Emodin, A Chinese Herbal Medicine, Inhibits Reoxygenation-Induced Injury in Cultured Human Aortic Endothelial Cells by Regulating the Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) and Endothelial Nitric Oxide Synthase (eNOS) Signaling Pathway

Background: Ischemia-reperfusion injury is associated with vascular dysfunction. The aim of this study was to investigate the role of emodin, a Chinese herbal medicine, in hypoxia-reoxygenation injury in cultured human aortic endothelial cells (HAECs) and its effects on the expression of the peroxisome proliferator-activated receptor-γ (PPAR-γ) and endothelial nitric oxide synthase (eNOS) signaling pathway.

Material/Methods: An in vitro hypoxia-reoxygenation model used cultured human aortic endothelial cells (HAECs). A colorimetric method evaluated the activity of peroxisome proliferator-activated receptor-γ (PPAR-γ). Phosphorylation of PPAR-γ and endothelial nitric oxide synthase (eNOS) were measured by Western blotting. Expression of inflammatory cytokines, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8 were evaluated by enzyme-linked immunosorbent assay (ELISA) and Western blotting. Nitric oxide (NO) production was detected by diamino-fluorescein-FM diacetate (DAF-FM DA) fluorescence. Immunoprecipitation was used to evaluate the molecular coupling of heat shock protein (HSP)90 and eNOS.

Results: Hypoxia-reoxygenation injury of HAECs reduced the activity and phosphorylation of PPAR-γ, eNOS, NO production, and HSP90/eNOS molecular coupling in a time-dependent manner. Hypoxia-reoxygenation increased the levels of inflammatory cytokines TNF-α, IL-6, and IL-8 in a time-dependent manner. Emodin treatment recovered PPAR-γ activity and phosphorylation, eNOS phosphorylation, and HSP90/eNOS coupling in HAECs in a concentration-dependent manner, which was reversed by the PPAR-γ inhibitor GW9662, and the eNOS inhibitor, L-NAME. The recovery of HSP90/eNOS coupling by emodin was impaired by GW9662 treatment.

Conclusions: An in vitro hypoxia-reoxygenation (ischemia-reperfusion injury) model of induction of endothelial cell inflammatory mediators showed that emodin recovered the PPAR-γ and eNOS pathway activity.

MeSH Keywords: Emodin • Endothelial Cells • Inflammation • PPAR gamma

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Background

Ischemia-reperfusion injury is sustained and irreversible cell damage resulting from restoration of blood flow following ischemia due to vascular compromise and reperfusion [1]. Multiple events are initiated by reperfusion within a very short period after the restoration of blood flow, which involves complex intracellular signaling transduction and expression of inflammatory mediators. Previous studies have shown that vascular endothelial cells are sensitive and vulnerable to ischemia-reperfusion injury [2]. Also, ischemia-reperfusion injury can induce and enhance the inflammatory responses in many cell types, including endothelial cells [3]. The inflammation associated with ischemia-reperfusion injury is associated with the deterioration endothelial integrity and recruitment of lymphocytes and platelets, which are the initiators of the arterial thrombosis [4]. Therefore, it is accepted that the expression of endothelial inflammatory cytokines and mediators is one of the critical pathological changes in conditions such as acute coronary syndrome (ACS), which leads to myocardial ischemia-reperfusion injury [5].

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is recognized as a factor exerting cardio-protective effects [6]. Several previous studies indicated that ischemia-reperfusion injury could inhibit the activation of the PPAR-γ signaling pathway by reducing the phosphorylation of PPAR-γ [7]. Also, some specific PPAR-γ agonists have been shown to attenuate ischemia-reperfusion cell injury in multiple organs such as brain, liver, heart, and lung, and that the PPAR-γ could further activate endothelial nitric oxide synthase (eNOS), which plays a critical role in maintaining and improving endothelial function in response to harmful stimuli [8]. Furthermore, eNOS was reported to suppress generation of inflammatory cytokines via nitric oxide (NO)-dependent mechanisms [9]. However, the involvement of PPAR-γ in ischemia-reperfusion vascular injury is poorly understood.

