BJA

Translocation of protein kinase C isoforms is involved in propofol-induced endothelial nitric oxide synthase activation

L. Wang[1][*][†], B. Wu[1][†], Y. Sun[2][3], T. Xu[1], X. Zhang[1], M. Zhou[1] and W. Jiang[1][*]

[1]*Department of Anesthesiology and* [2]*Department of Neurosurgery, School of Medicine, Shanghai Sixth Municipal Hospital, Shanghai Jiaotong University, Shanghai 200233, China.* [3]*Department of Neurosurgery, Shanghai Huashan Hospital, Fudan University, Shanghai, China*

*Corresponding authors. E-mail: jiangw@sjtu.edu.cn (W.J.); liwang1118@hotmail.com (L.W.)

Background. Previous studies have indicated that protein kinase C (PKC) may enhance endothelial nitric oxide synthase (eNOS) activation, although the detailed mechanism(s) remains unclear. In this study, we investigated the roles of PKC isoforms in regulating propofol-induced eNOS activation in human umbilical vein endothelial cells (HUVECs).

Methods. We applied western blot (WB) analysis to investigate the effects of propofol on Ser^{1177} phosphorylation-dependent eNOS activation in HUVECs. Nitrite (NO_2^-) accumulation was measured using the Griess assay. The phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway was examined by WB assay. Propofol-induced translocation of individual PKC isoforms in subcellular fractions in HUVECs was analysed using WB assay.

Results. In HUVECs, protocol treatment (1–100 μM) for 10 min induced a concentration-dependent increase in phosphorylation of eNOS at Ser^{1177}. The NO production was also increased accordingly. PKC inhibitors, bisindolylmaleimide I (0.1–1 μM), and staurosporine (20 and 100 nM), effectively blocked propofol-induced eNOS activation and NO production. Further analyses in fractionated endothelial lysate showed that short-term propofol treatment (50 μM) led to translocation of PKC-α, PKC-δ, PKC-ζ, PKC-η, and PKC-ε from cytosolic to membrane fractions, which could also be inhibited by both PKC inhibitors. These data revealed that the differential redistribution of these isozymes is indispensable for propofol-induced eNOS activation. In addition, Akt was not phosphorylated in response to propofol at Ser^{473} or Thr^{308}.

Conclusions. Propofol induces the Ser^{1177} phosphorylation-dependent eNOS activation through the drug-stimulated translocation of PKC isoforms to distinct intracellular sites in HUVECs, which is independent of PI3K/Akt-independent pathway.

Anaesthetic induction and maintenance with propofol are often accompanied with undesired peripheral vasodilatation and hypotension that may be harmful to patients with compromised cardiac reserve. Propofol has been reported to affect arterial and venous circulation both *in vivo*[1–3] and *in vitro*.[4][5] Studies of blood vessel models have suggested that propofol seems to influence cellular processes including calcium signalling,[6] sympathetic neurotransmission,[7] and the function of endothelium.[8] Propofol-induced hypotension also seems to be associated with its effects on the central nervous system.[4]

Endothelial cells (ECs) play a major role in the regulation of the vascular tone, through the production of several vasoactive mediators such as nitric oxide, prostacyclin, endothelin, and endothelial-derived hyperpolarizing factor.[9] Nitric oxide is a profound vasodilator. Constitutive production of nitric oxide by the endothelium maintains the vasculature in a state of vasodilatation. It has been suggested that propofol-induced hypotension is partly, if not all, related to the nitric oxide signalling,[10][11] although

[†]These authors contributed equally to this work.

the mechanism has not been fully determined. In ECs, NO is largely synthesized enzymatically by endothelial nitric oxide synthase (eNOS).[12] eNOS is carefully regulated not only at the transcriptional level but also by several post-translational mechanisms. For example, eNOS phosphorylation at Ser^{1177}(human)/^{1179}(bovine) by phosphatidylinositol 3-kinase (PI3K)-dependent Akt plays a critical role in its activation by various agonists, such as vascular endothelial growth factor.[13 14] However, Ser^{1177}/Ser^{1179} is the target of multiple protein kinases in addition to Akt.[13] Recent studies revealed that protein kinase C (PKC) is also important and responsible for regulation on eNOS function and NO production.[13 15–17]

PKCs are a family of at least 10 isoenzymes, grouped into three subclasses, the classical PKCs (α, βI, βII, and γ), the novel PKCs (δ, θ, ε, and η), and the atypical PKCs (λ and ζ).[18] It has been identified that the prominent PKC isoforms expressed in human umbilical vein endothelial cells (HUVECs) involve PKC-α, PKC-δ, PKC-ε, PKC-η, and PKC-ζ.[19 20] Previous studies have indicated that altered expression or enzymatic activity of PKCs is implicated in circulatory disturbance in coronary artery disease, arthrosclerosis, hypertension, myocardial ischaemia–reperfusion, and circulatory shock,[21–24] although little is known about its relation to propofol-induced vasorelaxation. To better understand the roles of individual PKC isoforms in mediating propofol-induced eNOS activation and NO production, we performed an *in vitro* study using cultured vascular ECs.

