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Upstream signaling of protein kinase C-ε in xenon-induced pharmacological preconditioning
Implication of mitochondrial adenosine triphosphate dependent potassium channels and phosphatidylinositol-dependent kinase-1

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Abstract

Xenon elicits preconditioning of the myocardium via protein kinase C-ε. We determined the implication of (1) the mitochondrial adenosine-triphosphate dependent potassium (K_{ATP}) channels and (2) the 3′phosphatidylinositol-dependent kinase-1 (PDK-1) in activating protein kinase C-ε.

For infarct size measurements, anaesthetized rats were subjected to 25 min of coronary artery occlusion followed by 120 min of reperfusion. Rats received xenon 70% during three 5-min periods before ischaemia with or without the K_{ATP} channel blocker 5-hydroxydecanoate or Wortmannin as PI_3K/PDK-1 inhibitor. For Western blot, hearts were excised at five time points after xenon preconditioning (Control, 15, 25, 35, 45 min).

Infarct size was reduced from 42±6% (mean±S.D.) to 27±8% after xenon preconditioning ($P<0.05$). Western blot revealed an increased activation of PKC-ε after 45 min and of PDK-1 after 25 min during xenon preconditioning. 5-hydroxydecanoate and Wortmannin blocked both effects.

PKC-ε is activated downstream of mitochondrial K_{ATP} channels and PDK-1. Both pathways are functionally involved in xenon preconditioning.

Keywords: Anaesthetic preconditioning; Xenon; Cardiac preconditioning; Mitochondrial adenosine triphosphate dependent potassium channel; Protein kinase C; 3′Phosphatidylinositol-dependent kinase-1

1. Introduction

The halogenated fluorocarbons used as volatile anaesthetics have been shown to mimic ischaemic preconditioning, the strongest endogenous protection mechanism of the heart (Cason et al., 1997). This so called pharmacological or anaesthetic preconditioning results in a pronounced myocardial protection against further cellular damage in vivo (Mullenheim et al., 2002, 2003). In this context, we could recently show that the "chemically" inert and anaesthetic gas xenon induces preconditioning of the heart (Weber et al., 2005a). Xenon has only minimal haemodynamic and cardiovascular side effects (Schmidt et al., 2001; Preckel et al., 2002a,b) in comparison with the more pronounced haemodynamic effects of the volatile anaesthetics (Preckel et al., 2002a,b; Rossaint et al., 2003). Therefore, this inert gas might become a suitable anaesthetic for patients at high-risk for perioperative cardiac events.

Concerning the underlying molecular mechanism of xenon preconditioning, protein kinase C (PKC)-ε and its downstream target p38 mitogen activated protein kinase (MAPK) were identified as key mediators in the signal transduction (Weber et al., 2005a). Moreover, downstream of PKC, the MAPK-activated protein kinase 2 (MAPKAPK-2), the small heat shock protein 27 (HSP27) and actin filaments are implicated in mediation of xenon-induced myocardial protection (Weber et al., 2005b).

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However, the signalling pathways leading to the activation of the key mediator enzyme PKC in xenon-induced preconditioning are completely unknown. There exist two putative pathways that may lead to activation of PKC: First, it might be activated by phosphorylation of a threonine or serine residue in the activation loop by the 3′phosphatidylinositol-dependent kinase-1 (PDK-1) (Parekh et al., 2000; Le Good et al., 1998), leading to subsequent autophosphorylation of PKC. Second, the enzyme can be activated by mitochondrial adenosine triphosphate dependent potassium channel (K_{ATP} channel) dependent release of free radicals (Gopalakrishna and Anderson, 1989; Gopalakrishna and Jaken, 2000).

It has been shown that free radicals are generated by opening of K_{ATP} channel channels in the heart (Pain et al., 2000) and this leads to activation of PKC. However, in turn PKC activation itself can cause opening of K_{ATP} channels. Consequently, there exists a strong controversy about the causal relationship between PKC and this activation pathway.

The present study aimed to clarify which of the above mentioned pathways leads to the pronounced activation of PKC-ε. We assume that there exists a causal relationship of K_{ATP} channels, PDK-1 and PKC-ε in the signalling cascade of xenon-induced preconditioning.

