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Sevoflurane-induced cardioprotection depends on PKC-α activation via production of reactive oxygen species

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Background. We previously demonstrated the involvement of the Ca^{2+}-independent protein kinase C-δ (PKC-δ) isoform in sevoflurane-induced cardioprotection against ischaemia and reperfusion (I/R) injury. Since sevoflurane is known to modulate myocardial Ca^{2+}-handling directly, in this study we investigated the role of the Ca^{2+}-dependent PKC-α isoform in sevoflurane-induced cardioprotective signalling in relation to reactive oxygen species (ROS), adenosine triphosphate-sensitive mitochondrial K^+ (mitoK^+_{ATP}) channels, and PKC-δ.

Methods. Preconditioned (15 min 3.8 vol% sevoflurane) isolated rat right ventricular trabeculae were subjected to I/R, consisting of 40 min superfusion with hypoxic, glucose-free buffer, followed by normoxic glucose-containing buffer for 60 min. After reperfusion, contractile recovery was expressed as percentage of force development before I/R. The role of PKC-α, ROS, mitoK^+_{ATP} channels, and PKC-δ was established using the following pharmacological inhibitors: Go6976 (GO; 50 nM), *n*-(2-mercaptopropionyl)-glycine (MPG; 300 μM), 5-hydroxydecanoic acid sodium (5HD; 100 μM), and rottlerin (ROT; 1 μM).

Results. Preconditioning of trabeculae with sevoflurane improved contractile recovery after I/R [65 (3)% (I/R+SEVO) *vs* 47 (3)% (I/R); $n=8$; $P<0.05$]. This cardioprotective effect was attenuated in trabeculae treated with GO [42 (4)% (I/R+SEVO+GO); $P>0.05$ *vs* (I/R)]. In sevoflurane-treated trabeculae, PKC-α translocated towards mitochondria, as shown by immunofluorescent co-localization analysis. GO and MPG, but not 5HD or ROT, abolished this translocation.

Conclusions. Sevoflurane improves post-ischaemic contractile recovery via activation of PKC-α. ROS production, but not opening of mitoK^+_{ATP} channels, precedes PKC-α translocation towards mitochondria. This study shows the involvement of Ca^{2+}-dependent PKC-α in addition to the well-established role of Ca^{2+}-independent PKC isoforms in sevoflurane-induced cardioprotection.

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Sevoflurane protects the myocardium from ischaemia and reperfusion (I/R) injury by eliciting a protective signal transduction cascade.[1] This cardioprotective signalling cascade relies on the production of reactive oxygen species (ROS), activation of protein kinase C (PKC), and opening of ATP-sensitive mitochondrial K^+ (mitoK^+_{ATP}) channels.[2–4] However, the exact mechanisms and target end-effector proteins of sevoflurane-induced cardioprotection remain to be elucidated.

In preconditioning-related signalling cascades, the Ca^{2+}-independent PKC isoforms PKC-δ and -ε have been considered most important,[4–7] whereas less evidence exists for the involvement of Ca^{2+}-dependent PKC isoforms. Nevertheless, in various cardioprotective

signalling cascades, Ca^{2+} has been demonstrated as a central mediator,[8–10] and evidence exists that Ca^{2+}-dependent iso-enzymes are activated during ischaemia[9 11] and other preconditioning stimuli, such as Ca^{2+}-induced preconditioning.[12] Interestingly, since volatile anaesthetics profoundly modulate myocardial Ca^{2+}-handling,[13] activation of Ca^{2+}-dependent PKC isoforms can be expected, but the exact relationship between Ca^{2+}-dependent PKC isoforms and volatile anaesthetic-induced preconditioning has not been established.

In this study, using isolated right ventricular trabeculae of the rat, we investigated whether sevoflurane-induced preconditioning involves activation of the Ca^{2+}-dependent PKC isoform, PKC-α. We specifically focused on the interaction of PKC-α with more established mediators of volatile anaesthetic-induced cardioprotective signalling, that is, ROS and mitoK^+_{ATP} channels, and especially the essential Ca^{2+}-independent PKC isoform PKC-δ.[14]