Emodin is one of the bioactive components extracted from Rheum palmatum L., which has been used as a medicinal herb in traditional Chinese medicine and has been used in the treatment of both ischemic disease and inflammatory disease for many decades [10]. The effects of emodin on PPAR-γ have been previously described [11,12]. Also, emodin has been found to suppress inflammation in a PPAR-γ-dependent manner [13].

The aim of this study was to investigate the role of emodin, a Chinese herbal medicine, in hypoxia-reoxygenation injury in cultured human aortic endothelial cells (HAECs) and its effects on the PPAR-γ and eNOS signaling pathway. In the current study, the in vitro hypoxia-reoxygenation model was used to simulate ischemia-reperfusion injury, and HAECs were exposed to hypoxia-reoxygenation injury. The expression of endothelial inflammatory cytokines and mediators was examined after emodin was administrated to hypoxia-reoxygenation exposed HAECs. To examine the involvement of the PPAR-γ and eNOS pathway, the effects of specific PPAR-γ and eNOS inhibitors were also studied.

Material and Methods

Cell culture, hypoxia-reoxygenation exposure, and treatments

Human aortic endothelial cells (HAECs) were obtained from the Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured with endothelial basal medium (EBM-2) supplemented with 5% fetal bovine serum (FBS) (Invitrogen) and an antibiotic mixture, in a humidified environment with 5% CO₂ and 95% normal air at 37°C.

Cells at a confluency of 80-90% were used for the subsequent experiments. The protocol of hypoxia-reoxygenation exposure was in accordance with previous studies [14]. Briefly, cultured HAECs were washed three times in phosphate buffered saline (PBS). The original medium was changed with a modified ischemia-mimetic solution (135 mM NaCl; 0.33 mM NaH₂PO₄; 8 mM KCl; 0.5 mM HEPES; 5 mM CaCl₂; 20 mM lactate; pH, 6.8). Cells were then transferred to a hypoxic atmosphere of 5% CO₂, 1% O₂, 94% N₂ and incubated for eight hours. After that, the medium was changed to the original medium and the cells were incubated under normoxia condition for 2 hours. Cells were treated with emodin (Sigma-Aldrich) at different concentrations (0, 5, 10, and 15 μmol/l) for 24 hours. Cells were also co-treated with the peroxisome proliferator-activated receptor-γ (PPAR-γ) inhibitor GW9662 (Sigma-Aldrich) at 5 μmol/l or endothelial nitric oxide synthase (eNOS) inhibitor No-Nitro-L-arginine methyl ester hydrochloride (L-NAME) (Sigma-Aldrich) at 100 μmol/l for 24 hours.

Nitric oxide (NO) production

The NO production in HAECs was determined using the NO-sensitive fluorescence probe diaminofluorescein-FM diacetate (DAF-FM DA) kit (Beyotime) according to the protocol provided by the manufacturer. HAECs were incubated with DAF-FM DA at a final concentration of 5 μmol/l at 37°C in a hypoxic atmosphere of 5% CO₂, 1% O₂, 94% N₂ and incubated for eight hours. After that, the cells were washed three times in PBS. Then the cells were excited at 495 nm and observed at 515 nm with an inverted fluorescence microscope.

Inflammatory cytokine detection

The concentrations inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8 in cell culture medium supernatant were determined by enzyme-linked immunosorbent assay (ELISA) with commercially available immunoassay kits.

Index terms: Peroxisome proliferator-activated receptor; Endothelial inflammation; Nitric oxide; Inflammatory cytokines; Hypoxia-reoxygenation injury; Emodin

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standard deviation (SD). Differences between groups were evaluated by the Student’s t-tests and ANOVA. The posthoc analysis was carried out by NSK tests. Statistical significance was considered to be p<0.05.

