Propofol inhibits lipopolysaccharide-induced lung epithelial cell injury by reducing hypoxia-inducible factor-1α expression

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Editor’s key points

- Propofol treatment reduced mortality in a mouse model of endotoxaemia.
- Propofol reduced hypoxia-inducible factor (HIF)-1α expression and cytokine expression mediated by lipopolysaccharide in human lung epithelial cells and in mice.
- Inhibition of HIF1α expression may be the molecular mechanism that allows propofol to protect against sepsis-induced lung injury.

Background. Lipopolysaccharide (LPS) may activate hypoxia-inducible factor (HIF)-1α, which up-regulates cytokine expression and the lethality of LPS-induced shock. We investigated the effect of propofol on HIF-1α expression and acute lung injury in LPS-treated mice.

Methods. A series of both positive and negative control experiments were performed. We injected BALB/C mice with propofol or vehicle i.p. immediately and 12 h after an LPS challenge. After 24 h, we examined the lung wet/dry weight ratio, neutrophil infiltration, and HIF-1α mRNA expression and inflammatory cytokines in the lung tissue. Survival was determined for 48 h after LPS injection. In vitro, we determined the responses of A549 cells, with and without HIF-1α silenced, to treatment with LPS alone and LPS plus propofol.

Results. Propofol prolonged survival and attenuated acute lung injury and decreased the expression of HIF-1α, interleukin (IL)-6, keratinocyte-derived chemokine, and tumour necrosis factor-alpha (TNF-α) in the lungs of endotoxaemic mice. In HIF-1α knockdown-A549 cells, LPS-induced TNF-α, IL-6, and the pro-apoptotic gene, BNIP3 expression and apoptosis were reduced. Propofol, but not an inhibitor of nuclear factor κB, reduced HIF-1α expression in LPS-stimulated A549 cells. Propofol also down-regulated, in A549 cells, the expression of IL-6, IL-8, and TNF-α, Bcl-2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), and apoptosis.

Conclusions. Propofol reduces apoptosis in LPS-stimulated lung epithelial cells by decreasing HIF-1α, BNIP3, and cytokine production. Using propofol to inhibit HIF-1α expression may protect against the acute lung injury caused by LPS-induced sepsis.

Keywords: acute lung injury; cytokines; hypoxia-inducible factor-1α; propofol; sepsis

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Septic shock most commonly affects the lung and frequently causes acute lung injury and acute respiratory distress syndrome.1 Apoptosis of lung epithelial cells is important in the pathogenesis of sepsis-induced acute lung injury.2 3 Propofol (2,6-di-isopropylphenol) is a widely used i.v. anaesthetic and sedative agent that is increasingly used to manage traumatic head injury, status epilepticus, delirium tremens, and status asthmaticus, and to sedate critically ill patients so as to facilitate intubation and ventilation.4 Propofol has anti-inflammatory and antioxidant effects: it suppresses the migration, phagocytosis, and oxidative ability of macrophages.5 In addition, it inhibits lipopolysaccharide (LPS)-induced nitric oxide production and biosynthesis of the inflammatory cytokines tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and IL-6 in macrophages.6 Propofol also reduces apoptosis and up-regulates endothelial nitric oxide synthase expression in hydrogen peroxide-stimulated human endothelial cells.7 8 Propofol attenuates endotoxin-induced acute lung injury in a murine model of sepsis.9–11 This protective effect may be via inhibition of the release of proinflammatory cytokines, free radicals, and nitric oxide.9 10 However, the molecular mechanisms are still unclear. Hypoxia-inducible factor (HIF)-1 is a transcription factor which is essential for regulating oxygen homeostasis.12

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It also regulates the expression of target genes important in angiogenesis, erythropoiesis, energy metabolism, and cell survival. HIF-1α is a heterodimer composed of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit. HIF-1α expression is determined by a balance between degradation and synthesis. The regulation of HIF-1α activity occurs at multiple levels: gene expression, intracellular localization of the HIF-1α/β complex, and transcriptional activity of HIF-1α. HIF-1α is constitutively hydroxylated by prolyl hydroxylases (PHDs) in normoxia; this allows it to be recognized by the von Hippel–Lindau ubiquitin ligase, which leads to polyubiquitination and proteasomal degradation.

