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ORIGINAL ARTICLE

The intravenous anesthetic propofol inhibits lipopolysaccharide-induced hypoxia-inducible factor 1 activation and suppresses the glucose metabolism in macrophages

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Abstract
Purpose Hypoxia-inducible factor 1 (HIF-1) is a master transcription factor of hypoxia-induced gene expression. Anesthetics and perioperative drugs have been reported to affect HIF-1 activity. However, the effect of propofol on HIF-1 activity is not well documented. In this study, we investigated the effect of propofol on HIF-1 activation using macrophage-differentiated THP-1 cells.
Methods Cells were exposed to lipopolysaccharide (LPS) under 20 or 1% O_2 conditions with or without propofol treatment. The cell lysate was subjected to Western blot analysis using anti-HIF-1α and HIF-1β antibodies. HIF-1-dependent gene expression was investigated by quantitative real-time reverse-transcriptase PCR analysis and luciferase assay. The amount of cellular lactate and ATP was assayed.
Results Propofol suppressed HIF-1α protein accumulation induced by LPS, but not by hypoxia in the THP-1 cells in a dose-dependent manner by inhibiting the neo-synthesis of HIF-1α protein. Induction of the HIF-1 downstream gene expression including glucose transporter 1, enolase 1, lactate dehydrogenase A, pyruvate dehydrogenase kinase-1 and vascular endothelial growth factor was inhibited by propofol. Propofol suppressed LPS-induced lactate accumulation and ATP content in THP-1 cells.
Conclusion Our experimental results indicate that propofol inhibits HIF-1 activation and downstream gene expression induced by LPS and suppressed HIF-1-dependent glucose metabolic reprogramming. HIF-1 suppression by propofol in macrophages may explain molecular mechanisms behind the inhibitory effect of propofol on cellular inflammatory responses.

Keywords Propofol · Hypoxia-inducible factor 1 · Macrophage · Glucose metabolism

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Introduction

Propofol (2,6-diisopropylphenol) is used not only for induction of general anesthesia, but also for patient sedation in the perioperative period [1]. On the other hand, long-time continuous administration of propofol is associated with an increase in the incidence of side effects, including suppression of cellular inflammatory responses [2, 3]. Studies on human neutrophils and leukocytes demonstrated that propofol has immunomodulatory effects. It is reported that suppression of nuclear factor κB (NF-κB) activity by propofol is one of the molecular mechanisms behind the suppression [3]. Moreover, a report describes that propofol suppresses macrophage functions including chemotactic and phagocytic activities [4]. However, the molecular mechanisms behind the inhibitory effect of propofol on cellular inflammatory responses are largely unknown.

Hypoxia induces expression of a select set of genes encoding glycolytic enzymes and glucose transporters that generate ATP under specific conditions such as anaerobic circumstances [5]. At the cellular level, the adaptation includes a switch of energy metabolism from oxidative

phosphorylation to anaerobic glycolysis, increased glucose uptake and the expression of stress proteins related to cell survival and death. At the molecular level, the adaptation involves changes in gene expression. The transcription factor hypoxia-inducible factor 1 (HIF-1) plays an essential role in the maintenance of O_2 homeostasis [6, 7]. HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β subunits that bind to specific regulatory sequences known as hypoxia response elements (HREs) [8].

We previously reported that the volatile anesthetic halothane inhibits the hypoxia-induced HIF-1 activation by distinct molecular mechanisms [9]. We also indicated the local anesthetics including lidocaine and bupivacaine or opioid receptor agonists do not affect HIF-1 activity within the clinically relevant dosage [10, 11]. On the other hand, isoflurane and xenon are reported to activate HIF-1 even under 20% O_2 conditions [12–14]. In addition, we previously reported that propofol reversibly inhibits HIF-1 activity and the gene expression mediated by HIF-1 by blocking the synthesis of the HIF-1α subunit under 20 or 5% O_2 conditions, but not under 1% O_2 conditions in the kidney-derived cell line HEK293 cells and primarily cultured human umbilical vein endothelial cells, and that the effect is not dependent on its hypnotic effect [15]. We also reported that LPS promotes HIF-1 activation by enhancing both HIF-1α protein expression through a translation-dependent pathway and HIF-1α transcriptional activity in THP-1 human myeloid cells that have undergone macrophage differentiation, but not in undifferentiated monocytic THP-1 cells by increasing the neosynthesis of HIF-1α protein in a reactive oxygen species (ROS)-dependent manner [16, 17]. Based on the evidence, we investigated the effects of propofol on HIF-1 activation in macrophages and the subsequent cellular events.