Methods

Animals and experimental set-up

This study was performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of VU University Medical Center. Our experimental set-up has previously been described in detail.[14 15] Briefly, male Wistar rats (250–400 g; Harlan, The Netherlands) were anaesthetized with sodium pentobarbital (80 mg kg^{-1}, i.p., Nembutal® Sanofi Sante BV). Subsequently, the heart was removed and trabeculae were isolated from the right ventricle (length 2–5 mm, diameter <0.2 mm) under contractile arrest conditions. Trabeculae were mounted in an airtight organ bath, superfused with normal Tyrode buffer consisting of 120 mM NaCl, 1.22 mM $MgSO_4 \cdot 7H_2O$, 1.99 mM NaH_2PO_4, 27.0 mM $NaHCO_3$, 5.0 mM KCl, 1 mM $CaCl_2$, and 10 mM glucose oxygenated with O_2 95% and CO_2 5%. The muscles were then stretched up to 95% of the optimal length determined by the force–length relationship. After stabilization (40 min at 27°C followed by 20 min at 24ºC), the initial developed force of contraction ($F_{dev,start}$) and the potentiated force ($F_{pot,start}$), as determined by a post-extrasystolic potentiation protocol (PESP), were recorded. PESP determines the contractile reserve of trabeculae by maximally filling the sarcoplasmic reticulum with Ca^{2+}.[16] Trabeculae failing to stabilize, spontaneously contracting trabeculae and trabeculae failing to show PESP ($F_{pot,start}$) were excluded.

Experimental protocol

Figure 1 (Groups A–E) demonstrates the experimental design for all groups exposed to the preconditioning protocol. Ischaemia was simulated (except time controls) by superfusion of hypoxic Tyrode (N_2 95% and CO_2 5%) without glucose and increasing the stimulation frequency to 1 Hz. Forty minutes after ischaemic rigor development, trabeculae were reperfused for 60 min with oxygenated normal Tyrode and basal stimulation conditions (0.2 Hz). The recovery of F_{dev} ($F_{dev,rec}$) was expressed as a percentage of the $F_{dev,start}$. Trabeculae were preconditioned for 15 min with normal Tyrode saturated with 3.8 vol% sevoflurane 30 min before simulated ischaemia and reperfusion (SI/R). After washout of sevoflurane,

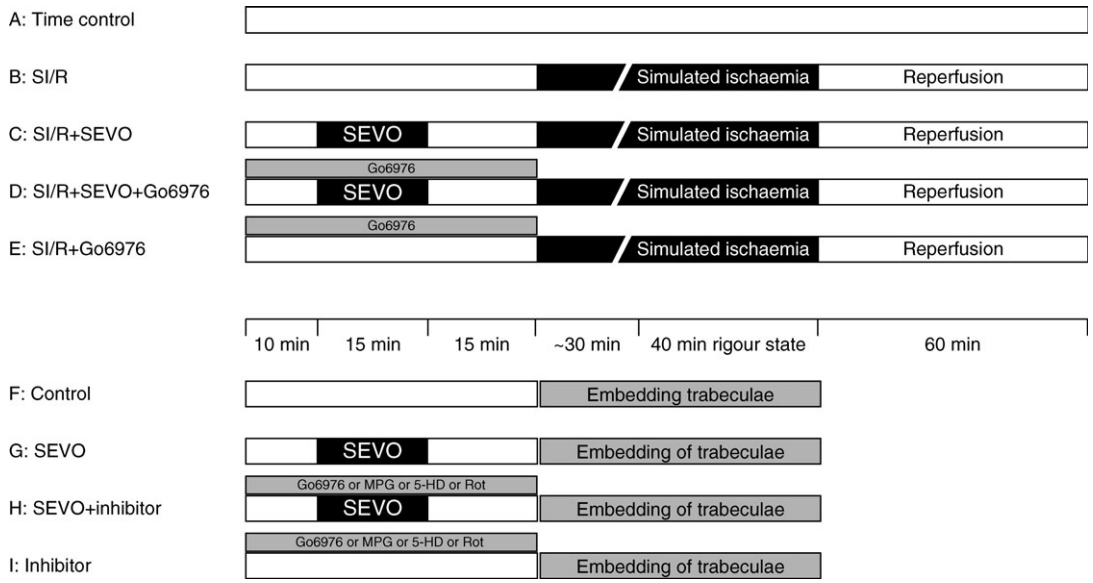

Fig 1 Overview of the randomized experimental design for trabeculae subjected to an I/R-protocol and contractile function measurements (A–E) or immunofluorescent analysis of PKC-α translocation (F–I). SI/R, simulated ischaemia and reperfusion; SEVO, sevoflurane; MPG, *n*-(2-mercaptopropionyl)-glycine (300 μM); 5-HD, 5-hydroxydecanoic acid sodium (100 μM); ROT, rottlerin (1 μM).

trabeculae were superfused for 15 min with normal Tyrode until SI/R. In a separate experimental group, sevoflurane preconditioning was preceded by addition of Go6976 (GO) (50 nM; PKC-α inhibitor; Biomol).