Under hypoxic conditions, HIF-1α accumulates, translocates into the nucleus, and determines the activity of HIF-1. HIF-1α participates in the transcriptional activation of hypoxia-responsive genes by binding to the hypoxia-response element in the promoter or enhancer region of these genes, and promotes the production of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-12, which reach harmful levels in the host during sepsis. HIF-1α is also activated by LPS and contributes to the cytokine activation and lethality of LPS-induced sepsis.

Propofol blocks the synthesis of the HIF-1α subunit during hypoxia and LPS stimulation in hepatic cells. LPS-induced sepsis may up-regulate HIF-1α, which is critical for cytokine activation and apoptosis, but the involvement of HIF-1α in septic lung epithelial cells is not clear. We hypothesized that LPS-induced HIF-1α expression contributes to lung epithelial cell apoptosis and that propofol protects against LPS-induced lung epithelial cell injury by decreasing HIF-1α expression.

**Methods**

**Animal model**

The animal experiments were done in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were housed in a temperature [25 ± 1°C] and humidity [60 ± 5%] controlled room and kept on a 12:12 light–dark cycle (light on at 06:00 h). Water and standard laboratory diet were ad libitum. To study the effect of propofol on acute lung injury in endotoxemia, we divided 6–8-week-old BALB/c mice into five groups for an LPS challenge. Awake mice in Group 1 (PBS control) received i.p. phosphate-buffered saline (PBS, pH 7.4). Group 2 received i.p. 30 mg kg⁻¹ LPS from *Escherichia coli* B55:5 (Sigma-Aldrich) as previously described. Group 3 (LPS+prop) received LPS plus 50 mg kg⁻¹ propofol immediately and 12 h after LPS injection (Diprivan 1%; Astra Zeneca, London, UK). Group 4 (LPS+InP) received LPS plus an equivalent volume of soyabean-oil emulsion, Intralipid 10% (Baxter Healthcare, Deerfield, IL, USA), immediately and 12 h after the LPS injection. Group 5 (prop) received propofol only (n=6 for all groups). This subhypnentic dosage of propofol used was based on previous studies.

Twenty-four hours after the LPS challenge, the mice were killed by cervical dislocation after brief anaesthesia, blood was collected via cardiac puncture and the lungs were harvested. Tissue specimens were immediately frozen in liquid nitrogen for RNA and protein extraction or fixed in formalin for histological examination. The serum was prepared and stored at −80°C before analysis. Another group of mice underwent the same endotoxic shock experiments and their lungs were harvested for lung-water content measurement by determining the ratio of the wet and dry weight of the lung tissue.

To determine the mortality of LPS-challenged mice with propofol treatment, we injected mice with LPS (30 mg kg⁻¹, i.p.) and then treated with early propofol (50 mg kg⁻¹, i.p., immediately and 12 h after LPS injection) or late propofol (50 mg kg⁻¹, i.p., 3 and 15 h after LPS injection) or equivalent volume of PBS as a vehicle control (n=6 per group). Survival was determined for 48 h after LPS injection.

**Histological examination**

After exsanguination, the lungs were fixed via intratracheal instillation with 10% neutral phosphate-buffered formalin. All specimens were embedded in paraffin. Serial sections (4 μm thick) were processed using haematoxylin and eosin stain. Neutrophil sequestration was assessed by averaging the number of neutrophils counted in 40 randomly selected microscopic fields (×1000) on each slide. Histological sections were evaluated by an examiner blinded to the source of the samples.

