Propofol selectively inhibits nuclear factor-κB activity by suppressing p38 mitogen-activated protein kinase signaling in human EA.hy926 endothelial cells during intermittent hypoxia/reoxygenation

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Abstract. Intermittent hypoxia/reoxygenation (IHR) induces proinflammatory cytokines, contributing to the pathogenic process of atherosclerosis associated with obstructive sleep apnea (OSA). Two transcription factors, nuclear factor-κB (NF-κB) and hypoxia-inducible factor-1 (HIF-1), have been indicated to mediate proinflammatory cytokines during IHR. The anti-inflammatory effects of propofol have attracted increasing attention in regard to the treatment of multiple diseases associated with inflammation. The present study examined whether propofol inhibits NF-κB and HIF-1 activity in vascular endothelial cells during IHR. EA.hy926 endothelial cells were exposed to IHR for 64 cycles with or without propofol treatment. Gene knockdown by transfection of siRNA against p38 mitogen-activated protein kinase (MAPK) was also used to investigate the molecular mechanisms. Compared with the control group, IHR exposure significantly induced the activation of NF-κB and HIF-1, enhanced the mRNA expression of proinflammatory cytokines and increased the activation of p38 MAPK. Propofol dose-dependently inhibited the IHR-induced activation of NF-κB, but not HIF-1, which was accompanied by decreased levels of proinflammatory cytokines. In addition, IHR-induced p38 MAPK activity was attenuated by propofol in a similar manner to the reduction in NF-κB activity. Furthermore, knockdown of p38 MAPK with siRNA significantly reduced the IHR-induced activation of NF-κB, while not affecting HIF-1. These data demonstrate that propofol selectively attenuates the IHR-induced activation of NF-κB, but not HIF-1, in vascular endothelial cells, and these beneficial effects are likely to be based on the inhibition of the p38 MAPK signaling pathway. Propofol may have the potential to prevent atherosclerosis in patients with OSA by inhibiting NF-κB-mediated inflammation in the vascular endothelium.

Introduction

Obstructive sleep apnea (OSA) is characterized by intermittent hypoxia/reoxygenation (IHR) as a result of repetitive episodes of complete or partial obstructions of the upper airway during sleep. IHR is an independent risk factor for the development of coronary and cerebral vascular diseases, two common consequences of atherosclerosis (1-3). The mechanisms by which hypoxic signaling accelerates the initiation and progression of atherosclerosis have yet to be fully elucidated.

Nuclear factor-κB (NF-κB) is a transcription factor that has crucial roles in inflammation, immunity, cell proliferation and apoptosis (4-6). Activation of NF-κB is controlled by the inhibitor of κB (I-κB), which retains NF-κB in the cytoplasm (7). Emerging evidence has revealed that the activation of NF-κB in the endothelium may contribute to the pathogenic process of atherosclerosis associated with IHR (8,9). NF-κB-mediated inflammatory pathways have integrated roles in classic atherosclerosis induced by a high-cholesterol diet (9). Patients with OSA have increased NF-κB activity in circulating neutrophils and monocytes, and elevated serum levels of NF-κB-dependent gene products (10-12). Furthermore, using an in vitro model in cultured cells exposed to repetitive hypoxia/reoxygenation, previous studies have demonstrated a selective and dose-dependent activation of NF-κB compared with adaptive hypoxia-inducible factor-1 (HIF-1)-dependent pathways, and the p38 mitogen-activated protein kinase (MAPK) signaling pathway is believed to mediate the activation of NF-κB during IHR (7).

Propofol (2,6-diisopropylphenol) is a potent intravenous hypnotic agent widely used for the induction and maintenance of anesthesia. In addition, propofol exhibits anti-inflammatory properties by decreasing the production of proinflammatory cytokines, altering the production of nitric oxide and...
inhibiting neutrophil function (13). A study reported that propofol inhibited the activation of p38 MAPK to exert its anti-inflammatory effect (14). However, it remains to be elucidated whether propofol inhibits NF-κB and HIF-1 activity in the vascular endothelial cells during IHR. In the present study, an in vitro model of human vein endothelial cells was employed to mimic IHR and evaluate the effects of propofol on the IHR-induced activation of NF-κB and HIF-1 and their molecular mechanisms.