Digital imaging fluorescence microscopy of PKC-α distribution

The sevoflurane-induced subcellular redistribution of PKC-α was studied by immunofluorescent staining followed by digital imaging fluorescence microscopy in trabeculae subjected to sevoflurane alone or in combination with GO (50 nM), *n*-(2-mercaptopropionyl)-glycine (MPG; 300 μM; ROS scavenger; Sigma-Aldrich), 5-hydroxydecanoic acid sodium (5-HD; 100 μM; mitoK^+_{ATP} inhibitor; Sigma-Aldrich), or rottlerin (ROT; 1 μM; PKC-δ inhibitor, Sigma-Aldrich), as described earlier.[14 17] Briefly, trabeculae subjected to an experimental protocol (Fig. 1, Groups F–I) were embedded in gelatin and cross-sections were subsequently fixed, stained for PKC-α [rabbit anti-protein kinase C-α (664–672) serum: aS-2416S (100% cross-reactivity with PKC-α and 0% cross-reactivity with all other PKC isozymes); Research and Diagnostic Antibodies, North Las Vegas, NV, USA] and counterstained for nuclei [4′,6-diamidino-2-phenylindole (DAPI); Vectashield, Vector Laboratories, Burlingame, CA, USA], and mitochondria (anti-GRP75; Stressgen, Ann Arbor, MI, USA), or the sarcolemma [wheat germ agglutinin (WGA); Molecular Probes, Leiden, The Netherlands]. The sections were analysed with a ZEISS Axiovert 200 Marianas™ inverted digital imaging microscope using Slidebook™ software (Slidebook™ version 4.1; 3I, Denver, CO, USA).

Statistical analysis

All values are given as mean (SEM). Force experiments were analysed using analysis of variance followed by a Tukey *post hoc* test. A *P*-value of <0.05 was considered to reflect a significant difference.

Results

General characteristics

Table 1 shows the general characteristics of trabeculae subjected to SI/R (except time controls). In the experimental group SI/R+GO, the time to rigor was slightly prolonged when compared with the time to rigour of the SI/R group. However, there was no correlation between the time to rigour and final contractile recovery, and therefore this difference did not bias our observations.

Table 1 General characteristics of trabeculae subjected to ischaemia and reperfusion. CSA, cross-sectional area; $F_{dev,start}$, initial developed force before the experiment; $F_{pot,start}$, potentiated force development before the experiment; $F_{pas,start}$, passive force before the experiment; SI/R, simulated ischaemia and reperfusion; SEVO, sevoflurane; GO, Go6976. *$P<0.05$ *vs* SI/R and SI/R+SEVO+GO

	CSA (mm^2)	$F_{dev,start}$ ($mN\ mm^{-2}$)	$F_{dev,start}/F_{pot,start}$ (%)	$F_{pas,start}$ ($mN\ mm^{-2}$)	Time to rigor (min)
Time control	0.06 (0.02)	51 (9)	62 (5)	2.1 (0.4)	
SI/R	0.04 (0.010	52 (8)	47 (5)	4.2 (1.3)	28 (3)
SI/R+SEVO	0.06 (0.01)	57 (10)	47 (6)	4.5 (1.1)	29 (2)
SI/R +SEVO+GO	0.05 (0.01)	43 (7)	46 (6)	2.6 (0.5)	27 (2)
SI/R+GO	0.02 (0.002)	60 (11)	48 (7)	2.8 (0.5)	40 (4)*

Sevoflurane-induced cardioprotection depends on PKC-α

At the end of the full experimental protocol (170 min), F_{dev} decreased to 79 (7)% [n=6; (time control)] whereas SI/R reduced the contractile recovery ($F_{dev,rec}$) to 47 (3)% [(SI/R); n=9; $P<0.05$ *vs* (time control)] (Fig. 2). Pre-treatment with sevoflurane improved the $F_{dev,rec}$ to 65 (3)% [(SI/R+SEVO); n=8; $P<0.05$ *vs* (SI/R)]. In trabeculae with PKC-α inhibition by GO, sevoflurane-induced cardioprotection was completely abolished [42 (4)% [(SI/R+SEVO+GO); n=10; $P<0.05$ *vs* (SI/R+SEVO)]. PKC-α inhibition by GO before SI/R did not show intrinsic activity on $F_{dev,rec}$ [40 (6)% (SI/R+GO); n=5, $P>0.05$ *vs* (SI/R)].