Materials and methods

Cell culture and reagents

THP-1 human myeloid leukemia cells (a gift from Dr. Kume at Kyoto University) were maintained in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 g/ml streptomycin. To promote differentiation of THP-1 cells to macrophage, cells were exposed to phorbol-12-myristate-13-acetate (PMA) at a final concentration of 50 nM for 12 h. PMA, lipopolysaccharide (LPS) from *E. coli* 055:B5 and 2-deoxyglucose (2-DG) were obtained from Sigma (St. Louis, MO, USA) [16, 17]. 2,6-Diisopropylphenol (propofol) and its isomer 2,4-diisopropylphenol and DMSO as solvent were obtained from Sigma [15]. The inhibitor of protein synthesis cycloheximide (CHX) and the cell-permeable proteasome inhibitor Z-Leu-Leu-Leu-al (MG132) were from Calbiochem (San Diego, CA, USA).

In our pilot studies, no significant cell death was observed at a variety of time points and doses of LPS and propofol using trypan blue exclusion dye assay.

Hypoxic treatment

Cells were maintained in a multi-gas incubator (APMW-36, Astec, Japan) and were exposed to hypoxia (1% O_2–5% CO_2–94% N_2) at 37°C [16, 18].

Immunoblot assays

Whole cell lysates were prepared using ice-cold lysis buffer [0.1% SDS, 1% NP40, 5 mM EDTA, 150 mM NaCl, 50 mM Tris–Cl (pH 8.0)] containing 2 mM dithiothreitol, 1 mM Na_3VO_4 and Complete Protease Inhibitor (Roche Diagnostics, Basel, Switzerland). Samples were centrifuged at 10,000×*g* to pellet cell debris. For HIF-1α and HIF-1β, 100-μg aliquots were fractionated by 7.5% SDS-polyacrylamide gel electrophoresis and subjected to immunoblot assay using mouse monoclonal antibodies against HIF-1α (BD Biosciences, San Jose, CA, USA) and HIF-1β (BD Biosciences) at 1:1,000 dilution, or β-actin (Sigma) at 1:2,000 dilution and HRP-conjugated sheep antibody against mouse IgG (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) at 1:2,000 dilution. A chemiluminescent signal was developed using ECL reagent (GE Healthcare Bio-Science Corp.) [19, 20].

Quantitative real-time reverse-transcriptase PCR (qRT-PCR) analysis

RNA was purified using RNeasy (Qiagen Inc., Madison, WI, USA) and treated by DNase. First-strand synthesis and real-time PCR reactions were performed using QuantiTect SYBR Green PCR Kit (Qiagen Inc.) following a protocol provided by the company. PCR reaction and detection were performed using Applied Biosystems 7300 real-time PCR system (Foster City, CA, USA). PCR primers were purchased from Qiagen Inc. The fold change in expression of each target mRNA relative to 18S rRNA was calculated [21].

Reporter gene assays

Transfection of THP-1 cells was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) following a protocol provided by the manufacturer. Plasmid p2.1 contains a 68-bp hypoxia response element (HRE) from the *ENO1* gene inserted upstream of an SV40 promoter in the luciferase reporter

plasmid pGL2-Promoter (Promega Inc.) [18]. Plasmid pVEGF-*KpnI* contains nucleotides −2274 to +379 of the human *VEGF* gene inserted into the luciferase reporter pGL2-Basic (Promega Inc.) [18]. The reporter gene plasmid and the control plasmid pRL-SV40 (Promega Inc.) containing a SV40 promoter upstream of *Renilla reniformis* luciferase coding sequences were pre-mixed and used. After treatment, the cells were harvested, and the luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega Inc.). The ratio of firefly to *Renilla* luciferase activity was determined. Normalized mean count ± SD of three independent transfections is shown as relative luciferase activity (RLA) [17].