Materials and methods

Cell culture and IHR. The human endothelial cell line EA.hy926, generated from a fusion of primary human umbilical vein endothelial cells (HUVECs), was purchased from the Cell Library of Shanghai Institutes for Biological Sciences (Shanghai, China). The EA.hy926 cells were grown and maintained in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and kept in a humidified incubator at 5% CO₂. For all experiments, the cells were grown to 50-70% confluency and starved for 1 day in 1% FBS medium prior to the different treatments. IHR exposure was performed in a computer-controlled incubator chamber connected to a BioSpherix OxyCycler (Biospherix, Redfield, NY, USA), as previously described (8). The cells were maintained at 37°C and 5% CO₂ in the hypoxic chamber, in which the O₂ levels were shifted between 1% for 10 min and 21% for 5 min. The cells were exposed to IHR for 64 cycles based on a previous study in which such cycles of IHR were determined to sufficiently induce the activation of NF-κB in HUVECs (8). The cells in the control group were maintained in normoxic conditions at 21% O₂ and 5% CO₂.

Propofol treatment. Pure propofol was purchased from Sigma-Aldrich (St. Louis, MO, USA) to exclude the effect of lipid emulsion. Prepared propofol was added to the medium at different concentrations of 0, 25, 50 or 100 µM for 30 min prior to IHR exposure, and kept in the medium throughout the entire experiment. This dose range for propofol was selected from a previous study and considered as the range of concentrations that was clinically relevant (15). Cells that were not treated with either propofol or IHR were used as a control. At the end of the experiments, a luciferase reporter assay, western blot analysis or quantitative polymerase chain reaction (qPCR) were performed, as described below.

Knockdown of p38 MAPK by siRNA. siRNA specifically targeting p38 MAPK and a control siRNA were purchased from Dharmacon (Lafayette, CO, USA). The cells were grown to 50% confluence in antibiotic-free medium, and p38 siRNA or control siRNA were transfected using Lipofectamine® transfection reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instructions. The cells were incubated at 37°C for 48 h to allow for maximal knockdown of the target gene and then exposed to the indicated cycles of IHR. A luciferase reporter assay or western blot analysis was performed, as described below.

Luciferase reporter assays for NF-κB and HIF-1 activity. Luciferase reporter assays were conducted, as described previously (7). Briefly, the cells were grown to 50-70% confluence on culture dishes and transiently transfected with NF-κB or HIF-1 luciferase reporter constructs and then co-transfected with a constitutively active Renilla luciferase reporter construct (pSV40-Renilla; Promega Corp., Madison, WI, USA). At 24 h post-transfection, the cells were lysed in luciferase cell lysis buffer (Promega Corp., Fitchburg, WI, USA). The luciferase activity was assessed by addition of an excess of luciferin/adenosine triphosphate (Promega Corp., Fitchburg, WI, USA) and luminometry (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). All the luciferase reporter values were normalized to the Renilla luciferase activity and expressed as a fold induction relative to the control group.

Western blot analysis. Cell homogenates were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked in Tris-buffered saline Tween-20 (TBST) with 5% skimmed milk and incubated overnight with primary rabbit polyclonal anti-IκBα, mouse monoclonal anti-phosphorylated IκBα (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), rabbit monoclonal anti-p38 MAPK, rabbit monoclonal anti-phosphorylated p38 MAPK (1:1,000 and 1:500, respectively; Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse monoclonal anti-β-actin (1:200; Santa Cruz Biotechnology, Inc.) antibodies. Next, the membrane was incubated for 1 h with secondary antibodies diluted with TBST. The signals of the detected proteins were visualised by an enhanced chemiluminescence (ECL) reaction system (Millipore, Billerica, MA, USA) and quantified by ImageJ software. (National Institutes of Health, Bethesda, MD, USA).

qPCR. Total RNA was prepared from the cells that remained in the wells by extraction with TRizol® reagent (Invitrogen, Carlsbad, CA, USA) and purification using an RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer’s instructions. RNA with an A260/A280 ratio between 1.8 and 2.0 was used for reverse transcription using the qScript cDNA kit (Quanta BioSciences, Gaithersburg, MD, USA). qPCR was performed in the ABI prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) using SYBR® Green (SuperArray Bioscience, Valencia, CA, US). The primer sequences were as follows: Tumor necrosis factor α (TNF-α) forward, 5'-CGAGTGACAGCAGGCTGAC-3' and reverse, 5'-GGTGTGGTGAGGAGCACAT-3'; interleukin-1β (IL-1β) forward, 5'-AACAGATGAAGTCTGCTTGC-3' and reverse, 5'-TGGAGAACACCACTTGTTGCTCCA-3'; interleukin-6 (IL-6) forward, 5'-AAATGCCAGCCTGAGCAAC-3' and reverse, 5'-AACAAACTCTGAGGTTGCCCATGCTAC-3'; and GAPDH forward, 5'-GGTGCTCAGCCTCAGCAGCAGC-3' and reverse, 5'-GGTGCTGCTTGTGGAATCTCA-3'. The values were normalized to GAPDH and the final concentration of mRNA was calculated using the formula x = 2^(-ΔΔCt), where x is the fold difference relative to the control.