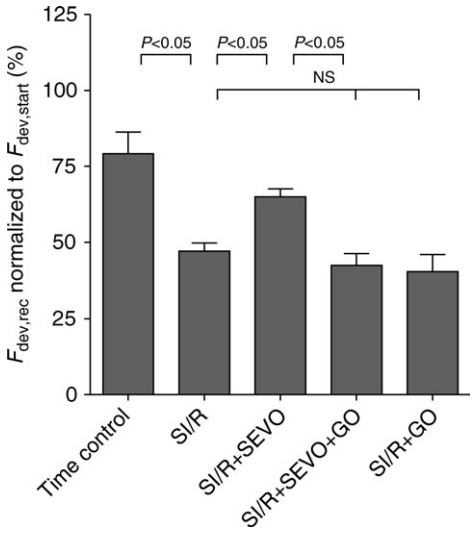

Fig 2 The role of PKC-α in sevoflurane-induced cardioprotection. Recovery of the developed force ($F_{dev,rec}$) is expressed as a fraction of the initial developed force ($F_{dev,start}$). Pretreatment with sevoflurane increased the post-ischaemic contractile force recovery and this protective effect was attenuated in trabeculae treated with Go6976 (50 nM). SI/R, simulated ischaemia and reperfusion; SEVO, sevoflurane; GO, Go6976. Data are mean (SEM); time control, n=6; SI/R, n=9; SI/R+SEVO, n=8; SI/R+SEVO+GO, n=10; SI/R+GO, n=5.

PKC-α translocation in response to sevoflurane

In order to elucidate further the role of PKC-α activation in sevoflurane-induced cardioprotection, the subcellular distribution of PKC-α was evaluated in direct response to sevoflurane. Figure 3A shows the translocation pattern of PKC-α in response to sevoflurane and sevoflurane in combination with GO in cross-sections of isolated trabeculae. In Figure 3A, green represents the specific PKC-α staining, blue the nuclear DAPI staining, and red the WGA staining of the sarcolemma. In control trabeculae (Fig. 3A, Control), PKC-α is distributed homogeneously in the cytosol. In response to sevoflurane, PKC-α predominantly translocated into distinct particles (Fig. 3A, SEVO, white arrows), which co-localized with mitochondrial heat shock protein GRP75 (Fig. 3B, white arrows point to corresponding particles). Correlation factors between the green channel (PKC-α) and red channel (GRP75 in mitochondrial matrix) were calculated to quantify sevoflurane-induced PKC-α translocation into the mitochondria. The correlation factor, as determined by the imaging software, in controls was 0.22 and increased by sevoflurane treatment to 0.59 (maximal correlation factor is 1). In addition, occasional larger patches (interstitial cells) and more linear, membrane-like staining of PKC-α (sarcolemmal or subsarcolemmal structures) were also seen. Most importantly, PKC-α translocation was not detected in trabeculae treated with GO (Fig. 3A, SEVO+GO; the correlation factor between PKC-α and GRP75 in mitochondria decreased to 0.32) and GO treatment itself did not affect PKC-α translocation (not shown). To identify whether PKC-α translocation occurs upstream or downstream of ROS-production, opening of mitoK$^+_{ATP}$ channels, or PKC-δ activation, PKC-α translocation was studied in trabeculae treated with MPG, 5-HD, or ROT, respectively (Fig. 4). In trabeculae exposed to MPG, the sevoflurane-induced translocation of PKC-α was abolished (Fig. 4, SEVO+MPG). Interestingly, sevoflurane-induced PKC-α translocation was not abolished by 5-HD (Fig. 4, SEVO+5HD) or ROT (Fig. 4, SEVO+ROT). These data suggest that sevoflurane activates PKC-α via ROS-production, but opening of mitoK$^+_{ATP}$ channels and activation of PKC-δ are not involved or act downstream of PKC-α activation.