Since to date there exist only few data addressing the exact molecular mechanism of preconditioning induced by the inert gas xenon, the results of the present study will contribute to elucidate the cellular mechanism of xenon-induced pharmacological preconditioning.

2. Materials and methods

The study was performed in accordance with the regulations of the German Animal Protection Law. Moreover, it was approved by the Animal Care Committee of the District of Düsseldorf. Male Wistar rats [(200–250 g), 10 per group for infarct size measurement, 4 per group for Western blot comparison, were anaesthetized by intraperitoneal S-ketamine injection (150 mg/kg)]. Animals had free access to food and water at all times before the start of the experiments.

2.1. Materials

Xenon was kindly provided by Messer Griessheim GmbH (Krefeld, Germany). Wortmannin, 5-hydroxydecanoate (5-HD) and monoclonal anti-α-tubulin mouse antibody were purchased from Sigma (Taufkirchen, Germany). The enhanced chemiluminescence protein detection kit was purchased from Santa Cruz (Heidelberg, Germany). Total PKC-ε rabbit polyclonal antibody was from Upstate (Charlottesville, USA). Peroxidase-conjugated goat anti-rabbit and donkey anti-mouse antibodies were from Jackson Immunolab (Dianova, Hamburg, Germany), phospho PKC-ε rabbit, phospho PDK-1 [Serine 241 and Tyrosine (373/376)] rabbit and total PDK-1 rabbit antibodies were from Cell Signaling (Frankfurt/M, Germany). During performance of the experiments the antibody detecting Tyrosine (373/376) phosphorylation of PDK-1 could not further be provided by Cell signaling. Therefore, not all experiments could be performed for both phosphorylation sites of this enzyme. Triphenyltetrazoliumchloride, Coomassie blue and all other materials were either purchased from Sigma (Taufkirchen, Germany) or Merck-Eurolab (Munich, Germany).

2.2. Animal preparation

Male Wistar rats (200–250 g) were anaesthetized by intraperitoneal S-ketamine injection (150 mg/kg). Further preparation and infarct size measurement by Triphenyltetrazoliumchloride staining were performed as described previously (Weber et al., 2005a; Toma et al., 2004). The area of risk and the infarcted area were determined by planimetry using SigmaScan Pro 5® computer software (SPSS Science Software, Chicago, IL) and corrected for dry weight.

2.3. Measurement of haemodynamic variables

Aortic pressure was digitized using an analogue to digital converter (PowerLab/8SP, ADInstruments Pty Ltd, Castle Hill, Australia) at a sampling rate of 500 Hz and were continuously recorded on a personal computer using Chart for Windows v5.0 (ADInstruments Pty Ltd, Castle Hill, Australia).

2.4. Experimental protocol for infarct size measurement

Rats were divided into six groups
Fig.1A:

Control group ($n=10$): rats received 25% oxygen plus 75% nitrogen during 3 times 5-min before they were subjected to 25 min of left coronary artery occlusion.

Xenon preconditioned group ($n=10$): rats received xenon 70% (equivalent to 0.43 minimal alveolar concentration (MAC) in rats) for three 5-min periods, interspersed with two 5-min washout periods 10 min before the 25 min coronary artery occlusion. The 30% rest gas consisted of 5% nitrogen and 25% oxygen.

Control with 5-HD group ($n=10$): 5-HD (5 mg/kg) was intravenously administered 45 min before the 25 min coronary artery occlusion.

Xenon + 5-HD group ($n=10$): xenon preconditioned rats received intravenous 5-HD (5 mg/kg) 10 min before xenon administration.

Control with Wortmannin group ($n=10$): Wortmannin (15 μg/kg) was administered intravenously 45 min before the 25 min coronary artery occlusion.

Xenon + Wortmannin group ($n=10$): xenon preconditioned rats received Wortmannin (15 μg/kg) intravenously 10 min before xenon administration.

2.5. Experimental protocol for Western blot

Rats were divided into 10 groups (Fig. 1B and C)
Fig.1B:

Control group ($n=4$): After surgical preparation, rats received 25% oxygen plus 75% nitrogen during 3 times 5-min before the hearts were excised.