Methods

Cell culture

The HUVEC line used in this study was purchased from the American Type Culture Collection (ATCC, CRL-1730; Passage 12). Cells were cultured in medium M199 (Gibco BRL, Life Technologies, NY, USA) supplemented with 3 ng ml^{-1} β-EC growth factor (Sigma, St Louis, MO, USA), 4 U ml^{-1} heparin, 10% fetal bovine serum (Invitrogen Corporation, Australia), 100 U ml^{-1} penicillin, and 100 μg streptomycin. Cells were cultured in a humidified 95% air–5% CO_2 incubator at 37°C. Culture media were replaced every 3 days.

Cell assay and treatment

CRL-1730 cell lines at Passages 14–18 were collected for assay. Cultured HUVECs were randomly assigned into one of the following three groups.

Group 1

To investigate the effects of propofol on phosphorylation of eNOS at Ser^{1177} (eNOS-pSer^{1177}), HUVECs were treated with 1, 10, 50, or 100 μM propofol (Astra Zeneca, Cheshire, UK) for 10 min at 37°C. Cells and parallel controls were lysed, and the levels of phospho- and total eNOS were determined by immunoblot assay.

Group 2

To investigate the effects of propofol on Akt and phospho-Akt expressions, HUVECs were treated with either solvent control or propofol (at the nearly maximal concentration of 50 μM) for 10 min at 37°C. Phospho-Akts (Akt-pSer^{478} and Akt-pThr^{308}) were analysed by immunoblots using specific antibodies.

Group 3

To assess the effects of propofol on PKC translocation from cytosolic to membrane fractions, HUVECs were first treated with potent PKC inhibitors, either bisindolylmaleimide I (Bis I, 0.1–1 μM) or staurosporine (20–100 nM) for 30 min. Treated cells along with parallel controls (treated with intralipid 10%) were then stimulated with or without propofol (50 μM) for 10 min at 37°C. Cell lysates including total lysate, cytosolic, and membrane fractions were prepared, and the expressions of eNOS, eNOS-pSer^{1177}, PKC-α, PKC-ε, PKC-ζ, PKC-η, and PKC-δ were analysed using immunoblotting. Parallel studies were performed using a diacylglycerol (DAG) analogue, phorbol myristate acetate (PMA, 1 μM).

Unless stated otherwise, drugs were purchased from Sigma Aldrich (Sigma, St Louis, MO, USA). PMA was dissolved in DMSO. The final concentration of DMSO was adjusted to 0.003% for each solution.

Total and subcellular fractionation of ECs

To collect total cell lysate, cells were immediately lysed after propofol or solvent control in buffer (pH 7.4) containing 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 10 μg ml^{-1} leupeptin, 60 μg ml^{-1} aprotinin, and 1 mM phenylmethanesulfonyl fluoride. Cell lysate was then centrifuged at 16 000g for 10 min, and the supernatants were collected. Fractionation of HUVECs was performed following protocols described in Ramzy and colleagues,[25] with minor modification. Briefly, ECs were collected with lysis buffer containing protease inhibitors, 20% glycerol, and 0.05% Triton X-100. Harvested cells were then centrifuged for 2 min at 800g, and the supernatant was collected as supernatant 1 (SUP1). The pellet was resuspended and centrifuged for 2 min in the same buffer. The supernatant was collected and added to SUP1. SUP1 was centrifuged at 100 000g for 30 min at 4°C, producing a pellet containing the total cellular membrane fraction and a supernatant as the cytosolic fraction.

Immunoblotting

Western blot (WB) analysis was performed on the total cell lysate, cytosolic, and membrane fractions. Protein concentrations were measured using the Bradford protein assay.[26] All samples were mixed with Laemmli sample buffer (sodium dodecyl sulphate, SDS) and placed in a boiling water bath for 5 min. Proteins (50 μg) were resolved in 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Blots were probed with antibodies against eNOS, eNOS-pSer[1177], PKC-α, PKC-ε, PKC-ζ and PKC-δ, respectively. Immunoblots were developed using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G and detected by enhanced chemiluminescence (Pierce, Rockford, IL, USA). Protein bands were quantitated with a densitometer. SDS–PAGE reagents and Bradford protein assay reagent were from Bio-Rad Laboratories (Hercules, CA, USA). All antibodies used in the present study were obtained from Cell Signaling Technology (Beverly, MA, USA).