Discussion

The present study shows for the first time that, in addition to the previously described role of the Ca^{2+}-independent PKC-δ isoform, sevoflurane-induced cardioprotection also involves activation of the Ca^{2+}-dependent isoform PKC-α. In addition, sevoflurane-induced translocation of PKC-α is dependent on the production of ROS, occurs upstream of opening of mitoK$^+_{ATP}$ channels, and is independent of PKC-δ activation.

Cardioprotective signalling elicited by pre-conditioning has been demonstrated to rely on PKC activation. However, the sequences of events and the specific PKC isoforms involved in this protective cascade have been widely discussed.[2 4 18] PKC-δ and -ε have been considered most important in ischaemic,[19] opioid,[20] and volatile anaesthetic-induced preconditioning.[6 7] Indeed, we previously demonstrated that sevoflurane-induced cardioprotective responses in our model depend on PKC-δ activation, whereas PKC-ε is not primarily involved. Furthermore, we showed that PKC-δ translocation in response to sevoflurane depends on ROS production, but not opening of mitoK$^+_{ATP}$ channels.[14] Importantly, the present data show for the first time that the Ca^{2+}-dependent PKC-α isoform is activated by sevoflurane and is also involved in sevoflurane-induced cardioprotective signalling. It has previously been shown that volatile anaesthetics are able to increase PKC-α activity in purified rat brain extracts and skeletal muscle.[21] In addition, PKC-α is activated, in response to other preconditioning stimuli, such as ischaemia,[11 22] phenylepinephrine,[23] Ca^{2+},[9] and opioids,[20] and specific PKC-α inhibition has been reported to abolish cardioprotection induced by ischaemic preconditioning (IPC).[24 25]

Our data suggest that PKC-α is activated by sevoflurane via the formation of ROS, but not opening of mitoK$^+_{ATP}$ channels. This ROS-dependent activation of PKC-α has been supported by Hoek and colleagues[25] demonstrating that PKC-α inhibition attenuated cardioprotection by IPC, but did not affect ROS-production during pre-conditioning. However, in contrast to our findings Hassouna and colleagues[24] showed that IPC and diazoxide-induced preconditioning involved PKC-α acting downstream of the mitoK$^+_{ATP}$ channels. In addition, it is reported that upon pre-conditioning with the mitoK$^+_{ATP}$ channel opener diazoxide, PKC-α translocated towards the sarcolemma.[26] Diazoxide and sevoflurane increase intracellular ROS production, and scavenging ROS with MPG attenuates subsequent pre-conditioning.[14 27] However, sevoflurane-induced ROS production has been reported to be independent of mitoK$^+_{ATP}$ channels,[28] and therefore, the explanation underlying these contradictory findings may relate to differences in the mechanism of ROS production.

How sevoflurane-induced PKC activation contributes to the improved contractile recovery after I/R injury remains speculative. PKC-α has been demonstrated to be actively involved in the regulation of cell survival and apoptosis,[29 30] in addition to direct involvement in the regulation of cardiomyocyte contractility.[31–34] Although others showed that PKC-α translocates towards the sarcolemma in response to Ca^{2+} and ischaemia,[9] we found that PKC-α translocated towards mitochondria. This implies that direct modification of contractile or Ca^{2+}-handling proteins in our model may be less likely. Interestingly, mitochondrial PKC-α has been associated with increased phosphorylation of the anti-apoptotic protein Bcl2.[35] Recently, Imahashi and colleagues[36] showed that modulation of myocardial energy metabolism by Bcl2 over expression