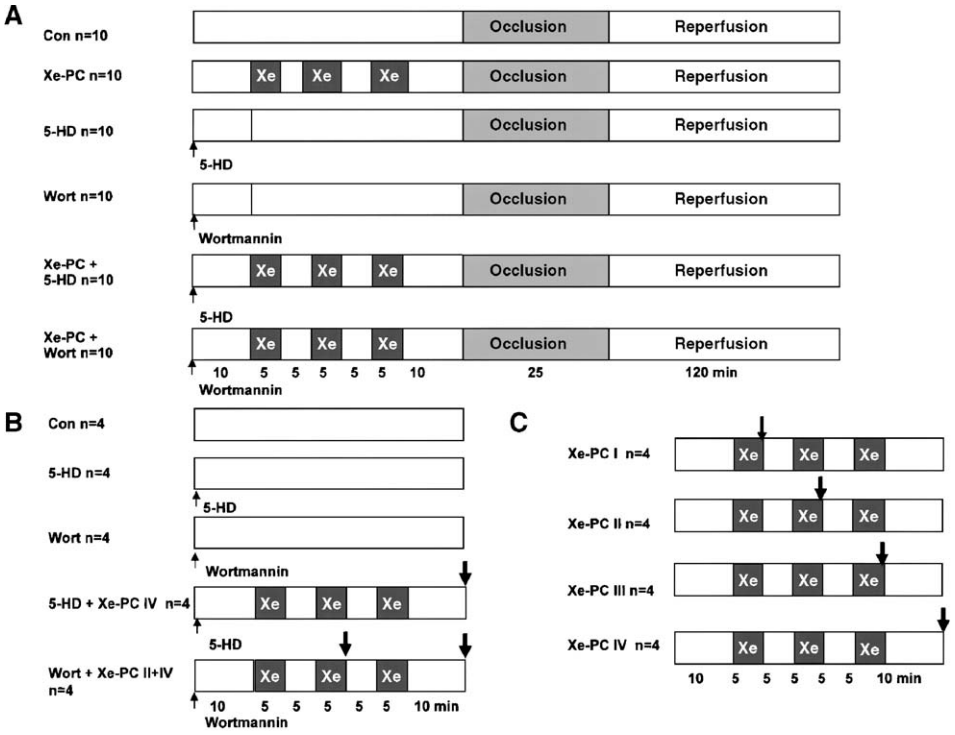

Fig. 1. Panel A. Experimental protocol for infarct size measurement. Con = control, Xenon preconditioning = Xe-PC, Wort = Wortmannin, 5-HD = 5-hydoxydecanoate. Panel B + Panel C. Experimental protocol for Western blot assay: Con = control, Xenon preconditioning = Xe-PC, Wort = Wortmannin, 5-HD = 5-hydoxydecanoate, Xe-PC I–IV = four different time points during the xenon preconditioning protocol.

Control with 5-HD group ($n=4$): 5-HD (5 mg/kg) was administered intravenously 45 min before the hearts were excised.

Xenon preconditioning IV + 5-HD group ($n+4$): xenon preconditioned rats received 5-HD (5 mg/kg) intravenously 10 min before xenon administration.

Control with Wortmannin group ($n=4$): Wortmannin (15 μg/kg) was administered intravenously 45 min before excision of the hearts.

Xenon preconditioning II and IV + Wortmannin group ($n=4$): xenon preconditioned rats received Wortmannin (15 μg/kg) intravenously 10 min before xenon administration and hearts were either excised after the second xenon preconditioning cycle or after the total of 45 min (Xe-PC IV).

Fig. 1C: Time course of xenon preconditioning:

Xenon preconditioned group I–IV (Xe-PC I–IV) (each, $n=4$): rats received xenon 70% for one (I), two (II), three (III) 5-min period or for three 5-min periods + 10 min washout before excision of the hearts.

