>
<td>10.8 ± 2.6</td>
<td>38.7 ± 2.3</td>
<td>0.50</td>
</tr>
<tr>
<td>GW0742</td>
<td>38.5 ± 3.1*</td>
<td>21.3 ± 1.6*</td>
<td>42.8 ± 1.4*</td>
<td>0.62*</td>
</tr>
<tr>
<td>DMSO control</td>
<td>50.2 ± 2.2</td>
<td>10.5 ± 1.3</td>
<td>38.4 ± 1.5</td>
<td>0.49</td>
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*P < 0.05 vs. the normal or control groups.
Fig. 4—Effect of PPARβ down-regulation on fibroblast morphology and proliferation changes induced by heat injury [(A): Fibroblasts were transfected with PPARβ shRNA plasmid prior to heat injury. PPARβ mRNA expression in fibroblasts of the normal, shRNA and shRNA control treated group was quantified and normalized with β-actin. Values expressed as mean ± SEM of three independent experiments. *P<0.05 vs. the normal control group; (B): PPARβ protein expression in fibroblasts from the above three groups were quantified and normalized with GAPDH. *P<0.05 vs. the control group. Results shown are representative of three independent experiments; (C): Transmission electron microscopic observation (x10,000). After heat injury followed by a 24 h recovery period, the nucleoli of fibroblasts that received the shRNA plasmid could not be observed and there was much more vacuolar degeneration of the mitochondria into the cytoplasm with some evidence of severe cell fracture compared to the normal or shRNA control groups after heat injury (↓- damaged structure); and (D): By immunoblot, after 24 h fibroblasts were treated with heat injury (52°C for 30 s), PCNA levels were quantified as the ratio of the density of PCNA to that of β-tubulin. *P<0.05 vs. the normal, control group. Values expressed as the mean ± SEM of three independent experiments]
plasmid transfection reagent, PPARβ shRNA plasmid or control shRNA plasmid were delivered into cultured fibroblasts. The transfer of shRNA plasmid was able to inhibit PPARβ mRNA and protein expression by >50% and >40%, respectively. The control shRNA plasmid did not alter PPARβ expression (Fig. 4A and B). As shown in Fig. 4C, the decreased expression of PPARβ exacerbated the destructive effects of heat injury, compared to the control shRNA group. After heat injury followed by a 24 h recovery period, the cell proliferation index in the PPARβ shRNA-treated group was significantly lower than that of the normal control and shRNA control cells, indicating that PPARβ stimulated the proliferative response (Table 3). Consistent with this observation, lower levels of PCNA were found in the PPARβ shRNA-treated group after heat injury, compared with both control groups (Fig. 4D). No toxic effects were observed after shRNA transfection (cell survival data >90%).

**Discussion**

In the latest clinical treatment of excisional wounds, autogeneic epidermis is frequently used to cover the wound and preserve the degenerative dermis. This is in contrast with the traditional treatment strategy, whereby excisional wounds are also covered, but the dead and degenerated tissue is removed rather than preserved. Clinical observation and animal experiments both support the latest treatment strategy and suggest that autologous skin transplantation heals in a scarless fashion and that the heat-injury degeneration of fibroblasts recovers gradually\(^{16-18}\).

Significant attention has been paid to mechanisms that promote dermal fibroblast migration, which allows the cells to infiltrate in from the wound edges. Heat injury-stimulated fibroblast degeneration and proliferative capacities, on the other hand, have not been well-defined. Therefore, we established a heat injury dermal fibroblast cell model by transiently treating cells with hot water. The establishment of this model allows us to explore the potential mechanisms of heat injury-induced degeneration of fibroblasts. This model closely recapitulates the clinical setting, where higher temperatures and shorter durations are typical\(^{19-21}\).

We found that the survival rate of heat-treated (52°C for 30 s) fibroblasts was 49.6%, nearly half a lethal dose. Significant morphological damage was observed in heat-treated fibroblasts, especially within the first 24 h. Therefore, we selected this model to examine the effects of PPARβ. The upregulation of PPARβ lasts over the entire healing process and is an important mediator of keratinocyte survival. The expression of PPARβ has been shown to be triggered by the activation of stress. Our results in this study indicated that heat injury could also induce the upregulation of PPARβ in fibroblasts. The PPARβ agonist GW0742 protected fibroblasts from heat injury. Likewise, shRNA against PPARβ significantly inhibited PPARβ expression and enhanced heat-induced injury. These results confirmed that PPARβ upregulation could protect fibroblasts by prolonging survival in the heat injury setting.