Lactate assay

Conditioned medium from triplicate macrophage cell cultures was harvested and assayed for lactate content by colorimetric detection (Sigma) according to the manufacturer's instructions. Sample values were calculated from a lactate standard curve and normalized to cell lysate protein content [17, 22].

Measurement of intracellular ATP

Cells were harvested, and cellular ATP content was determined using the CellTiter-Glo luminescent cell viability assay (Promega Inc.). Briefly, THP-1 cells were seeded in duplicate at 1×10^6 in 6-cm dishes. Cells were treated with indicated reagents. Cells were harvested on ice following two washes with ice-cold PBS, scraped into ice-cold PBS and then spun at 1,000×*g*. Cells were resuspended in a Tricine buffer. The diluted cells were immediately lysed for 5 min at room temperature with the cell lysis reagent. Following lysis, the whole-cell extract was used to measure luciferase activity. In order to normalize the ATP to cellular protein per sample, the protein content of each cell extract was determined by a Bradford assay. The molar amount of ATP corresponding to each sample was determined based on ATP standards versus the relative luciferase units. Next, the molar amount of ATP per microgram of protein produced by each cell line was determined for each experiment ($n = 5$ samples/cell line/treatment over the course of three experiments). Following deletion of the highest and lowest statistical outliers, these data ($n = 3$) were averaged for each cell line, and the average free ATP values ± SD were determined [17, 22].

Data analysis

All the experiments were done at least three times (unless mentioned otherwise), and representative blots are shown. Data were collected and expressed as mean ± SD. Significance test (one-way ANOVA followed by Dunnett's post hoc test) was performed using the Prism version 4 application.

Results

Effect of propofol on HIF-1α and HIF-1β protein expression

To examine the effect of propofol on HIF-1 activity, macrophage-differentiated THP-1 cells were treated with or without propofol under 20% O_2 or 1% O_2 conditions for 4 h. HIF-1α protein levels were low under 20% O_2 conditions, and increased markedly in response to LPS treatment or 1% O_2 conditions (Fig. 1a, lanes 2 and 4); 50 μM of propofol did inhibit the LPS-induced expression of HIF-1α under 20% O_2 conditions (lane 3). In contrast, hypoxic induction of HIF-1α protein accumulation was not suppressed by propofol treatment (lane 5). Expression of HIF-1β or β-actin was not suppressed either by hypoxic treatment or by propofol.

To investigate if the effect of the propofol on HIF-1α protein accumulation is concentration-dependent or not, THP-1 cells were treated by LPS under 20% O_2 for 4 h with 50 or 100 μM propofol and 100 μM 2,4-diisopropylphenol. Propofol inhibited induction of HIF-1α protein expression in a dose-dependent manner. 2,4-Diisopropylphenol also inhibited HIF-1α protein induction (Fig. 1b).

Next, the effect of propofol on HIF-1α protein synthesis was examined. Exposure of cells to the proteasome inhibitor Z-Leu-Leu-Leu-al (MG132) causes cells to accumulate HIF-1α protein (Fig. 1c, lane 2) because MG132 inhibits the proteasome activity; 100 μM propofol (lane 4) as well as the translation inhibitor cycloheximide (CHX) blocking ongoing protein synthesis (lane 3) inhibited the accumulation of HIF-1α induced by MG132 treatment.

Effect of the propofol on HIF-1-dependent gene expression

We investigated the effect of propofol on LPS-induced HIF-1-mediated gene expression in macrophage-differentiated THP-1 cells. The mRNA expression of genes was assayed using quantitative real-time RT-PCR technique. LPS treatment under 20% O_2 conditions or exposure to 1% O_2 conditions statistically significantly induced glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase-1 (PDK-1) mRNA expression compared to 20% O_2 conditions without LPS treatment in THP-1 cells. The mRNA of HIF-1α was induced by LPS treatment, but not under 1% O_2 conditions.