**Immunohistochemical staining**

All paraffin-embedded tissues were dewaxed followed by antigen retrieval in 10 mM of sodium citrate at 93°C for 20 min. Immunohistochemistry was done using the mouse anti-HIF-1α monoclonal antibody, diluted 1:100 (Santa Cruz Biotechnology Inc., USA) in blocking reagent at 4°C overnight. The primary antibody was omitted for negative controls. The slides were then treated consecutively with 1:200 horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG (Chemicon International, Inc., Temecula, CA, USA) and incubated for 2 h at room temperature. Slides were then incubated using an AEC substrate kit (Vector Laboratories, Burlingame, CA, USA), and the colour reaction was allowed to develop for 5–10 min. After washing slides were counterstained with Mayer’s haematoxylin (ThermoShandon, Pittsburgh, PA, USA) and mounted.

**Cytokines**

The serum levels of mouse TNF-α and the levels of human TNF-α, IL-6, and IL-8 from culture medium were analysed using ELISA kits (R&D Systems, Minneapolis, MN, USA) as previously described. Results are the means of duplicate assays.
Cell culture and reagents
The human lung epithelial cell line A549 (American Type Culture Collection, Rockville, MD, USA) was maintained in 100 mm dishes with RPMI-1640 medium (Gibco BRL, Life Technologies, Inc., USA) supplemented with 10% (v/v) fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2. Stock solutions of ~100 μM of propofol (Sigma-Aldrich Co., USA) in PBS were prepared by diluting 100 mM of propofol, dissolved in 100% dimethylsulfoxide (DMSO, Sigma-Aldrich). The iron chelator desferrioxamine (DFX) was from Sigma (St Louis, MO, USA). The nuclear factor κB (NFκB) inhibitor (E3-[4-(methylphenyl)sulfonyl]-2-propenenitrile (BAY11-7082) was from Calbiochem (San Diego, CA, USA). Control A549 cells were treated with PBS containing 0.1% DMSO.

Knockdown of HIF-1α gene expression
To determine the effect of HIF-1α on decreasing downstream or related gene expression under LPS stimulation, and whether propofol regulates HIF-1α expression in lung epithelial cells, we silenced HIF-1α gene expression in A549 cells using lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) and a silencing vector (pGIPZ Lentiviral shRNAmir, Thermo Scientific, USA). A pGIPZ non-silencing vector was used for negative controls. After the A549 cells had been incubated for 12 h in the transfection mixture, the medium was changed, and the cells were incubated for another 24 h to allow maximum target gene inhibition. The HIF-1α-silenced A549 cells were then treated and analysed.

Reverse transcriptase–polymerase chain reaction
Total RNA was extracted using a reagent (RNA-Bee, Tel-Test Inc., Friendswood, TX, USA) and subsequently underwent reverse transcription. IL-6, IL-8, keratinocyte-derived chemokine (KC, an IL-8 homologue in mice), TNF-α, HIF-1α, and BNIP-3 (Bcl-2/adenovirus E1B 19 kDa interacting protein 3) were amplified using polymerase chain reaction (PCR) with gene-specific primers (Table 1). PCR products were visualized on 2% agarose gels containing ethidium bromide. 18S-rRNA gene-specific primers (Table 1). PCR products were visualized on 2% agarose gels containing ethidium bromide. Western blotting densities were analysed using the Bio-Profil program and expressed as a fold increase relative to PBS controls.

Apoptosis analysis
Apoptosis was evaluated using the Annexin V Apoptosis Detection kit (BD Biosciences) and the propidium iodide (PI) with flow cytometry. After the indicated treatments, cells were resuspended in binding buffer and incubated in the dark with fluorescein isothiocyanate-conjugated Annexin V and PI for 15 min at room temperature. The cells were analysed using a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, CA, USA) equipped with a doublet discriminating module and Windows Multiple Document Interface (WinMDI) version 2.8 (The Scripps Research Institute, La Jolla, CA, USA). Annexin-V-positive cells were considered apoptotic. Hoechst 33342 staining was also used to assess apoptosis. Culture plates were briefly washed three times with PBS and fixed with 3.7% paraformaldehyde. Hoechst 33342 solution (final concentration 0.002%) was added to the wells for a 30 min incubation. The cells were observed using a fluorescence microscope (IX70; Olympus Taiwan Co., Ltd, Taiwan), and images were collected using the microscope fitted with an Olympus America camera and MagnaFire 2.1 software.