Detection of NO

In order to estimate the amount of NO production, we adopted the Griess assay to measure the accumulated nitrite (NO_2^-), a stable breakdown product of NO. During the assay, medium aliquots were mixed with equal volumes of the Griess reagent and incubated at room temperature for 15 min. The azo dye production was then analysed by a spectrophotometer with absorbance set at 540 nm. Sodium nitrite was used as a standard.

Statistical analysis of data

Results shown in the blots are representative of three or more independent experiments. Densitometry data and NO products were analysed using one-way ANOVA with a significance level of $P < 0.05$.

Results

Propofol-induced Ser[1177] phosphorylation and activation of eNOS

Using cultured HUVECs, we first tested whether propofol phosphorylates eNOS at Ser[1177], a catalytically positive regulatory site. HUVECs were treated with 1, 10, 50, or 100 μM propofol, respectively, for 10 min. As shown in Figure 1A, propofol induced a rapid increase in eNOS phosphorylation at Ser[1177] in a concentration-dependent manner. The evident concentration was approximal at 10 μM, with a maximal effect at 50 μM. Treatment with propofol for a short period (10 min) did not change the level of total eNOS in HUVECs. The increase in eNOS phosphorylation was accompanied by concomitant augmentation of NO production (Fig. 1B), which could be inhibited by the eNOS inhibitor *N*-nitro-L-arginine methyl ester (Fig. 1C).
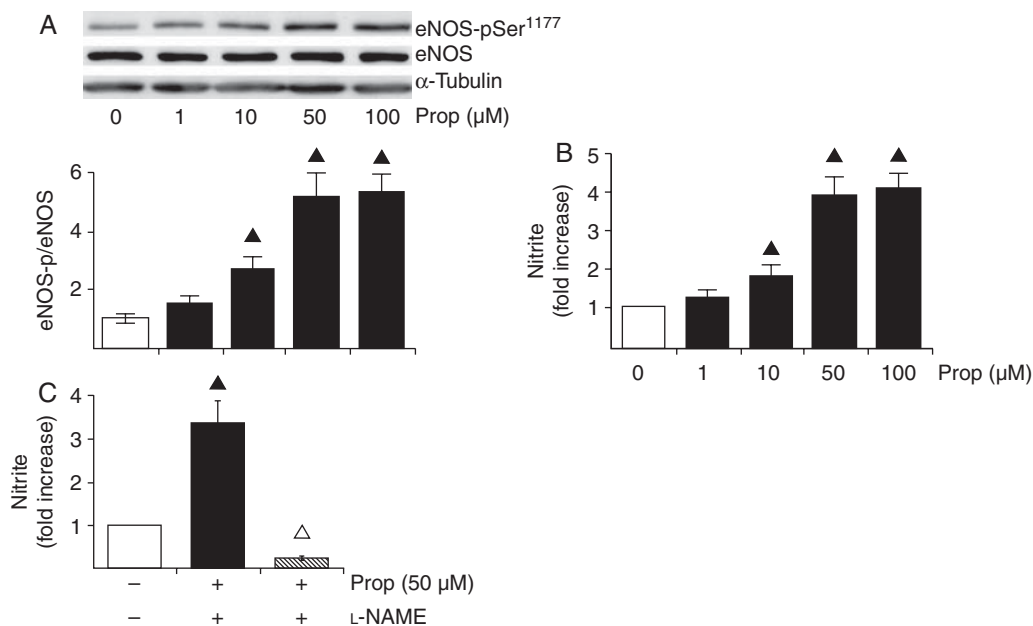

Fig 1 The effect of propofol (Prop) on eNOS phosphorylation (eNOS-pSer[1177]) and NO production. HUVECs were treated with the indicated concentrations of propofol for 10 min, and (A) phosphorylation of eNOS at Ser[1177] was determined using immunoblot analysis with phospho-specific antibody. The bar graphs below are densitometry analyses of the ratio of phosphorylated eNOS to total eNOS with the vehicle control set as 1. (B) Production of nitrite in collected culture media was measured by the Griess assay. The nitrite concentration in the vehicle control culture medium was set as 1. (C) Cultures were pretreated with the eNOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 100 μmol litre^{-1}) for 30 min before exposure with propofol (50 μM) for 10 min and nitrite production were measured by the Griess assay. The data shown are mean (SD) from three separate experiments (*n*=6). ▲$P < 0.05$ *vs* intralipid controls. △$P < 0.05$ between propofol alone and L-NAME plus propofol group.

According to the above observation, a concentration at 50 μM of propofol was adopted in the following assays.