PPARβ is necessary to skin wound healing, as demonstrated in PPARβ-null mice which exhibit significantly delayed skin wound healing\(^{22}\). Activation of PPARβ or the use of PPARβ ligands to promote keratinocyte survival and proliferation also likely to influence later stages of wound repair, including scar formation and tissue remodeling and hair follicle

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**Table 3—Cell cycle of PPARβ shRNA-treated fibroblasts**

[Fibroblasts were treated with heat injury and recovered for 24 h and then transfected with PPARβ shRNA or control shRNA plasmids. A flow cytometry assay showed that cell proliferation index in those cells transfected with the PPARβ shRNA plasmid was significantly lower than that of the cells transfected with the control shRNA plasmid and normal cells. The percentage of cells in the G0/G1 phase in the PPARβ shRNA plasmid-transfected cells was significantly higher than in the normal and control groups. The percentage of cells in the S and G2/M phases in the PPARβ shRNA plasmid-transfected cells was lower than in the normal and control groups. Values expressed as the mean ± SEM of three independent experiments]

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<tbody>
<tr>
<td>Normal</td>
<td>50.3 ± 2.3</td>
<td>12.5 ± 2.1</td>
<td>36.5 ± 2.4</td>
<td>0.49</td>
</tr>
<tr>
<td>shRNA</td>
<td>76.5 ± 1.8*</td>
<td>7.8 ± 1.2*</td>
<td>13.6 ± 0.8*</td>
<td>0.22*</td>
</tr>
<tr>
<td>shRNA control</td>
<td>50.7 ± 1.8</td>
<td>13.0 ± 0.7</td>
<td>36.1 ± 1.5</td>
<td>0.49</td>
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*P<0.05 vs. the normal or control groups.
Additionally, activation of PPARβ has been shown to reduce apoptosis in a variety of cells, including human umbilical vein endothelial cells and HaCaT keratinocytes\(^\text{11-13}\). In response to epithelial injury, cytokine signaling leads to increased expression of PPARβ\(^\text{25}\). Our results were consistent with the above results and provided additional evidence that upregulation of PPARβ increased cell viability after heat injury. We found that blocking the activation of PPARβ significantly affected the viability of fibroblasts after heat injury.

GW0742 and other PPARβ ligands have been demonstrated inhibit cell proliferation in both human and mouse lung fibroblasts\(^\text{15}\). Treprostinil sodium has been shown to reduce the remodeling associated with pulmonary hypertension, and these effects may be mediated by activating PPARβ\(^\text{15}\). In contrast, following heat injury in the skin, the expression of PPARβ also increases and results in an increase in cell proliferation. Using TESS software, we identified multiple heat shock factor 1 (HSF1) sequences in the promoter area of the PPARβ gene. One difference between the lung injury model and our model was that we used heat to induce the injury response. A linkage between the heat shock element and the peroxisome proliferator response element in the promoters of heat shock genes has been identified by investigating the binding sites for PPAR family members in a human epithelial cell line\(^\text{26}\). PPAR-HSF1 co-immunoprecipitation studies have revealed the nature of these interactions\(^\text{27}\). HSF1 and PPAR in combination both regulate heat shock target genes. Under heat stress conditions, HSF1 and PPARβ activate the stress-inducible transcription of Hsp genes and binds to DNA containing the the 5′-nGAAn-3’ sequence of the heat shock element to protect against heat injury\(^\text{28,29}\). Therefore, possibly upregulation and activation of PPARβ in heat injury-denatured skin fibroblasts resulted in an opposite cell proliferation response, compared with hypertension-induced remodeling lung fibroblast responses.

Additionally, the increase in ligand-activated PPARβ is coincident with the upregulation of integrin-linked kinase (ILK) and 3-phosphoinositide-dependent protein kinase-1 (PDK-1), and downregulation of phosphatase and tensin homologue (PTEN) expression, leading to an accumulation of phosphorylated Akt. The increased level of phosphorylated Akt can turn cells away from an apoptotic pathway, promoting cell survival\(^\text{30,31}\). Furthermore, there is evidence that the PPARβ pathway interacts with TGFβ signaling during wound healing and that the combination provides a synergistic mechanism that allows the successful re-epithelialization of the wounded area\(^\text{32}\). While we noticed cellular damage consistent with mitochondrial dysfunction, we did not isolate mitochondria and measure free radical production in the isolated mitochondria.

In conclusion, using a novel heat injury model, we demonstrated that heat injury upregulated the expression of PPARβ in dermal fibroblasts. Activation of PPARβ by GW0742 enhanced the protective response after heat injury and PPARβ downregulation exacerbated the damage induced by heat injury. Our study indicated that the mechanism contributing to the PPARβ induced protective response in dermal fibroblasts involved promoting fibroblast survival and proliferation after heat injury. From our results, we can conclude that PPARβ may be a therapeutically viable option for treating skin damage induced by heat injury.

**Acknowledgments**

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