Results

Hypoxia-reoxygenation exposure increased the levels of inflammatory cytokines in human aortic endothelial cells (HAECs) and cell culture medium supernatant

The results are shown in Figure 1. The hypoxia-reoxygenation exposure significantly increased expression levels of inflammatory cytokines including tumor necrosis factor (TNF-α), interleukin (IL)-6 and IL-8 in cultured human aortic endothelial cells (HAECs) in a time-dependent manner. Also, the concentrations of these inflammatory cytokines were increased by hypoxia-reoxygenation exposure in the supernatant of the culture medium of HAECs in a time-dependent manner.

Hypoxia-reoxygenation exposure inhibited the activation of peroxisome proliferator-activated receptor-γ (PPAR-γ), endothelial nitric oxide synthase (eNOS), nitric oxide (NO) signaling as well as the PPAR-γ/heat shock protein (HSP)90 interaction in cultured HAECs

The inhibitory effect of hypoxia-reoxygenation exposure on the PPAR-γ/eNOS/NO pathway is shown in Figure 2. The PPAR-γ activity was reduced by hypoxia-reoxygenation exposure in a time-dependent manner. Also, the phosphorylation levels of PPAR-γ and eNOS were also decreased by hypoxia-reoxygenation exposure in a time-dependent manner. The association between HSP90 and eNOS were impaired by hypoxia-reoxygenation exposure. As a result, the NO production in HAECs was reduced by hypoxia-reoxygenation exposure in a time-dependent manner.

Emodin significantly reduced the expression of inflammatory cytokines in HAECs exposed to hypoxia-reoxygenation and culture medium supernatant which was impaired by inhibitors of PPAR-γ and eNOS

The effects of emodin on inflammatory cytokine levels in HAECs exposed to hypoxia-reoxygenation are shown in Figure 3. Emodin decreased the levels of TNF-α, IL-6, and IL-8 in HAECs exposed to hypoxia-reoxygenation and culture medium supernatant in a concentration-dependent manner. However, co-treatment with both the PPAR-γ inhibitor GW6992, and the eNOS inhibitor L-NAME, significantly impaired the inhibitory effects of emodin on inflammatory cytokines production in HAECs exposed to hypoxia-reoxygenation.
Emodin recovered the activation of PPARγ/eNOS/NO signaling in HAECS exposed to hypoxia-reoxygenation, which was impaired by inhibitors of PPARγ and eNOS. As shown in Figure 4A, emodin incubation significantly recovered the PPARγ activity in HAECS exposed to hypoxia-reoxygenation in a concentration-dependent manner, which was impaired by both the PPARγ inhibitor GW6992, and the eNOS inhibitor L-NAME. As shown in Figure 4B, emodin incubation significantly recovered the NO production in HAECS exposed to hypoxia-reoxygenation, in a concentration-dependent manner, which was reversed by PPARγ and eNOS inhibitors. As demonstrated in Figure 4C, emodin treatment increased phosphorylation levels of PPARγ and eNOS in HAECS exposed to hypoxia-reoxygenation in a concentration-dependent manner, which was also impaired by PPARγ and eNOS inhibitors.

**Emodin restored PPARγ - HSP90 interaction in HAECS exposed to hypoxia-reoxygenation that was impaired by inhibitors of PPARγ**

The results were shown in Figure 5. Emodin treatment increased the association between HSP90 and eNOS in a concentration-dependent manner, which was impaired by the PPARγ inhibitor GW6992, rather than eNOS inhibitor L-NAME.