2.6. Separation of membrane and cytosolic fraction

For tissue fractionation and subsequent Western blot assay, another 10 groups (see Fig. 1B + C, each $n=4$) were subjected to similar treatment as described above, but the hearts were excised at the end of the last washout period. Tissue specimens were prepared for protein analysis to investigate PKC-ε activation and distribution (membrane-, cytosolic-fraction) within the tissues. The excised hearts were frozen in liquid nitrogen. Subsequently, a tissue fractionation was performed that was adapted from the literature (Kang et al., 1999; Mackay and Mochly-Rosen, 2001). The frozen tissue was pulverized and dissolved in lysis buffer containing: Tris base, ethyleneglycol-*bis*-(β-aminoethylether)-*N*, *N*, *N′*, *N′*-tetraacetic acid (EGTA), sodium fluoride (NaF) and sodium vanadate (Na_3VO_4) (as phosphatase inhibitors), a freshly added protease inhibitor mix (aprotinin, leupeptin and pepstatin), 100 μM/ml okadaeic-acid and dithiothreitol (DTT). The solution was vigorously homogenized on ice (Homogenisator, IKA) and then centrifuged at 1000 *g*, 4 °C, for 10 min. The supernatant, containing the cytosolic fraction, was centrifuged again at 16000 *g*, 4 °C, for 15 min to clean up this fraction. The remaining pellet was resuspended in lysis buffer containing 1% Triton X100, incubated for 60 min on ice and vortexed. The solution was centrifuged at 16000 *g*, 4 °C, for 15 min and the supernatant was collected as membrane fraction.

2.7. Western blot analysis

After protein determination by the Lowry method (Lowry, 1951), equal amounts of protein were mixed with loading buffer containing Tris–HCl, glycerol and bromphenol blue. Samples were vortexed and boiled at 95 °C before being subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% SDS electrophoresis gel). The proteins were separated by electrophoresis and then transferred to a polyvenylidene fluoride (PVDF) membrane by tank blotting. Unspecific binding of the antibody was blocked by incubation

with 5% fat dry milk powder solution in Tris buffered saline containing Tween (TBS-T) for 2 h. Subsequently, the membrane was incubated over night at 4 °C with the respective first antibody at indicated concentrations. After washing in fresh, cold TBS-T, the blot was subjected to the appropriate horseradish peroxidase conjugated secondary antibody for 2 h at room temperature. Immunoreactive bands were visualized by chemiluminescence detected on X-ray film (Hyperfilm ECL, Amersham) using the enhanced chemiluminescence system Santa Cruz. Equal loading of the protein to the SDS Page gel was ensured by Coomassie blue staining (Coomassie brillant blue®, Serva electrophoresis GmbH, Heidelberg, Germany) of each gel. The blots were quantified using a Kodak Image station® (Eastman Kodak Comp., Rochester, NY) and the results are presented as a ratio of phosphorylated protein to total protein [average light intensity, AVI]. The values are expressed as *x*-fold compared with the control value.

2.8. Statistical analysis

Data are expressed as means±standard deviation (S.D.). Group comparisons were analyzed by one way analysis of variance between groups (ANOVA) followed by Bonferroni's correction for multiple comparisons. A $P<0.05$ was considered statistically significant.

3. Results

3.1. Infarct size measurement

Xenon preconditioning reduced the infarct size compared with controls (27±8% vs. 42±6% of area at risk, $P<0.05$, Fig. 2). The blocker of the mitochondrial K_{ATP} channels 5-HD abolished the cardioprotective effect of xenon preconditioning (40±12%, $P<0.05$ vs. xenon preconditioning, Fig. 2). 5-HD alone had no effect on infarct size. The second inhibitor, Wortmannin (15 μg/kg), a blocker of the PI_3K/PDK-1 pathway, had no effect on infarct size in unpreconditioned hearts (39±13%), but abolished the cardioprotection of xenon preconditioning (41±8%, $P<0.05$ vs. xenon preconditioning, Fig. 2). These data demonstrate an involvement of both targeted pathways in the preconditioning effect of xenon.