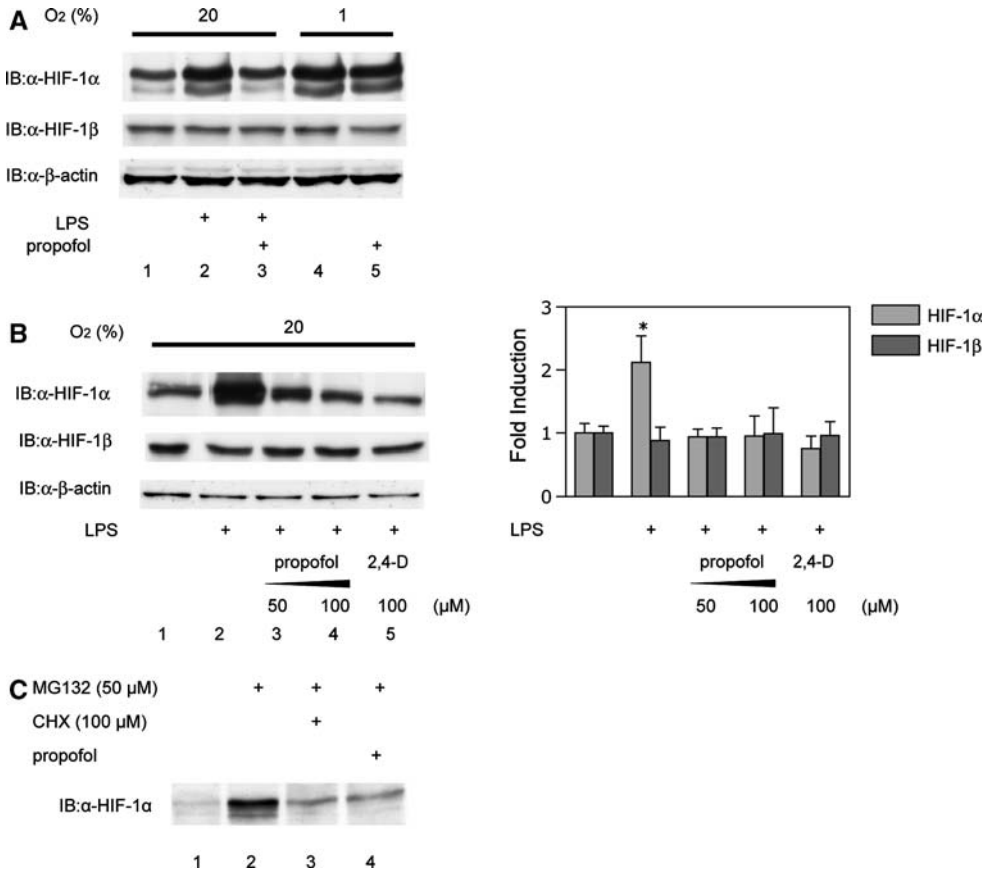

Fig. 1 Propofol inhibits LPS-induced HIF-1 protein expression in macrophage-differentiated THP-1 cells. **a** THP-1 cells were exposed to 20% O_2 or 1% O_2, or 20% O_2 with 50 μg/ml LPS with or without 50 μM propofol for 4 h and harvested for immunoblot assays using anti-HIF-1α, -HIF-1β or -β-actin antibody (Sigma). **b** THP-1 cells were exposed to 20% O_2 with 50 μg/ml LPS with or without 50 or 100 μM propofol or 100 μM 2,4-diisopropylphenol for 4 h and harvested for immunoblot assays using anti-HIF-1α, -HIF-1β or -β-actin antibodies (Sigma) (*left panel*). Experiments were repeated three times. Representative immunoblots are shown. Intensity of the respective HIF-1α and HIF-1β band was analyzed densitometrically, and fold induction to 20% O_2 condition with no treatment is plotted accordingly as mean ± SD. $^*P < 0.05$ compared to no treatment in each treatment regimen (one-way ANOVA followed by Dunnett's post hoc test) (*right panel*). **c** THP-1 cells were treated with 100 μM CHX, 100 μM propofol and 50 μM MG132 for 4 h under 20% O_2 conditions. Lysates were prepared and were subjected to immunoblot assay using anti-HIF-1α antibody

Fifty micromolar propofol suppressed the basal expression of mRNA of GLUT1, LDHA and PDK-1 induced by 50 μM LPS under 20% O_2 conditions. Expression of HIF-1α mRNA by itself was induced by LPS, but the induction was not significantly affected by 50 μM propofol (Fig. 2a).

The effect of propofol on HIF-1 activity was also investigated in THP-1 cells using two hypoxia response element (HRE)-luciferase reporter constructs. Propofol inhibited the LPS-induced HRE-dependent gene expression in a dose-dependent manner, as well as 2,4-diisopropylphenol in both enolase1- and VEGF-derived reporter constructs (Fig. 2b).