**Discussion**

Ischemia-reperfusion injury is accepted as one of the contributors to many cardiovascular diseases by bringing irreversible damage to the endothelium by inducing cell apoptosis and inflammation [15]. The inflammatory cytokines generated in vivo by ischemia-reperfusion injury would recruit immune cells, which would further participate in exacerbating blood vessel barrier dysfunction by direct inflammatory cell infiltration and promoting thrombosis by activating circulating platelets [16]. Ischemia-reperfusion injury has been defined as the restoration of blood flow after total occlusion of blood vessels, and hypoxia-reoxygenation is accepted as a model that mimics ischemia-reperfusion injury in vitro [17,18].

Inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8 are considered to be typical inflammatory factors in vascular inflammation. TNF-α causes oxidative stress in vascular endothelial cells and activates several pro-inflammatory nuclear factors such as NF-κB, which further induces synthesis of more inflammatory cytokines [19]. IL-6 is involved in increasing vascular permeability under clinical situations that include myocardial infarction and stroke [20]. IL-8 plays a role in platelets aggregation and activation contributing...
Figure 2. Cultured human aortic endothelial cells (HAECs) exposed to hypoxia-reoxygenation and expression of peroxisome proliferator-activated receptor-γ (PPAR-γ) and endothelial nitric oxide synthase (eNOS). (A) Columns indicate the detected optical density (O.D) at 450nm indicating peroxisome proliferator-activated receptor-γ (PPAR-γ) activities in cultured HAECs exposed to hypoxia-reoxygenation for 0, 12, and 24 hours, respectively. (B) The left side shows the captured images of DAF-FM DA fluorescent stain of HAECs. Columns on the right part indicated the fluorescent intensities of DAG-FM DA stain of HAECs exposed to hypoxia-reoxygenation for 0, 12, and 24 hours, respectively. (C) The immunoblots of pPPAR-γ, PPAR-γ, p-eNOS, eNOS and GAPDH in HAECs are shown on the left. Columns on the right show the relative phosphorylation levels of PPAR-γ (white columns) and eNOS (black columns) in cultured HAECs exposed to hypoxia-reoxygenation for 0, 12, and 24 hours, respectively. (D) The immunoblots on the right show the immunoprecipitation analysis of heat shock protein (HSP)90/eNOS interaction in HAECs. Columns on the right indicate the eNOS/HSP90 association (normalized to HSP90) in HAECs exposed to hypoxia-reoxygenation for 0, 12, and 24 hours, respectively. Differences were significant (p<0.05) between a. and b. Differences were significant (p<0.05) between b. and c.
to the formation of thrombosis [21]. In this study, hypoxia-reoxygenation was performed to treat cultured human aortic endothelial cells (HAECs). The results showed that the levels of inflammatory cytokines, including TNF-α, IL-6, and IL-8 in cell culture supernatants of HAECs, and in HAECs exposed to hypoxia-reoxygenation occurred in a time-dependent manner. These results suggested that the vascular inflammatory response was stimulated during ischemia-reperfusion injury.

Nitric oxide (NO) is an important effective small molecule in the cardiovascular system participating in proliferation, apoptosis, and oxidative stress of the vascular endothelium [22]. NO plays a critical role in the development, maintenance, and regulation of the vascular inflammatory status in cardiovascular diseases, including atherosclerosis, stroke, and ischemia-reperfusion injury [23]. The formation of NO is catalyzed by endothelial nitric oxide synthase (eNOS), which is activated by phosphorylation. PPAR-γ has been considered as a nuclear factor exerting anti-inflammatory effects when activated [24]. Loss of PPAR-γ activity might result in elevation of levels of inflammatory cytokines in ischemic diseases such as cerebral ischemia [25]. Also, previous studies have shown that PPAR-γ activation stimulated the synthesis and release of NO, and so eNOS was identified as a downstream molecular target of PPAR-γ [26].