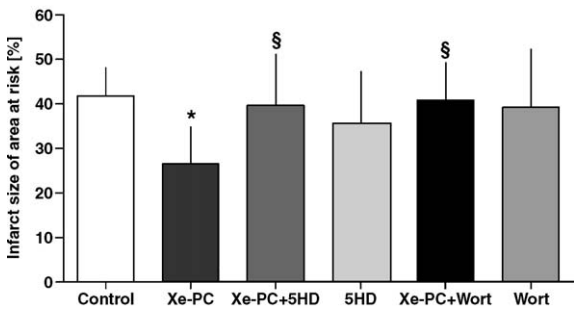

Fig. 2. Infarct size. Histogram shows the infarct size (percent of area at risk) of controls, xenon preconditioning (Xe-PC), 5-hydroxydecanoate (5-HD) alone, Wortmannin alone, xenon preconditioning+5-HD and Xe-PC+Wortmannin group. Data show means±S.D., *$P<0.05$ vs. control group, §$P<0.05$ vs. xenon preconditioning.

During the procedure, no significant differences in heart rate and aortic pressure were observed between the experimental groups during baseline, ischaemia or the reperfusion period (data not shown).

3.2. Causal relationship between PKC-ε activation and K_{ATP} channels in xenon preconditioning

From our recent investigations we know that PKC-ε phosphorylation and translocation is significantly increased after the whole preconditioning protocol with xenon (e.g. 45 min after the start of the experiment=xenon preconditioning IV) (Weber et al., 2005a). Thus, PKC-ε phosphorylation was determined at time point Xe-PC IV using a phospho-specific antibody against PKC-ε. Xenon leads to a marked phosphorylation of PKC-ε compared with controls after 45 min (1.4±0.6 vs. 1.0±0.5, $P<0.05$, Fig. 3A). Changes in phosphorylation of PKC-ε were not due to different amounts of PKC-ε as the Western blot using an antibody against total PKC-ε (Fig. 3A, lower Western blot) showed a uniform distribution of total PKC-ε. 5-HD alone had no significant effect on PKC-ε phosphorylation (0.9±0.3, $P>0.05$ vs. control), but completely abolished the effect of xenon on PKC-ε phosphorylation (0.8±0.5, $P<0.05$ vs. xenon preconditioning).

PKC-ε is not only activated by phosphorylation, but also by translocation to membranes. Both activation steps can occur in parallel and also independently of each other. Western blot assay of fractionated tissue (Fig. 3B) clearly revealed that xenon preconditioning increased the translocation of PKC-ε to membrane regions (ratio membrane to cytosol PKC-ε: 5.4±3.1 vs. 1.0±0.6 in controls, $P<0.05$, Fig. 3B). Again, blockade of mitochondrial K_{ATP} channels abolished this effect (1.5±0.4 vs. 5.4±3.1 after xenon preconditioning IV) while 5-HD alone had no effect on translocation of PKC-ε (0.7±0.5 vs. controls). α-Tubulin detection served as loading control (Fig. 3B, lower Western blot). The absence of PKC activation after xenon preconditioning in the presence of a mitochondrial K_{ATP} channel blockade suggests that opening of the mitochondrial K_{ATP} channels occurs upstream in the signaling cascade of xenon-induced preconditioning.

3.3. Time dependent activation of PDK-1 during xenon preconditioning protocol

For Western blot of PDK-1, hearts were excised at five different time points (each $n=4$): without further treatment (Con), after the first (xenon preconditioning I, 15 min), the second (xenon preconditioning II, 25 min) and the third period of xenon preconditioning (xenon preconditioning III, 35 min) or directly before ischaemia (xenon preconditioning IV, 45 min). The phosphorylation of PDK-1 at Tyrosine (373/376) is important for the catalytic activity of this enzyme. Moreover, PDK-1 becomes phosphorylated at various Serine sites and the autophosphorylation at Serine 241 on the activation loop is critically important for complete activation of PDK-1. Therefore, we investigated both phosphorylation sites [(Serine 241 and Tyrosine (373/376)] of PDK-1 in the time course series. The results