Effects of propofol on cellular energy metabolism

Next, we examined the effects of propofol on cellular energy metabolism using THP-1 cells (Fig. 3a). The lactate levels in culture media increased in response to LPS, and the increase was suppressed by treatment with propofol in a dose-dependent manner. In addition, we assayed ATP levels in THP-1 cells. Propofol reduced intracellular ATP content significantly, as well as the glycolytic inhibitor 2-DG with or without LPS treatment (Fig. 3b).

Discussion

In this report, we demonstrated an inhibitory effect of the intravenous anesthetic propofol on LPS-induced HIF-1 activation in THP-1 macrophage. Propofol suppressed HIF-1α protein expression and HIF-1 activation induced by LPS and inhibited glycolysis-dependent energy metabolism (Fig. 4).

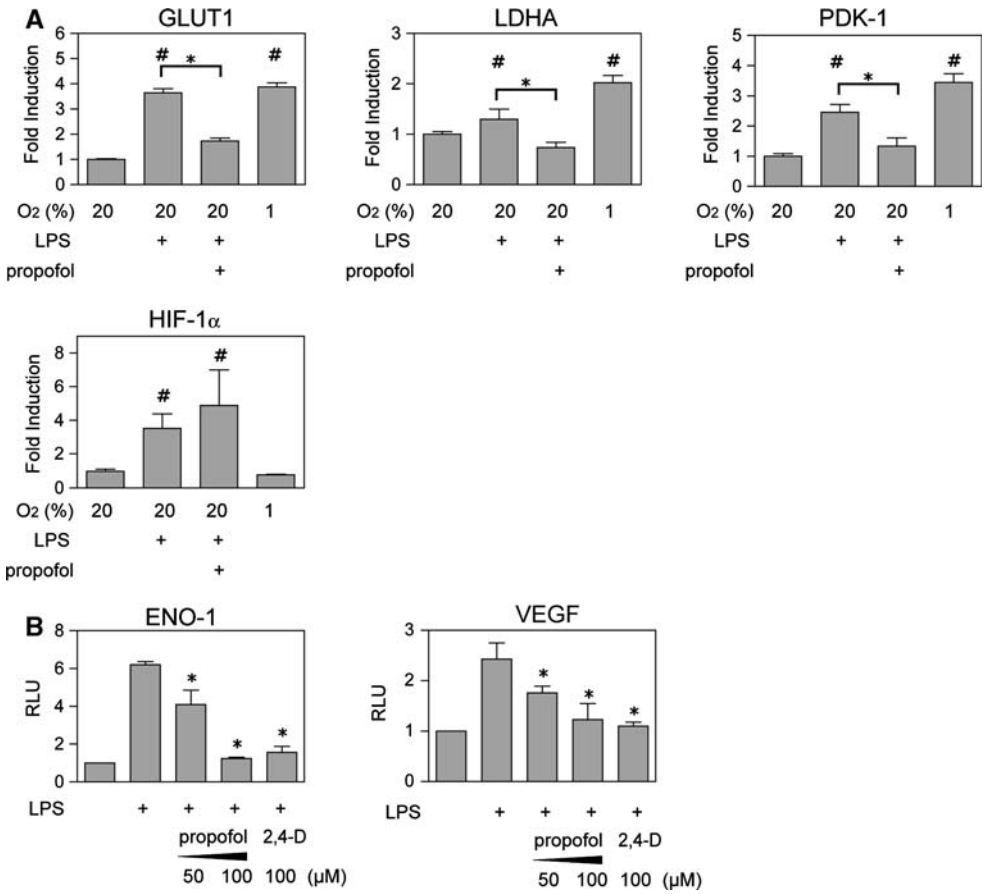

Fig. 2 Effect of propofol on LPS-induced HIF-1-dependent gene expression. **a** THP-1 cells were exposed to 20 or 1% O_2 with or without 50 μg/ml LPS and 50 μM propofol for 24 h, and total RNA was isolated. Expressions of mRNA of glucose transporter 1 (GLUT1), lactate dehydrogenase A (LDHA), pyruvate dehydrogenase kinase, isozyme 1 (PDK-1) and HIF-1α were analyzed by real-time RT-PCR using specific primer pairs. The fold induction was calculated on the value of non-hypoxic untreated cells. Each value was obtained as a mean of triplicate PCR reactions. Results shown represent mean ± SD of three independent experiments. $^{*}P < 0.05$ between indicated columns. $^{\#}P < 0.05$ compared to columns of 20% O_2 consitions without any treatment (one-way ANOVA followed by Dunnett's post hoc test). **b** THP-1 cells were transfected with p2.1 reporter or pVEGF-*KpnI* plasmid. After 6 h incubation, cells were treated with 50 μg/ml LPS or indicated concentrations of propofol or 2,4-diisopropylphenol (2,4-D) under 20% O_2 conditions for 18 h and harvested for luciferase assays. The relative luciferase activity (RLA) was based on the value of non-hypoxic untreated cells. Results shown represent mean ± SD of three independent transfections. $^{*}P < 0.05$ compared to LPS(+)/test reagent (−) (one-way followed by Dunnett's post hoc test)