Statistical analysis
SigmaPlot 9.0 (Systat Software Inc., Richmond, CA, USA) was used for all statistical analyses. Data are median and the corresponding inter-quartile range (IQR) or individual raw data points as indicated. Multiple parametric comparisons were

<p>| Table 1 | Primer pairs used in this study. m, mouse; h, human. *The same primer pair was used to amplify the mHIF-1α gene. KC, keratinocyte-derived chemokine; BNIP3, Bcl-2/adenovirus E1B 19 kDa interacting protein 3 |</p>
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analysed using the Bio-Profil program and expressed as a fold increase relative to PBS controls.
performed by one-way analysis of variance, followed by Dunnett’s post hoc test. Comparisons between two groups were performed by Student’s t-test. Comparisons among mortality rates of the groups were made with the Kaplan–Meier and the Mantel–Cox methods. Statistical significance was set at $P<0.05$.

**Results**

**Survival, lung injury, and HIF-1α and cytokine production in LPS-treated mice**

Three hours after the LPS-challenge injections, mice manifested lethargy, piloerception, diarrhoea, huddling, and malaise, clear signs of endotoxic shock. Twenty-four hours post-treatment, body weight was significantly lower, and lung wet/dry weight ratio, serum TNF-α, and lung neutrophil sequestration were significantly higher in the LPS and LPS+InP groups than in the PBS and LPS+prop groups (Fig. 1). Mortality rates 48 h after LPS injection were 83, 17, and 50% for the PBS, early, and late propofol treatment groups, respectively (Fig. 2A). The mortality rates for both early and late propofol groups were lower than the PBS group; the early propofol group had significantly lower mortality than the other groups. Western blotting showed that, compared with the PBS group, HIF-1α expression was significantly higher in the LPS and LPS+InP groups, but compared with the LPS group, HIF-1α expression was significantly lower in the LPS+prop group (Fig. 2a). HIF-1α staining was stronger in lung epithelial cells in the LPS group (arrowheads), but weakly stained in the control (arrows) and LPS+prop (arrows) groups (Fig. 2c). LPS treatment significantly induced lung IL-6, KC, TNF-α, and HIF-1α mRNA expression (Fig. 2b), which was attenuated by propofol treatment (Fig. 2a).

**HIF-1α, cytokine production, and apoptosis in lung epithelial cells**

Western blotting showed that HIF-1α expression was up-regulated in ns-shRNA A549 cells 1 h after LPS treatment (Fig. 3a). Reverse transcriptase–PCR (RT–PCR) showed that the endogenous expression of HIF-1α mRNA was lower in HIF-1α knockdown-A549 cells (HIF-1α shRNA) than in control (ns-shRNA)-A549 cells 8 h after they had been treated with LPS (200 ng ml$^{-1}$) or PBS (Fig. 3b). mRNA expression for HIF-1α, TNF-α, IL-6, and BNIP3 was significantly higher in LPS-treated ns-shRNA-A549 cells than in LPS-treated

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**Fig 1** Body weight (a), lung wet/dry weight ratio (b), serum TNF-α (c), and neutrophil sequestration in lungs (d) in mice ($n=6$) 24 h after LPS. Data are median, IQR, and full range. *$P<0.05$ vs the PBS group; **$P<0.05$ vs the LPS group.
HIF-1α shRNA-A549 cells (Fig. 3B). Inconsistent with mRNA expression, the protein levels of TNF-α and IL-6 in cultured medium of LPS-treated ns-shRNA-A549 cells were higher than of LPS-treated HIF-1α shRNA-A549 cells (Fig. 3C). More apoptosis was seen in ns-shRNA-A549 cells than in HIF-1α shRNA-A549 cells 24 h after LPS treatment (Fig. 3D and E).