Statistical analysis. Values are expressed as the mean ± standard error from at least three independent experiments. Each treatment was performed in the triplicate culture wells.
Statistically significant values were tested by one-way analysis of variance followed by Bonferroni's post-hoc test for multiple comparisons. P<0.05 was used to indicate a statistically significant difference.

Results

Effects of propofol on IHR-induced NF-κB and HIF-1 activity. Using luciferase reporter assays, the NF-κB and HIF-1 signaling pathway activity in HUVECs was measured in response to propofol treatment. As shown in Fig. 1, 64 cycles of IHR significantly induced the activation of NF-κB and HIF-1 compared with the control group. Propofol at 25 and 50 µM dose-dependently inhibited the NF-κB activity compared with IHR alone. High-dose propofol (100 µM) did not further reduce NF-κB activity (Fig. 1A). By contrast, neither a high nor a low dose of propofol affected the IHR-induced HIF-1 activity (Fig. 1B).

To examine whether propofol inhibited the activation of NF-κB through regulating its inhibitor, IκBα, in the cytoplasm, the protein levels of total and phosphorylated IκBα were assessed (Fig. 2). IHR alone caused a significant increase in IκBα phosphorylation (Fig. 2B) and a decrease in the total IκBα levels (Fig. 2C), and these changes were prevented by propofol in a dose-dependent manner.

Effects of propofol on IHR-induced proinflammatory cytokines. Due to NF-κB being a key component in the regulation of inflammation, the present study examined whether propofol treatment reduces IHR-induced expression of proinflammatory cytokines in HUVECs. qPCR demonstrated that the mRNA expression of the proinflammatory cytokines TNF-α, IL-1β...
and IL-6, were markedly enhanced by IHR compared with the control group (Fig. 3). Propofol at 50 or 100 µM significantly inhibited the increase of mRNA expression of TNF-α, IL-1β and IL-6 in the HUVECs exposed to IHR.

**Effects of propofol on p38 MAPK.** It was hypothesized that propofol attenuates the IHR-induced activation of NF-κB and its downstream proinflammatory cytokines by inhibiting p38 MAPK signaling. To test this hypothesis, the protein levels...
of total and phosphorylated p38 MAPK were determined by western blot analysis (Fig. 4). Compared with the control group, IHR increased the protein levels of phosphorylated p38 MAPK (Fig. 4B). Propofol at 25 and 50 µM dose-dependently reduced the protein levels of phosphorylated p38 MAPK in the HUVECs exposed to IHR, and high-dose propofol (100 µM) had no further effect on the phosphorylation of p38 MAPK. By contrast, the protein levels of total p38 MAPK were not affected by either IHR or propofol among the groups (Fig. 4C).

Effects of p38 siRNA on IHR-induced NF-κB and HIF-1 activity. To further confirm that propofol selectively reduces the NF-κB activity through inhibition of p38 MAPK signaling, the effects of p38 MAPK knockdown with siRNA were examined on the IHR-induced NF-κB and HIF-1 activity. Compared with the control siRNA, p38 siRNA effectively produced a knockdown of p38 MAPK by 84% in the HUVECs (Fig. 5), which was accompanied by a significant reduction in the IHR-induced activation of NF-κB (Fig. 6A), but not HIF-1 (Fig. 6B).

Discussion

The novel findings of the present study are summarized as follows: i) Propofol selectively inhibited the activation of NF-κB, but not HIF-1, in the HUVECs during IHR; ii) the reduced activation of NF-κB by propofol was accompanied by decreases in the levels of proinflammatory cytokines; and iii) the inhibitory effect of propofol on IHR-induced NF-κB activity was likely be based on the suppression of the p38 MAPK signaling pathway.