As indicated in Figs. 1c and 2a, propofol suppressed HIF-1α protein accumulation in LPS-stimulated macrophages by not decreasing expression of HIF1α mRNA; 50 μM propofol inhibited HIF-1α protein accumulation induced by the strong proteasome inhibitor MG132 as well as 100 μM CHX (Fig. 1c). We demonstrate that propofol neither affects HIF-1α-hyroxylase activity nor facilitates degradation of HIF-1α by proteasome [15]. Consequently, it is highly probable that propofol inhibits translation of HIF-1α protein from mRNA in THP-1 cells as well as in the case of HEK293 cells and HUVECs. Because LPS induces HIF-1α expression in macrophages that is ROS-dependent and in which the induction is inhibited by the antioxidant *N*-acetylcysteine [17], it is possible that propofol suppresses LPS-induced HIF-1α accumulation through its antioxidant activity [23]. Because 2,4-diisopropylphenol, which does not have an anesthetic effect, exerted a suppressive effect on HIF-1α protein, propofol may work as an HIF-1 inhibitor in an manner independent of its hypnotic effect. In Fig. 2a, LPS significantly induced HIF-1α mRNA, but 1% O_2 exposure did not. This is consistent with previous reports [7, 16, 17], including the original one [8].

Propofol is reported to inhibit lactate production. In addition, a report describes that propofol suppresses macrophage functions and ATP synthesis [4, 24]. In fact, we observed that propofol decreased cellular lactate production (Fig. 3a). In addition, we demonstrated that propofol

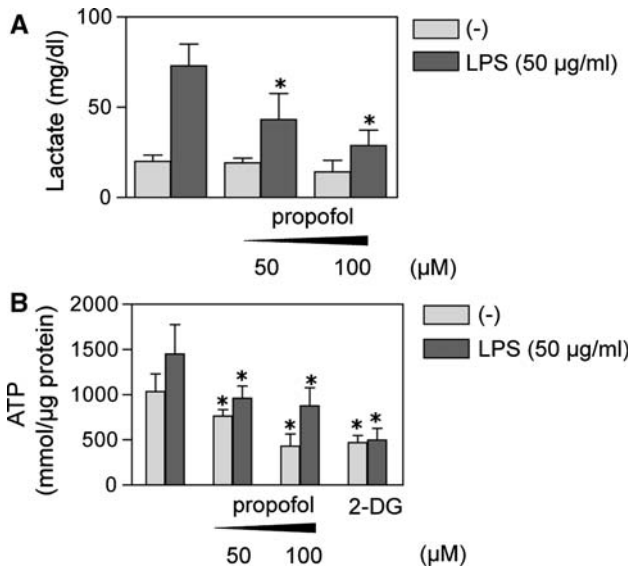

Fig. 3 Glycolysis and ATP generation in THP-1 macrophages are affected by propofol. **a** Lactate concentrations in supernatant of THP-1 cells were measured under indicated treatment for 24 h. Values were normalized to total protein content. Results shown represent mean ± SD of three independent transfections. $*P < 0.05$ compared to each control condition (no propofol with or without LPS treatment) (ANOVA followed by Dunnett's post hoc test). **b** THP-1 cells were cultured under indicated treatment for 24 h. Cell lysates were harvested and intracellular ATP concentrations measured by means of a luciferase-based chemiluminescent assay. Values were normalized to total protein content. Results shown represent mean ± SD of three independent transfections. $*P < 0.05$ compared to each control condition (no propofol with or without LPS treatment) (ANOVA followed by Dunnett's post hoc test)