**HIF-1α, BNIP3, and cytokine production in LPS-stimulated lung epithelial cells in vitro**

To investigate the possible mechanism whereby propofol reduces apoptosis, we analysed the effect of propofol on HIF-1α expression in A549 cells treated for 2 h with LPS (200 ng ml⁻¹) with or without propofol at the indicated concentrations. HIF-1α protein was significantly lower in cells treated with 50 and 100 μM propofol (Fig. 4A). RT-PCR also showed that IL-6, IL-8, TNF-α, and HIF-1α mRNA expression 4–8 h after LPS treatment (Fig. 5A) and BNIP3 mRNA expression were dose-dependently reduced in propofol-treated cells (Fig. 5A). Protein levels of IL-6, IL-8, TNF-α in culture medium 12 h after LPS treatment were also reduced by the NFκB inhibitor, BAY11-7082, and propofol (Fig. 5C). As LPS up-regulates HIF-1α and cytokines through the TLR4 pathway, we next determined if propofol reduced...
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HIF-1α by inhibiting NFκB activation via TLR4 pathway. Propofol, but not BAY11-7082, reduced HIF-1α in LPS-stimulated A549 cells (Fig. 4a).

We next investigated whether the protective effect of propofol was related to HIF-1α expression. HIF-1α shRNA-A549 cells and ns-shRNA-A549 cells were treated with PBS (containing 0.1% DMSO), LPS (200 ng ml⁻¹), or LPS+propofol (50 μM). The percentage of apoptotic cells after 24 h was significantly higher in all LPS-treated ns-shRNA-A549 cells than in all LPS-treated HIF-1α shRNA-A549 cells. However, it was significantly lower in LPS+propofol-treated ns-shRNA-A549 cells than in LPS-only-treated ns-shRNA-A549 cells (Fig. 6a). In HIF-1α shRNA-cells, the percentage of apoptotic cells was no different between LPS+propofol-treated and

Fig 3  (a) HIF-1α protein in PBS- or LPS-treated ns-shRNA-A549 cells and HIF-1α knockdown-A549 cells. (b) Expression of HIF-1α, TNF-α, IL-6, and BNIP-3 mRNA in PBS- or LPS-treated ns-shRNA-A549 cells and HIF-1α shRNA-A549 cells. (c) TNF-α and IL-6 levels in culture medium from PBS- or LPS (200 ng ml⁻¹)-treated ns-shRNA-A549 cells and HIF-1α shRNA-A549 cells. Apoptosis in PBS- or LPS-treated ns-shRNA-A549 cells and HIF-1α shRNA-A549 cells. (d) Hoechst staining and (e) flow cytometry with Annexin V and PI staining. (All n=3.) Data are individual raw data points. *P<0.05 vs the PBS group; #P<0.05 vs the ns-shRNA group.
LPS-only-treated cells. To confirm whether the protective effect of propofol was mediated by HIF-1α expression, we treated A549 cells with DFX, which up-regulates HIF-1α expression, and examined the effect of propofol on HIF-1α expression and cell injury. HIF-1α expression was lower in A549 cells treated with propofol than in those treated with LPS or DFX alone (Fig. 6B). Moreover, the percentage of apoptosis was lower in cells treated with propofol than in those treated with LPS or DFX alone (Fig. 6C).

Discussion

We found that, in a murine model of endotoxic shock, a sub-hypnotic dose of propofol down-regulated the expression of HIF-1α and the inflammatory cytokines IL-6, IL-8, and TNF-α in lung epithelial cells, and reduced lung injury. In vitro, propofol reduced the expression of HIF-1α and its downstream pro-apoptosis gene, BNIP3, and apoptosis in LPS- and DFX-treated lung epithelial cells. We hypothesize that the mechanism whereby propofol protects LPS-induced acute lung injury, at least in part, is via down-regulation of HIF-1α expression.

Although the effect of propofol on down-regulation of hypoxia-mediated HIF-1α expression in A549 cell was reported previously,26 in our study, we determined the effect of propofol on HIF-1α expression in non-hypoxia-induced lung epithelial cell injury in sepsis. We provide evidence that inhibition of HIF-1α expression may be the molecular mechanism by which propofol protects against acute lung injury in endotoxic shock. To be more relevant to the clinical setting, we investigated the outcome of propofol treatment in LPS-treated mice and found differing survival outcomes depending on the timing of propofol treatment after LPS injection. This result agrees with Taniguchi and colleagues,27 where early post-treatment with propofol resulted in a lower mortality rate and cytokine production than late post-treatment. Whether the different times of propofol treatment after endotoxaemia would have different effects on HIF-1α activity awaits further investigation.