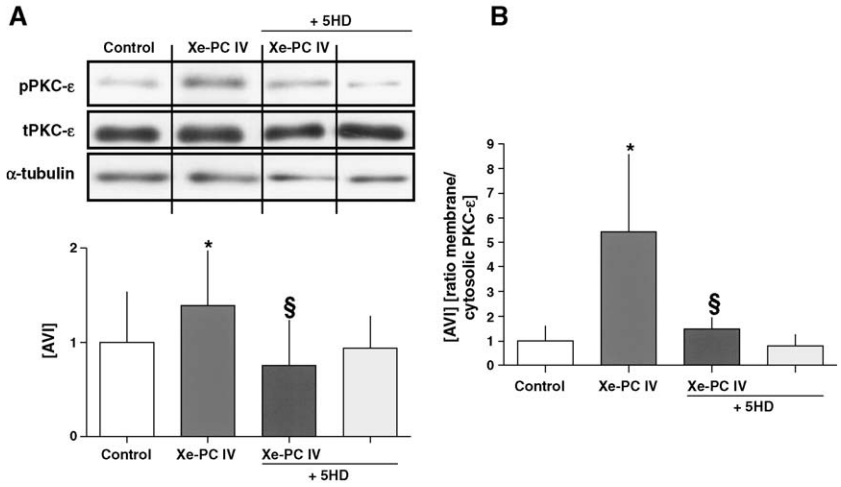

Fig. 3. Panel A. Phosphorylation of PKC-ε after K_{ATP} channel blockade. One representative Western blot experiment of cytosolic fraction of control and xenon (Xe-PC IV) treated hearts in the presence or absence of 5-hydroxydecanoate (5-HD) (each $n=4$) is shown. Upper panel shows phosphorylated form of PKC-ε, lower panel total PKC-ε. The histogram presents densitometric evaluation as average light intensity (AVI). Data show ratio of phosphorylated versus total PKC-ε (means ± S.D.). The values are expressed as *x*-fold compared with the control values. α-Tubulin served as internal standard. *$P<0.05$ vs. control, §$P<0.05$ vs. xenon preconditioning. Panel B. Translocation of PKC-ε after K_{ATP} channel blockade. Membrane and cytosolic fraction of control, Xe-PC IV, Xe-PC IV + 5-HD and 5-HD alone hearts (each $n=4$) were immunoblotted using antibodies against PKC-ε or α-tubulin. The histogram presents densitometric evaluation as average light intensity (AVI). Data are means ± S.D. and the ratio of membrane PKC-ε to cytosolic PKC-ε is shown. The values are expressed as *x*-fold compared with the control values. *$P<0.05$ vs. control group and §$P<0.05$ vs. xenon preconditioning.

demonstrate that both phosphorylation sites were influenced time dependently by xenon preconditioning (Fig. 4A + B), [Serine 241 phosphorylation: xenon preconditioning I: 1.5 ± 0.5, xenon preconditioning II: 1.5 ± 0.6, xenon preconditioning III: 1.4 ± 0.7, xenon preconditioning IV: 1.0 ± 0.2 vs. Con 1.0 ± 0.4 and Tyrosine (373/376): Xenon preconditioning I: 0.9 ± 0.4, xenon preconditioning II: 1.6 ± 1.1, xenon preconditioning III: 1.1 ± 0.7, xenon preconditioning IV: 0.9 ± 0.8 vs. Con 1.0 ± 0.8]. These findings show that xenon preconditioning time dependently activates PDK-1 and that this activation occurs before PKC-ε is activated.

3.4. Causal relationship between PKC-ε and PDK-1

To determine how xenon mediated PKC-ε activation is causally linked to the observed increase of PDK-1 phosphorylation,