Heat shock protein (HSP)90 has been reported to be involved in the regulation of ischemia-reperfusion as HSP90 binds to eNOS to maintain the catalytic activity of eNOS. This coupling between HSP90 and eNOS is largely dependent on the activity of PPAR-γ [27]. In this study, the hypoxia-reoxygenation exposure was shown to decrease the activity and phosphorylation of PPAR-γ in cultured HAECs in a time-dependent manner, leading to the interruption of molecular coupling between HSP90 and eNOS. The phosphorylation level of eNOS was impaired, which resulted in the reduced production of NO and increased levels of inflammatory cytokines in HAECs. These

Figure 3. Cultured human aortic endothelial cells (HAECs) exposed to hypoxia-reoxygenation, expression of inflammatory cytokines, and treatment with emodin and inhibitors of peroxisome proliferator-activated receptor-γ (PPAR-γ) and endothelial nitric oxide synthase (eNOS). The upper panel of left part of this figure shows the immunoblots of tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8 in cultured human aortic endothelial cells (HAECs) exposed to hypoxia-reoxygenation. Columns in the lower panel of the left part of this figure indicate the relative expression levels (normalized to GAPDH) of TNF-α (light blue columns), IL-6 (deep blue columns) and IL-8 (blue columns) in HAECs exposed to hypoxia-reoxygenation treated with emodin and inhibitors of PPAR-γ/eNOS respectively. Columns on the right part of this figure indicated the detected concentrations of TNF-α (light blue columns), IL-6 (deep blue columns) and IL-8 (blue columns) in culture supernatant of HAECs exposed to hypoxia-reoxygenation co-treated with emodin and inhibitors of PPAR-γ/eNOS respectively. Differences were significant (p<0.05) between a. and b. Differences were significant (p<0.05) between b. and c. Differences were significant (p<0.05) between d. and c. Differences were significant (p<0.05) between e. and d. Differences were significant (p<0.05) between f. and e. Differences were significant (p<0.05) between g. and e.
Figure 4. Cultured human aortic endothelial cells (HAECs) exposed to hypoxia-reoxygenation, expression of inflammatory cytokines, and treatment with emodin and inhibitors of peroxisome proliferator-activated receptor-γ (PPAR-γ) and endothelial nitric oxide synthase (eNOS). (A) Columns show the detected O.D. at 450nm indicating peroxisome proliferator-activated receptor-γ (PPAR-γ) activities in cultured human aortic endothelial cells (HAECs) exposed to hypoxia-reoxygenation co-treated with emodin and inhibitors of PPARγ/endothelial nitric oxide synthase (eNOS), respectively. (B) The left part of this panel demonstrates the captured fluorescent images of diaminofluorescein-FM diacetate (DAF-FM DA) fluorescence staining of HAECs. Columns on the right part of this panel indicated the fluorescent intensities of DAG-FM DA stain of HAECS exposed to hypoxia-reoxygenation co-treated with emodin and inhibitors of PPARγ/eNOS, respectively. (C) The immunoblots of p-PPAR, PPAR, p-eNOS, eNOS and GAPDH are demonstrated on the right part of this panel. Columns on the right part indicate the relative phosphorylation levels of PPAR-γ (light blue columns) and eNOS (deep blue columns) in HAECs exposed to hypoxia-reoxygenation co-treated with emodin and inhibitors of PPARγ/eNOS, respectively. Differences were significant (p<0.05) between a. and b. Differences were significant (p<0.05) between b. and c. Differences were significant (p<0.05) between d. and c. Differences were significant (p<0.05) between e. and d. Differences were significant (p<0.05) between f. and e. Differences were significant (p<0.05) between g. and e.
Emodin, a Chinese herbal medicine, inhibits reoxygenation-induced injury…

Figure 5. Cultured human aortic endothelial cells (HAECs) exposed to hypoxia-reoxygenation and analysis of heat shock protein (HSP90) and endothelial nitric oxide synthase (eNOS). The upper part of this figure demonstrates the immunoprecipitation analysis of HSP90/eNOS in HAECs. Columns on the lower part indicated the eNOS/Hsp90 association (normalized to HSP90) in HAECs exposed to hypoxia-reoxygenation for 0, 12, and 24 hours, respectively. Differences were significant (p<0.05) between a and b. Differences were significant (p<0.05) between b and c. Differences were significant (p<0.05) between d and c. Differences were significant (p<0.05) between e and d. Differences were significant (p<0.05) between f and e.

results showed that hypoxia-reoxygenation induced the inflammatory response in HAECs. Loss of activation of PPAR-γ was critically involved in the process that leads to the impairment of eNOS activity by disrupting the molecular interaction between eNOS and HSP90.