Propofol-induced phosphorylation and activation of eNOS is independent of the PI3K/Akt pathway

Previous studies have suggested that in cultured ECs, the eNOS phosphorylation at Ser^{1177} and subsequent eNOS
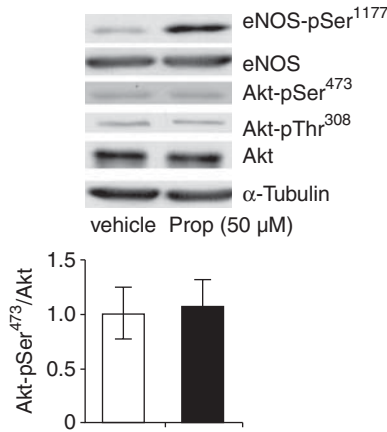

Fig 2 The role of PI3K/Akt pathway in propofol-induced phosphorylation of eNOS. HUVECs were stimulated with propofol (Prop; 50 μM) for 10 min. Phosphorylation of Akt at Ser^{473} and Thr^{308}, and eNOS at Ser^{1177} was determined using immunoblot analysis with phospho-specific antibodies. However, there is no significant difference in total and phosphor-Akt (Ser^{473} and Thr^{308}) expressions between basal control and propofol-treated groups ($P>0.05$). The data shown are mean (SD) from three separate experiments ($n=6$).

activation can be mediated by PI3K/Akt activation in response to several distinct eNOS activators such as vascular endothelial growth factor.[27] [28] In order to investigate whether the eNOS activation induced by propofol is dependent of PI3K/Akt, HUVECs were treated with 50 μM propofol for 10 min and underwent immunoblot analyses for Akt phosphorylation at Ser^{478} and Thr^{308} (Fig. 2). However, we did not observe an increase in either total Akt or phospho-Akt (at Ser^{473} and Thr^{307}), which is consistent with the speculation that PI3K/Akt signalling pathway may not be involved in propofol-induced eNOS phosphorylation in HUVECs.[29]

PKC is involved in propofol-induced phosphorylation and activation of eNOS

In order to test whether PKC signalling is involved in propofol-induced eNOS phosphorylation, we treated HUVECs with $0.1-1$ μM Bis I (a potent PKC inhibitor) for 30 min before they were exposed to 50 μM propofol for 10 min. As shown in Figure 3A, the propofol-induced increase in eNOS phosphorylation at Ser^{1177} was largely inhibited by Bis I in a concentration-dependent manner. In addition, Bis I also inhibited eNOS phosphorylation at Ser^{1177} induced by 1 μM DAG analogue PMA (Fig. 3B). Similar results were also observed when cells were treated with another PKC inhibitor staurosporine at 20 or 100 nM (data not shown). Moreover, pretreatment with Bis I also inhibited both propofol- and PMA-stimulated NO production
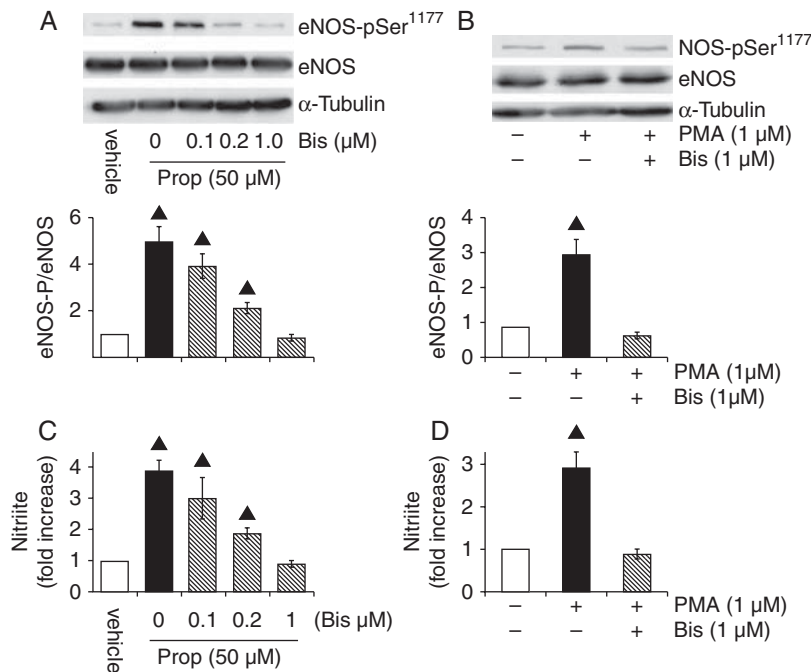