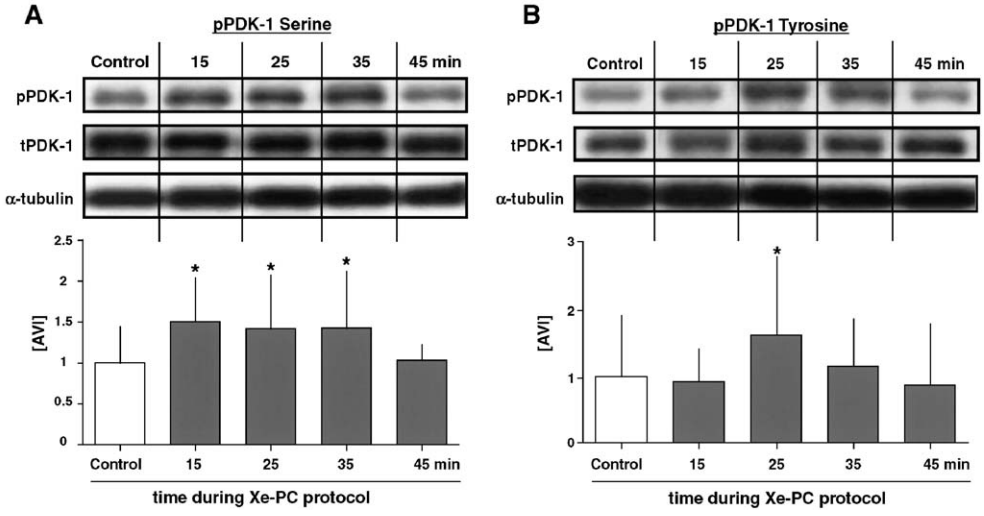

Fig. 4. Panel A. Time dependent phosphorylation of PDK-1 at Serine 241 during the xenon preconditioning protocol. One representative Western blot experiment of cytosolic fraction of control and xenon (Xe-PC time I–IV) (each $n=4$) is shown. Upper panel shows phosphorylated form of PDK-1 (Serine 241), lower panel total PDK-1. The histogram presents densitometric evaluation as average light intensity (AVI). Data show ratio of phosphorylated versus total PDK-1. Data are means ± S.D. The values are expressed as *x*-fold compared with the control values. α-Tubulin served as internal standard. *$P<0.05$ vs. control. Panel B. Time dependent phosphorylation of PDK-1 at Tyrosine (373/376) during xenon preconditioning protocol. One representative Western blot experiment of cytosolic fraction of control and xenon treated hearts (Xe-PC time I–IV) (each $n=4$) is shown. Upper panel shows phosphorylated form of PDK-1 Tyrosine (373/376), lower panel total PDK-1. The histogram presents densitometric evaluation as average light intensity (AVI). Data show ratio of phosphorylated versus total PDK-1. Data are means ± S.D. The values are expressed as *x*-fold compared with the control values. α-Tubulin served as internal standard. *$P<0.05$ vs. control.