Emodin is one of the bioactive components extracted from *Rheum palmatum* L., which has been used as a traditional Chinese herbal medicine for decades in Eastern Asia [28]. Emodin was reported in previous studies to exert endothelial protective effects in various diseases involving vascular endothelium injuries brought by hyperglycemia, irradiation, and cytotoxic drugs [29,30]. Emodin has also been considered to be an anti-inflammatory agent in infectious diseases, autoimmune diseases and allotransplantation [31,32]. Emodin was also described as an activator of PPAR-γ in previous investigations [13]. In the present study, emodin incubation recovered the activity and phosphorylation level of PPAR-γ in HAECs exposed to hypoxia-reoxygenation in a concentration-dependent manner. Also, the phosphorylation of eNOS was also increased in HAECs exposed to hypoxia-reoxygenation after incubation of emodin. We also found that the emodin incubation increased the molecular interaction between HSP90 and eNOS. As a result, the synthesis and secretion of NO were elevated when treated with emodin, whereas the inflammatory cytokines in HAECs were suppressed in HAECs exposed to hypoxia-reoxygenation. The treatment of inhibitors of PPAR-γ and eNOS impaired the anti-inflammatory effects of emodin on HAECs exposed to hypoxia-reoxygenation. Also, PPAR-γ inhibitor treatment impaired the molecular HSP90/eNOS association rather than eNOS inhibitor treatment. These data indicated that emodin inhibited the expression of endothelial inflammatory cytokines and mediators in a concentration-dependent manner. Recovery of PPAR-γ activity, which reinforced the coupling of HSP90 and eNOS was one of the molecular mechanisms of the anti-inflammatory effects of emodin in ischemia-reperfusion-induced endothelial injury.

This study had several limitations. Firstly, the current study was conducted in vitro. Though the *in vitro* hypoxia-reoxygenation model could simulate the *in vivo* ischemia-reperfusion injury model, the physiological and pathological changes were not the same. Thus, it would be more persuasive if an *in vivo* investigation was conducted. Second, the molecular mechanisms of the effects of eNOS on inflammation were not thoroughly investigated, and it is still unclear whether this effect was NO-dependent or NO-independent. Further studies using NO inhibition methods would be helpful.

Conclusions

The findings of this study showed that emodin, a Chinese herbal medicine had a protective effect on hypoxia-reoxygenation damage in cultured human aortic endothelial cells (HAECs) *in vitro*, by deactivating the peroxisome proliferator-activated receptor-γ (PPAR-γ) and endothelial nitric oxide synthase (eNOS) signaling pathway. This study investigated the possible molecular mechanism of endothelial cell injury due to ischemia-reperfusion and the expression of endothelial cell inflammatory mediators, and the therapeutic effect of emodin. The findings suggest that deactivation of the PPAR-γ and eNOS signaling pathway was critical to the expression of endothelial cell inflammatory cytokines due to hypoxia-reoxygenation in cultured
HAECs. Emodin was shown to recover the PPAR-γ activity in HAECs exposed to hypoxia-reoxygenation, by the molecular interaction of HSP90 and eNOS. As a result, the NO production was increased and the inflammation was suppressed. We believe that our data would not only deepen our current understanding of endothelial injury induced by ischemia-reperfusion injury, but also provide a novel theoretical basis for potential clinical application of emodin in vascular diseases in the future.

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