The propofol administered in vivo in our study was prepared in a lipid emulsion. There are several reports that lipids are immunosuppressants,28 although other studies of animal models of sepsis suggest that propofol but not Intralipid decreased oxidative injury.10 29 30 We also found that Intralipid did not reduce acute lung injury or HIF-1α expression in LPS-challenged mice.

We found that a high level of HIF-1α expression was concomitant with alveolar epithelial cell injury in LPS-treated mice, and that LPS-induced HIF-1α contributed to the up-regulation of cytokine and BNIP3 expression and to apoptosis in A549 cells. Others have reported that HIF-1α is involved in ATP production, cell proliferation, and apoptosis in alveolar epithelial cells,31 and that, in hypoxic A549 cells, HIF-1α promotes activity of the glycolysis pathway and
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Propofol reduces HIF-1α expression in LPS-stimulated ns-shRNA-A549 cells but not in LPS-stimulated HIF-1α shRNA-A549 cells. Thus, HIF-1α production contributed to LPS-induced apoptosis of lung epithelial cells, which was attenuated by propofol treatment. However, in HIF-1α knockdown cells, LPS-alone induced less apoptosis, and propofol did not further reduce LPS-induced HIF-1α production and apoptosis. Taking all these findings together, we hypothesize that the mechanism of the protective effect of propofol on LPS-induced lung epithelial cell injury, at least in part, is the down-regulation of HIF-1α expression.

LPS-induced HIF-1α transcription has been shown to be independently regulated through NFκB activation via TLR4 and is now believed to be a putative pathway for the non-hypoxia-dependent up-regulation of HIF-1α seen in the context of sepsis. Previous studies also showed that inhibition of NFκB attenuated LPS-induced NFκB-dependent gene expression but did not affect HIF-1α-dependent gene expression. In this study, the effect of the NFκB inhibitor attenuated LPS-induced cytokine production, but had no effect on HIF-1α expression. In addition, propofol, but not the NFκB inhibitor, reduced HIF-1α expression in LPS-stimulated A549 cells, which was consistent with previous studies. According to these observations, LPS-induced HIF-1α expression in lung epithelial cells is not regulated via...
Although we found that propofol down-regulated LPS-induced cytokines and HIF-1α mRNA expression in lung epithelial cells, in a hepatic cell hypoxia model, propofol inhibited HIF-1α accumulation but did not influence HIF-1α mRNA expression. The report suggested that hypoxia-induced HIF-1α expression was suppressed by the translation of HIF-1α mRNA into protein because propofol is reported to suppress MAPK activity, which is critical for regulating the rate of HIF-1α translation. This discrepancy may be attributable to the different cell types used, to the fact that hypoxia and LPS have different regulatory effects on the HIF-1α gene, or both.

Finally, we found that the expression of HIF-1α and the BNIP3 gene, an HIF-1α target gene that activates apoptosis, was higher, as was apoptosis, in ns-shRNA-A549 cells but not HIF-1α shRNA-A549 cells after LPS stimulation. This may mean that increased HIF-1α and BNIP3 expression is an apoptotic pathway in LPS-treated lung epithelial cells.

In summary, propofol inhibited LPS-induced up-regulation of HIF-1α expression in lung epithelial cells. LPS-induced HIF-1α expression exacerbates acute lung epithelial cell injury in sepsis. HIF-1α expression is associated with the expression of the BNIP3 gene and the proinflammatory cytokines IL-6, IL-8, and TNF-α, which leads to apoptosis in LPS-stimulated lung epithelial cells. The inhibition of HIF-1α expression may be the molecular mechanism that allows propofol to protect against acute lung injury in endotoxic shock.

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Conflict of interest

None declared.

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