Fig 3 The effect of PKC inhibitor on propofol-induced phosphorylation of eNOS. HUVECs were pretreated with Bis I ($0.1-1$ μM) for 30 min before stimulation with (A) propofol (Prop; 50 μM) and (B) PMA (1 μM) for 10 min. Phosphorylation of eNOS at Ser^{1177} was determined using immunoblot analysis with phospho-specific antibody. The bar graphs below are densitometry analyses of the ratio of phosphorylated eNOS to total eNOS. (C and D) NO production was measured as described in the Methods section. The data shown are mean (SD) from three separate experiments ($n=6$). ▲$P<0.05$ vs vehicle control.
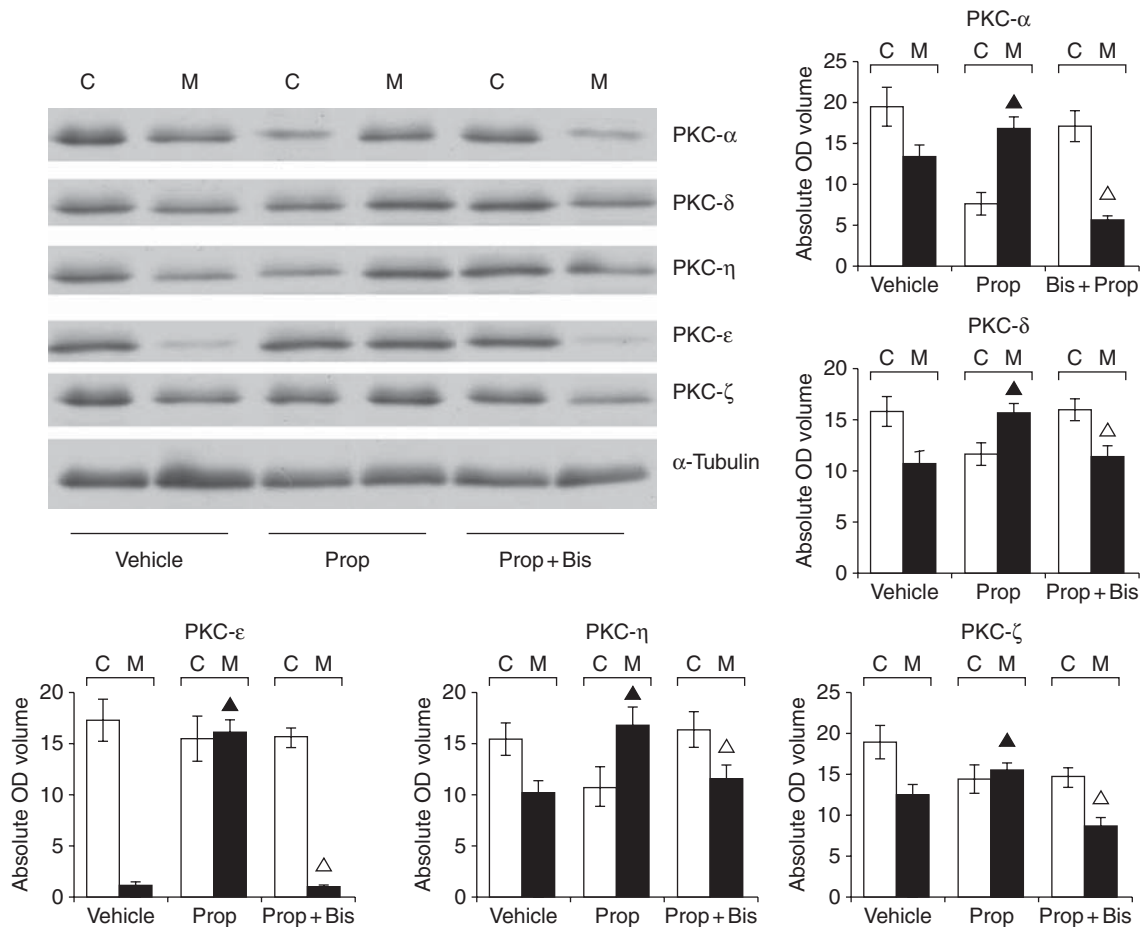
Fig 4 Effect of propofol on translocation of PKC isoforms from cytosolic to membrane fractions. HUVECs were treated with propofol (Prop; 50 μM) in the presence or absence of Bis I (1 μM; preincubation for 30 min) for 10 min, and PKC isoforms including PKC-α, PKC-δ, PKC-ε, PKC-η, and PKC-ζ in HUVECs cytosolic (C) and membrane (M) fractions were determined by the WB method using individual-specific antibody. The bar graphs right and below are the absolute optical density volume of each of the PKC isoforms observed. The data shown are mean (SD) from four separate experiments (*n*=8). ▲*P*<0.05 *vs* basal membrane fraction and △*P*<0.05 *vs* propofol alone treated membrane fraction.