IHR is a hallmark feature of OSA and is associated with atherosclerosis, which is a chronic inflammatory disease involving a plethora of cell types and multiple pathological processes (16,17). Atherogenesis is triggered by vascular endothelial dysfunction that is characterized by a pro-inflammatory state of the endothelium (18,19), leading to endothelial apoptosis (19,20). NF-κB is a well-known redox-sensitive transcription factor involved in numerous pathological conditions, including inflammatory processes and cell apoptosis. In resting cells, NF-κB is predominantly localized in the cytoplasm in a complex with I-κB, which undergoes phosphorylation, ubiquitination and degradation upon stimulation, leading to the translocation of NF-κB into the nucleus followed by transcription of a battery of genes (21). Increasing evidence suggests that IHR may induce the activation of NF-κB and the release of proinflammatory cytokines. In addition to NF-κB, HIF-1, a transcription factor that is essential for regulating oxygen homeostasis, also regulates the expression of target genes, including proinflammatory cytokines (22). HIF-1 is a heterodimer composed of an oxygen-regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit. Under hypoxic conditions, HIF-1α accumulates, translocates into the nucleus and determines the activity of HIF-1, which promotes the production of inflammatory cytokines. HIF-1 activity has been shown to be regulated through the NF-κB pathway (22). In the present study, 64 cycles of IHR significantly induced activation of NF-κB and HIF-1 in the HUVECs, accompanied by increased mRNA expression of TNF-α, IL-1β and IL-6. These results were consistent with previous findings in vivo and in vitro, demonstrating that NF-κB and HIF-1 are activated or produced in response to IHR and contribute to the expression...
of proinflammatory cytokines. Thus, inhibition of the proinflammatory cytokines by suppressing NF-xB or HIF-1 activity in the vascular endothelium may be crucial to prevent atherosclerosis in patients with OSA.

The anti-inflammatory effects of propofol, an intravenous general anesthetic agent, have attracted attention in the studies of multiple diseases associated with inflammation. An in vitro study has demonstrated that propofol post-conditioning inhibits the activation of NF-xB induced by hypoxia/reoxygenation and protects cardiomyocytes against apoptosis (15). Other studies reported that propofol suppresses the activation of HIF-1 induced by lipopolysaccharides or hypoxia in macrophages (24), alveolar epithelial cells (25) and lung epithelial cells (22). However, the effects of propofol on NF-xB and HIF-1 activity in vascular endothelial cells subjected to IHR have not been previously assessed. In the present study, using HUVECs, propofol was demonstrated to dose-dependently inhibit IHR-induced NF-xB activity by suppressing the phosphorylation of I-xBα. However, propofol did not change the HIF-1 activity. In addition, the reduced NF-xB activity caused by propofol was accompanied by decreased mRNA expression of proinflammatory cytokines. These observations indicate that the anti-inflammatory effects of propofol in HUVECs during IHR are mainly due to the inhibition of the NF-xB pathway, which may have a dominant role over the HIF-1 pathway in regulating proinflammatory cytokines in the vascular endothelial cells. A previous study revealed that inhibition of the NF-xB pathway resulted in reduced HIF-1 activity in mouse embryonic stem cells, indicating that HIF-1 is downstream of the NF-xB pathway (26). Furthermore, propofol, at a similar dose range to that used in the present study, was reported to inhibit NF-xB and HIF-1 activity induced by lipopolysaccharides in lung epithelial cells (22). The discrepancy between the current data and previous findings may be due to the different cell types or methods used for studying the activation of NF-xB and HIF-1.

p38 MAPK is critical for the production of NF-xB-dependent proinflammatory cytokines. A previous study demonstrated that IHR activated NF-xB via activation of the p38 MAPK signaling pathway. The hypothesis that propofol may reduce IHR-induced NF-xB activity in HUVECs by suppressing p38 MAPK activity was tested. The data of the present study revealed that the phosphorylation of p38 MAPK was dose-dependently inhibited by propofol in a similar manner to the reduction in NF-xB activity. Furthermore, the knockdown of p38 MAPK with p38 siRNA led to a significant reduction in IHR-induced NF-xB activity. Together, these results indicate that the reduced NF-xB activity caused by propofol acts through the inhibition of the p38 MAPK signaling pathway in HUVECs exposed to IHR. Indeed, propofol has been identified to attenuate the lipopolysaccharide-induced production of proinflammatory cytokines and monocyte chemotactic protein-1 by inhibiting the phosphorylation of p38 MAPK in human THP-1 cells (14).

In conclusion, the present study demonstrated that propofol attenuates the IHR-induced activation of NF-xB, but not HIF-1, in vascular endothelial cells, and that these beneficial effects are possibly based on the inhibition of the p38 MAPK signaling pathway. Propofol may have the potential to prevent atherosclerosis in patients with OSA by inhibiting NF-xB-mediated inflammation in the vascular endothelium.

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References