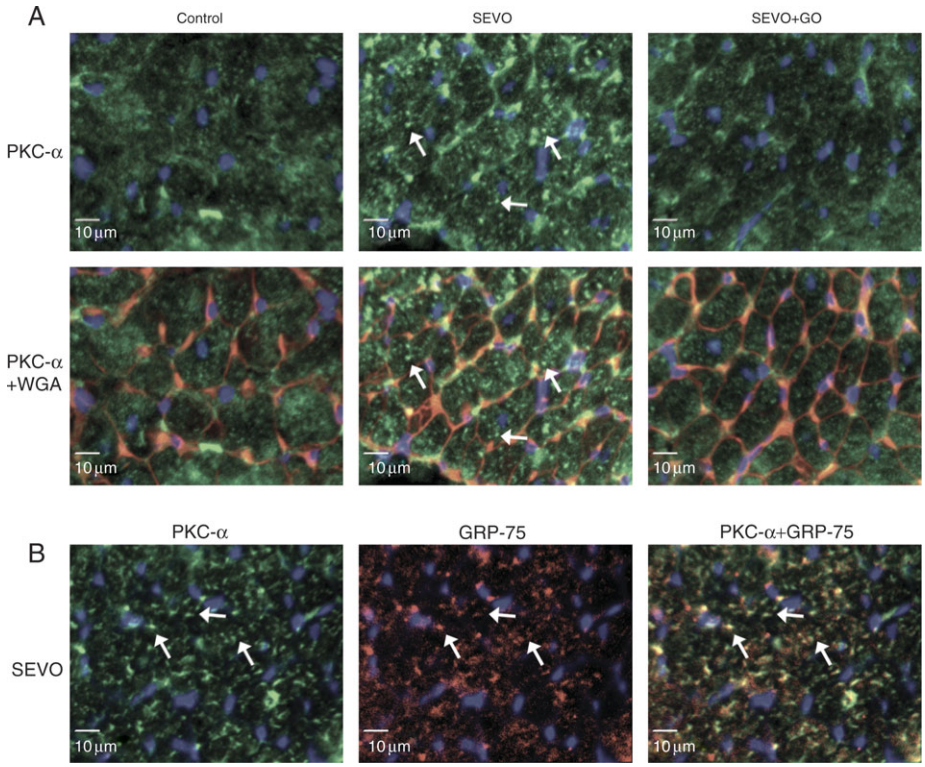

Fig 3 PKC-α translocation in response to sevoflurane in cross-sections of isolated rat trabeculae. (A) In all panels, green represents specific PKC-α staining, blue represents DAPI staining of the nuclei and red represents WGA staining of the sarcolemma. In control trabeculae, PKC-α is predominantly located in the cytosol. After sevoflurane exposure, PKC-α is detected in circumscript dots located within the cytosol (SEVO, white arrows). This sevoflurane-induced PKC-α translocation is effectively attenuated by Go6976 (50 nM) (SEVO+GO). Go6976 itself had no intrinsic effect on PKC-α translocation. (B) PKC-α colocalizes with the mitochondria after sevoflurane-induced preconditioning. In these panels, green represents specific PKC-α staining, blue the DAPI staining of the nuclei, and red the specific antibody against mitochondrial heat-shock protein GRP75. After sevoflurane preconditioning, PKC-α (PKC-α panel, white arrows) translocates towards mitochondria (GRP75 panel, white arrows) indicated by yellow in co-localization analyses (PKC-α+GRP75 panel, white arrows). SEVO, sevoflurane; GO, Go6976.