(Fig. 3C and D), which is consistent with earlier reports indicating that PKC is involved in NO production.[30][31]

Propofol-induced translocation of PKC isoforms from cytosolic to membrane fractions

We next investigated the effect of propofol on translocation of PKC isoforms from cytosolic to membrane fractions. First, we determined the isoenzyme expression profile in HUVECs by WB. As shown in the first column of Figure 4, before HUVECs were exposed to propofol, PKC-α, PKC-δ, PKC-η, and PKC-ζ were largely expressed within the cytosolic fraction, although their expression could also be detected in the membrane fraction. PKC-ε was exclusively associated with the cytosolic fraction. After HUVECs were treated with 50 μM propofol, all five isoforms translocated from cytosolic to membrane fractions (Fig. 4, Column 2). Pretreatment with 1 μM Bis I potently attenuated the propofol-induced translocation of all five isoforms (Fig. 4, Column 3). The propofol-induced translocation of PKC isoforms from

cytosolic to membrane fractions were also companied with the increase in phosphorylation of eNOS at Ser[1177], which could be inhibited by Bis I (Fig. 3A). These data suggest that the translocation of PKC isoforms from cytosolic to membrane fractions is required for Ser[1177] phosphorylation and activation of eNOS by propofol.

Discussion

In this study, we found that the translocation of individual PKC isoforms from cytosolic to membrane fractions induced by propofol is involved in propofol-stimulated eNOS Ser[1177] phosphorylation. This was associated with no change of Akt phosphorylation, suggesting that propofol may modulate eNOS in ECs through an Akt-independent pathway. These findings are consistent with a previous publication dealing with phosphorylation of eNOS by propofol.[29]

The activation of eNOS catalytic function by Ser[1177] phosphorylation is due to inhibition of calmodulin dissociation from eNOS and the enhancement of the internal

rate of eNOS electron transfer.[32] Ser[1177] phosphorylation can be catalysed by numerous kinases, including kinase Akt (protein kinase B), the cyclic AMP-dependent protein kinase (PKA), AMP-activated protein kinase, PKG, and calcium/calmodulin-dependent protein kinase II (CaM kinase II).[17 30 33 34] The contribution of these kinase pathways and their interactions are currently under investigation. Yet, it is clear that different extracellular stimuli activating distinct kinase pathway may lead to eNOS phosphorylation.

From our study, we confirmed that propofol increases eNOS phosphorylation at Ser[1177]. Ser[1177], in the reductase domain, is a key regulatory site for eNOS activity. By using dominant-negative mutants and overexpressing PKC-α or PKC-δ in wild-type animals, previous studies have demonstrated that PKCs phosphorylate eNOS Ser[1177/1179] in ECs.[13 15 17]

Although the relationship between propofol and PKCs activation has been suggested in tissues such as brain and cardiac muscle,[31 35] the roles of PKCs isoforms in mediating eNOS activation by propofol have never been reported in cultured ECs. We found that a potent PKC inhibitor, Bis I, diminished propofol-induced eNOS Ser[1177] phosphorylation. Similar results were also observed when cells were treated with another PKC inhibitor staurosporine at 20 or 100 nM (data not shown). Moreover, pretreatment with Bis I inhibited both propofol- and PMA-stimulated NO production (Fig. 3c and d), which is consistent with earlier reports indicating that PKC is involved in NO production.[13 15 17] These data lead to the conclusion that propofol induces eNOS activation, primarily via activation of PKC in EC. Moreover, data from our laboratory showed that propofol induces a subcellular translocation of PKC isoforms that is related to the activation of eNOS and NO production in HUVECs. This may add worthy information to the understanding of the cellular mechanism of propofol-induced hypotension.

All PKCs share extensive homology in their catalytic domains with each displaying low-substrate selectivity.[21 36] Yet, they exert pleiotropic effects by dispersing to different subcellular compartments.[21] Like phosphorylation and acylation, subcellular localization affects PKC activity[21 36] and may help generate the apparent temporal selectively. In this study, propofol (50 μM) caused a rapid increase in eNOS Ser[1177] phosphorylation within a period of 10–30 min, which reached a peak at 10 min (data not shown).

A subcellular translocation of PKC isoforms from the cytosolic to membrane fraction associates with activation of PKC isoforms and is facilitated by receptors for activated C kinases.[31] The binding of the PKC isoform to its specific receptor for activated C kinase is also critical to the phosphorylation of substrate proteins that are specific for the PKC isoform.