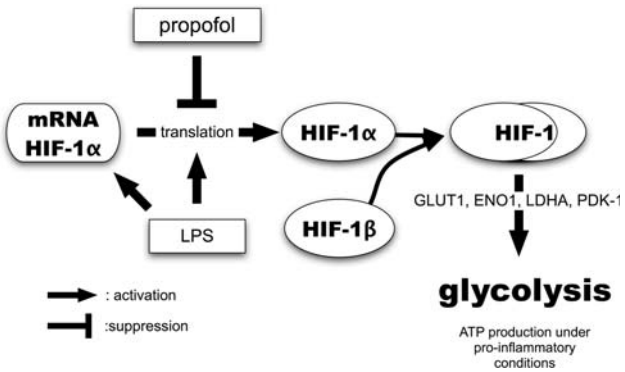

Fig. 4 Involvement of propofol in HIF-1 activity and glucose metabolism in macrophages LPS induced HIF-1α protein expression by increasing the mRNA accumulation and translation of HIF-1α from the mRNA in macrophages. Propofol does not affect the mRNA expression, but suppresses the translation to inhibit HIF-1 activity, which plays an essential role in glucose metabolism in cells responsible for innate immunity

inhibited LPS-elicited induction of mRNA expression of molecules that play critical roles in glucose metabolism (Fig. 2a, b). GLUT proteins are integral plasma membrane proteins and transport glucose through the plasma membrane. GLUT1 is responsible for the low level of basal glucose uptake required to sustain respiration in all cells. Enolase, also known as phosphopyruvate dehydratase, is a metalloenzyme responsible for the catalysis of 2-phosphoglycerate to phosphoenolpyruvate, a step of glycolysis. The conversion from pyruvate to lactate is a critical reaction for supplying NAD+ to promote the glycolytic pathway and is mediated by LDHA, whose expression is under regulation by HIF-1 [25]. Thus, propofol decreases the energy supply in the cells at least partially by the suppression of the glycolytic pathway, which is attributable to the decreased level of HIF-1 activity. Another interesting finding of our report is that propofol inhibits the LPS-induced increase of expression of pyruvate dehydrogenase kinase isozyme 1 (PDK-1), a kinase-regulating activity of pyruvate dehydrogenase (PDH) [26–28]. PDH is a part of a mitochondrial multienzyme complex that contributes to transforming pyruvate into acetyl-CoA by a process called pyruvate decarboxylation [29]. The enzymatic activity is regulated by a phosphorylation/dephosphorylation cycle. Phosphorylation of PDH by a specific PDK results in the inactivation of PDH causing reprogramming from an aerobic metabolism to an anaerobic one. Propofol suppresses the metabolic reprogramming of glucose and ATP production of LPS-activated macrophages in which mitochondrial respiration plays little roles in cellular energy charge. In fact, results using 2-DG in Fig. 3b shows that ATP production of differentiated THP-1 cells are dependent largely on the glycolytic pathway. Propofol inhibited ATP accumulation with or without LPS treatment (Fig. 3b). As we reported previously in HEK293 cells and HUVECS, propofol inhibits HIF-1 activity under normoxic conditions [15]. In addition, in THP-1 cells, 50 μM propofol inhibits basal or constructive HIF-1 activity (data not shown). The energy failure of macrophage results in profound impairment of myeloid cell aggregation, motility, invasiveness and bacterial killing [22]. On the other hand, HIF-1 has been identified as an essential factor in the development of LPS-induced sepsis in mice [30]. Inhibition of HIF-1 activity by propofol may thus represent a novel therapeutic target for LPS-induced sepsis.

In conclusion, we demonstrated that propofol suppressed LPS-induced HIF-1 activation and ATP production of macrophages. We note that we exclusively used macrophage-differentiated THP-1 cells and that our results should be confirmed in more physiological systems such as peripheral monocyte-derived macrophages and in vivo settings.

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