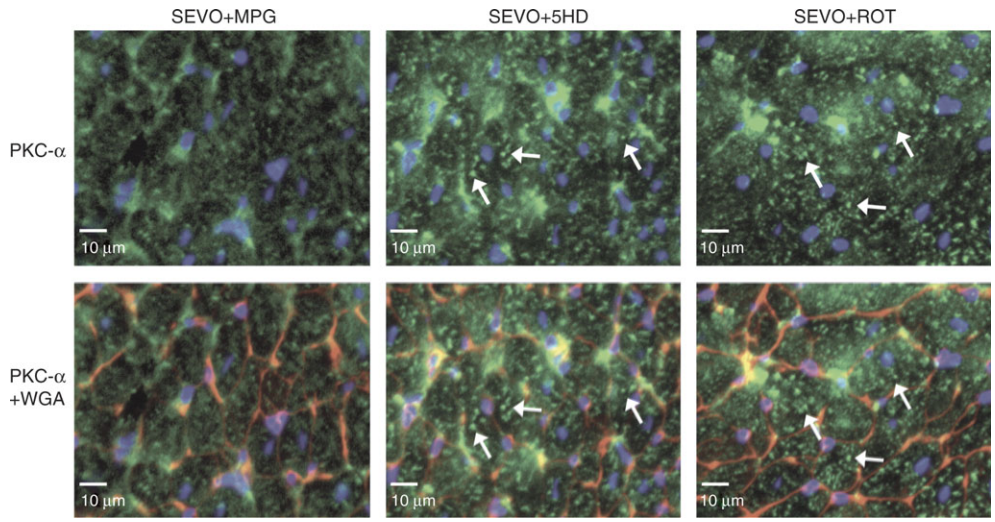

Fig 4 Sevoflurane-induced PKC-α tranlocation in relation to common mediators of cardioprotective signal transduction pathways. In all panels, green represents specific PKC-α staining, blue the DAPI staining of the nuclei, and red the WGA staining of the sarcolemma. Sevoflurane-induced a translocation of PKC-α from the cytosol towards the mitochondria (Fig. 3). This translocation was reduced by scavenging ROS during preconditioning with MPG (300 μM) (SEVO+MPG panel). Interestingly, sevoflurane-induced PKC-α translocation was not attenuated by 5HD (100 μM) (SEVO+5HD panel) or by ROT (1 μM) (SEVO+ROT panel).

contributed to increased recovery after I/R. We therefore speculate that this may be among the potential mitochondrial targets of PKC-α. As we previously demonstrated,[14] PKC-δ translocated to the sarcolemma in response to sevoflurane preconditioning, but the exact target remains speculative. Target proteins may include Ca^{2+}-handling proteins, which could contribute to a reduction in Ca^{2+} overload during I/R. We recently demonstrated that PKC-δ translocation in response to sevoflurane was dependent on the reverse mode of the Na^{+}/Ca^{2+}-exchanger illustrating the intimate relationship between Ca^{2+}-handling and cardioprotective signalling.[37] However, other potential targets in the sarcolemma have been suggested, including PKC-δ-dependent sensitization of the sarcolemmal ATP-sensitive potassium channel.[38] Future studies in our laboratory will be aimed to examine the relative role of the different PKC-isoforms involved in volatile anaesthetic-induced cardioprotection.

Serial signalling steps between different PKC-isoforms have been reported to occur in several cardioprotective signalling cascades. Recently, Inagaki and Mochly-Rosen[39] showed that ethanol-induced cardioprotection relies on both PKC-δ and PKC-ϵ activation. In addition, serial PKC signalling was also suggested in human atrial appendages with respect to PKC-ϵ and PKC-α.[24] In contrast, the present study shows that PKC-α activation is independent of PKC-δ, although others showed that PKC-α increases PKC-δ protein levels via stabilization of PKC-δ mRNA.[40] Therefore, serial PKC signalling with respect to PKC-α and PKC-δ seems to be less likely in our model, because of the acute and parallel activation of different isoforms by sevoflurane.

Finally, the finding that the Ca^{2+}-dependent PKC-α isoform contributes to sevoflurane-induced protective signalling may be relevant for the clinical induction of cardioprotection in the pathological myocardium. Alterations in myocardial Ca^{2+}-handling and expression of PKC-isoforms have been demonstrated in diseased myocardium, that is, because of heart failure. PKC-α activity is dose-dependently modified by Ca^{2+},[41] and likewise its activity and expression are increased in failing myocardium.[42] This may contribute to alterations in sevoflurane-induced cardioprotective signalling in diseased hearts.

Study limitations

This study largely depends on the specificity of the indolocarbazole compound GO. GO has been described to discriminate effectively between Ca^{2+}-dependent PKC isoforms with no effect on the novel PKC isoforms.[43] In the present study, we focused on PKC-α, as representative of the Ca^{2+}-dependent PKC isoforms, in the signalling cascade of sevoflurane-induced protective signalling. The specificity of GO was confirmed as PKC-α translocation induced by sevoflurane was abolished by this inhibitor. However, with this approach, we cannot entirely exclude the involvement of the other Ca^{2+}-dependent isoform PKC-β.

In summary, in addition to the previously described role of the Ca^{2+}-independent PKC-δ isoform, sevoflurane-induced preconditioning also depends on activation of the Ca^{2+}-dependent PKC-α isoform. During sevoflurane-induced preconditioning, PKC-α translocates towards the mitochondria. This mitochondrial translocation of PKC-α is dependent on the production of ROS, but is independent of opening of mitoK^{+}_{ATP} channels and PKC-δ. Although the exact relationship between PKC-α and PKC-δ remains to be established, the co-involvement of the Ca^{2+}-dependent PKC-α isoform in sevoflurane-induced cardioprotection may be of clinical relevance for the development of cardioprotective strategies in the diseased myocardium with altered Ca^{2+} handling.

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