Previous studies have confirmed that the major PKC isoforms expressed in HUVECs are PKC-α, PKC-δ, PKC-η, PKC-ζ, and PKC-ε, with some controversy over the existence of PKC-βII.[20 21] Protein kinase may vary among different mammals and cells from different vascular beds. In this study, we identified the presence of PKC isoforms expressed in HUVECs, which are PKC-α, PKC-δ, PKC-η, PKC-ζ, and PKC-ε. PKC-ε was expressed within the cytosolic fraction in ECs before propofol treatment, whereas PKC-α, PKC-δ, PKC-η, and PKC-ζ are expressed in both cytosolic and membrane fractions. Propofol caused translocation of all five isoforms from cytosolic to membrane fractions, providing indirect support for their activation.

Our data showed that PKC isozyme localization is related to eNOS phosphorylation at Ser[1177], although the underlying mechanism remains unclear. Previous studies have shown that both PKC-α and PKC-δ can phosphorylate eNOS at Ser[1179/1177],[13 15] suggesting the possibility of a direct interaction between PKC isoforms and eNOS.

In conclusion, we have shown that propofol can cause eNOS activation by increasing phosphorylation of eNOS at Ser[1177] (via an Akt-independent pathway), which is responsible for the endothelial-derived NO production. Propofol stimulates translocation of PKC-α, PKC-δ, PKC-η, PKC-ζ, and PKC-ε to distinct intracellular sites in HUVECs that may be a fundamentally important cellular mechanism of propofol-induced eNOS activation and subsequent NO production.

Conflict of interest

None declared.

Funding

References

1 Turner RJ, Gatt SP, Kam PC, Ramzan I, Daley M. Administration of a crystalloid fluid preload does not prevent the decrease in arterial blood pressure after induction of anaesthesia with propofol and fentanyl. *Br J Anaesth* 1998; **80**: 737–41

2 Baumert JH, Hein M, Hecker KE, Satlow S, Neef P, Rossaint R. Xenon or propofol anaesthesia for patients at cardiovascular risk in non-cardiac surgery. *Br J Anaesth* 2008; **100**: 605–11

3 Koch M, Backer DD, Vincent JL, Barvais L, Hennart D, Schmartz D. Effects of propofol on human microcirculation. *Br J Anaesth* 2008; **101**: 473–8

4 Wang X. Propofol and isoflurane enhancement of tonic gamma-aminobutyric acid type A current in cardiac vagal neurons in the nucleus ambiguus. *Anesth Analg* 2009; **108**: 142–8

5 Cheng TH, Chen JJ, Chen CH, Wong KL. Effects of propofol on cyclic strain-induced endothelium-1 Expression in human umbilical vein endothelial cells. *Anesthesiology* 2009; **110**: 74–80

6 Yang M, Ding X, Murray PA. Differential effects of intravenous anesthetics on capacitive calcium entry in human pulmonary

artery smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2008; **294**: L1007–12

7 Ebert TJ. Sympathetic and hemodynamic effects of moderate and deep sedation with propofol in humans. *Anesthesiology* 2005; **103**: 20–4

8 Gragasin FS, Davidge ST. The effects of propofol on vascular function in mesenteric arteries of the aging rat. *Am J Physiol Heart Circ Physiol* 2009; **297**: H466–H474

9 Galley HF, Webster NR. Physiology of the endothelium. *Br J Anaesth* 2004; **93**: 105–13

10 Bodelsson G, C;Sandstrom K, Wallerstedt SM, Hidestal J, Tronebrandt K, Bodelsson M. Effects of propofol on substance P-induced relaxation in isolated human omental arteries and veins. *Eur J Anaesth* 2000; **17**: 20–8

11 Corcoran TB, O'Shea A, Engel A, Shorten GD. The influence of propofol on P-selectin expression and nitric oxide production in re-oxygenated human umbilical vein endothelial cells. *Acta Anaesthesiol Scand* 2006; **50**: 348–54

12 Yetik-Anacak G, Catravas JD. Nitric oxide and endothelium: history and impact on cardiovascular disease. *Vasc Pharmacol* 2006; **45**: 268–79

13 Motley ED, Eguchi K, Patterson MM, Palmer PD, Suzuki H, Eguchi S. Mechanism of endothelial nitric oxide synthase phosphorylation and activation by thrombin. *Hypertension* 2007; **49**: 577–83

14 Sugawara T, Ayer R, Jadhav V, Chen W, Tsubokawa T, Zhang JH. Simvastatin attenuation of cerebral vasospasm after subarachnoid hemorrhage in rats via increased phosphorylation of Akt and endothelial nitric oxide synthase. *J Neurosci Res* 2008; **86**: 3635–43

15 Partovian C, Zhuang Z, Moodie K, *et al.* PKCα activates eNOS and increases arterial blood flow in vivo. *Circ Res* 2005; **97**: 482–7

16 Rask-Madsen C, King GL. Differential regulation of VEGF signaling by PKC-alpha and PKC-epsilon in endothelial cells. *Arterioscler Thromb Vasc Biol* 2008; **28**: 919–24

17 Sud N, Wedgwood S, Black SM. Protein kinase Cδ regulates endothelial nitric oxide synthase expression via Akt activation and nitric oxide generation. *Am J Physiol Lung cell Mol Physiol* 2008; **294**: L582–L591

18 Nishizuka Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* 1988; **334**: 661–5

19 Gliki G, Abu-Ghazaleh R, Jezequel S, Wheeler-Jones C, Zachary I. Vascular endothelial growth factor-induced prostacyclin production is mediated by a protein kinase C (PKC)-dependent activation of extracellular signal-regulated protein kinases 1 and 2 involving PKC-delta and by mobilization of intracellular Ca^{2+}. *Biochem J* 2001; **353**: 503–12

20 Lorenzi O, Frieden M, Villemin P, Fournier M, Foti M, Vischer UM. Protein kinase C-delta mediates von Willebrand factor secretion from endothelial cells in response to vascular endothelial growth factor (VEGF) but not histamine. *J Thromb Haemost* 2008; **6**: 1962–9

21 Gaudreault N, Perrin RM, Guo M, Clanton CP, Wu MH, Yuan SY. Counter regulatory effects of PKCβII and PKCδ on coronary

endothelial permeability. *Arterioscler Thromb Vasc Biol* 2008; **28**: 1527–33

22 Bock-Marguette I, Shrivastava S, Pipes GC, *et al.* Thymosin beta4 mediated PKC activation is essential to initiate the embryonic coronary developmental program and epicardial progenitor cell activation in adult mice in vivo. *J Mol Cell Cardiol* 2009; **46**: 728–38

23 Palaniyandi SS, Inaqaki K, Mochly-Rosen D. Mast cells and epsilonPKC: a role in cardiac remodeling in hypentension-induced heart failure. *J Mol Cell Cardiol* 2008; **45**: 779–86

24 Sivaraman V, Hausenlov DJ, Kolvekar S, *et al.* The divergent roles of protein kinase C epsilon and delta in simulated ischaemia-reperfusion injury in human myocardium. *J Mol Cell Cardiol* 2009; **46**: 758–64

25 Ramzy D, Rao V, Tumiati LC, *et al.* Elevated endothelin-1 level impair nitric oxide homeostasis through a PKC-dependent pathway. *Circulation* 2006; **114**: I-319–26

26 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein dye binding. *Analytical Biochem* 1976; **72**: 248–54

27 Kou R, SenBanerjee S, Jain MK, Michel T. Differential regulation of vascular endothelial growth factor receptors (VEGFR) revealed by RNA interference: interactions of VEGFR-1 and VEGFR-2 in endothelial cell signaling. *Biochemistry* 2005; **44**: 15064–73

28 Sud N, Kumar S, Wedgwood S, Black SM. Modulation of PKCδ signaling alters the shear stress-mediated increases in endothelial nitric oxide synthase transcription: role of STAT3. *Am J Physiol Lung cell Mol Physiol* 2009; **296**: L519–26

29 Wang B, Luo T, Chen D, Ansley DM. Propofol reduces apoptosis and up-regulates endothelial nitric oxide synthase protein expression in hydrogen peroxide-stimulated human umbilical vein endothelial cells. *Anesth Analg* 2007; **105**: 1027–33

30 Kan WH, Hsu JT, Schwacha MG, Choudhry MA, Bland KI, Chaudry IH. Estrogen ameliorates trauma-hemorrhage-induced lung injury via endothelial nitric oxide synthase-dependent activation of protein kinase G. *Ann Surg* 2008; **248**: 294–302

31 Wickley PJ, Ding X, Murray PA, Damron DS. Propofol-induced activation of protein kinase C isoforms in adult rat ventricular myocytes. *Anesthesiology* 2006; **104**: 970–7

32 Dudzinski DM, Michel T. Life of history of eNOS: Partners and pathway. *Cardiovasc Res* 2007; **75**: 247–60

33 Grossini E, Molinari C, Mary DA, *et al.* Urocortin II induces nitric oxide production through cAMP and Ca^{2+} related pathways in endothelial cells. *Cell Physiol Biochem* 2009; **23**: 87–96

34 Mount PF, Lane N, Venkatesan S, *et al.* Bradykinin stimulates endothelial cells fatty acid oxidation by CaMKK-dependent activation of AMPK. *Atherosclerosis* 2008; **200**: 28–36

35 Lu CW, Lin TY, Chiang HS, Wang SJ. Facilitation of glutamate release from rat cerebral cortex nerve terminal by subanesthetic concentration propofol. *Synapse* 2009; **63**: 773–81

36 Newton AC. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem Rev* 2001; **101**: 2353–6