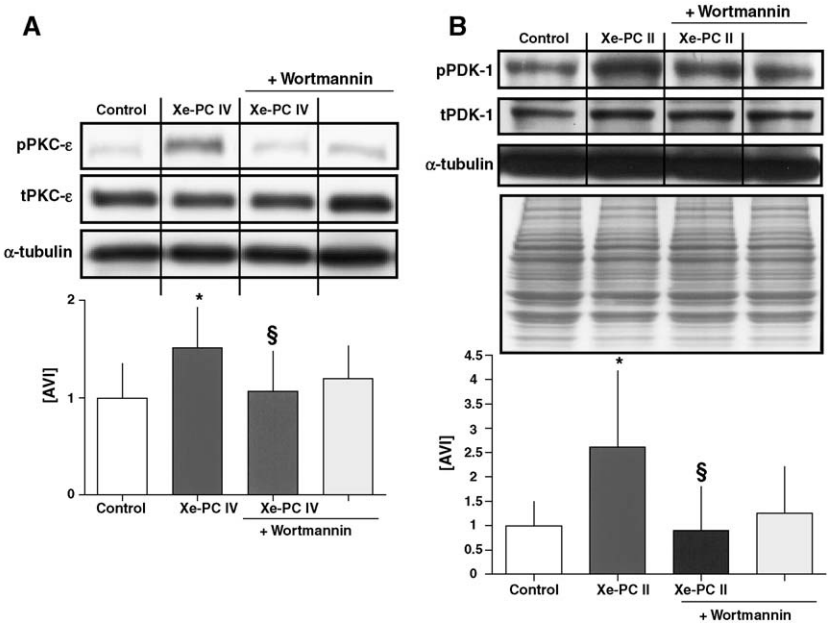

Fig. 5. Panel A. Causal relationship between PKC-ε and PDK-1 activation in xenon preconditioning. One representative Western blot experiment of cytosolic fraction of control and xenon (Xe-PC II) treated hearts in the presence or absence of Wortmannin (each $n=4$) is shown. Upper panel shows phosphorylated form of PKC-ε, lower panel total PKC-ε. The histogram presents densitometric evaluation as average light intensity (AVI). Data show ratio of phosphorylated versus total PKC-ε (means±S.D.). The values are expressed as x-fold compared with the control values. α-Tubulin served as internal standard. $^{*}P<0.05$ vs. control, $^{\S}P<0.05$ vs. xenon preconditioning. Panel B. Confirmation of effective blockade of PDK-1 by Wortmannin. One representative Western blot experiment of cytosolic fraction of control and xenon (Xe-PC time II) treated hearts in the presence or absence of Wortmannin (each $n=4$) is shown. Upper panel shows phosphorylated form of PDK-1 (Serine 241), lower panel total PDK-1. The histogram presents densitometric evaluation as average light intensity (AVI). Data show ratio of phosphorylated versus total PDK-1. Data are means±S.D. The values are expressed as x-fold compared with the control values. α-Tubulin served as internal standard. $^{*}P<0.05$ vs. control. Equal loading on the gel was confirmed by coomassie blue staining of the gel.

we determined phosphorylation of PKC-ε (time point IV, as described above) by immunoblotting hearts that were pretreated with the PI_3K/PDK-1 inhibitor Wortmannin (15 μg/kg). Wortmannin in fact abrogated the increased phosphorylation of PKC-ε detected after xenon administration (1.1±0.4 vs. xenon treatment 1.5±0.4, $P<0.05$, Fig. 5A) while Wortmannin alone had no effect on PKC-ε phosphorylation ($P>0.05$ vs. control, Fig. 5A). Since the blocker Wortmannin only indirectly blocks PDK-1 kinase via its upstream kinase PI_3K, we confirmed the inhibition of PDK-1 by immunoblotting phospho PDK-1 (at time II, which was the maximum for both phosphorylation sites, see Fig. 4A + B). As shown in Fig. 5B, pretreament with Wortmannin completely abolished the increase of PDK-1 phosphorylation (0.9± 0.8 vs. Xenon preconditioning II 2.6±1.5), demonstrating a sufficient blockade of the target enzyme. These results demonstrate that PDK-1 activation is indeed located upstream of PKC-ε in the signalling cascade of xenon-induced preconditioning.

4. Discussion

The phenomenon, known as pharmacological preconditioning induced by anaesthetics (anaesthetic preconditioning) has been described in vitro and in vivo but to date the mechanism by which anaesthetics and especially the "inert" gas xenon mediates the cardioprotection is poorly understood.

The anaesthetic *gas* xenon induces pharmacological preconditioning in the rat heart in vivo to the same extent as ischaemic preconditioning (Weber et al., 2005a). One of the key enzymes mediating this strong cardioprotection is PKC-ε (Weber et al., 2005a). The present study aimed to identify potential upstream "activators" of PKC-ε in pharmacological preconditioning of the heart exerted by the noble gas xenon. The main findings of our study are that (1) the opening of mitochondrial K_{ATP} channels and activation of PDK-1 are both functionally involved in the cardioprotection elicited by xenon, (2) that blockade of mitochondrial K_{ATP} channels results in the complete loss of xenon-induced phosphorylation and translocation of PKC-ε, (3) that xenon preconditioning induces a time dependent phosphorylation of PDK-1 and finally (4) that PKC-ε is no longer activated after blockade of PI_3K (phosphatidylinositol-3-kinase)/PDK-1 pathway by Wortmannin. Taken together, these data show that both putative activation pathways for the key enzyme PKC-ε are at least in part responsible for the initiation of PKC-ε phosphorylation and translocation and are located upstream of PKC-ε in the signalling pathway. Most importantly, both signalling steps (opening of mitochondrial K_{ATP} channels and activation of the PI_3K/PDK-1 pathway) are functionally relevant for xenon-induced cardioprotection.

In the heart, PKC activation itself can cause opening of K_{ATP} channels. This fact has led to a controversy about the causal relationship between PKC and this activation pathway. K_{ATP} channels are also functionally involved in anaesthetic preconditioning as demonstrated by Toller et al. (2000). For xenon, the implication of K_{ATP} channels in the cardioprotection had not been investigated before. It has been shown that release of free radicals after opening of the mitochondrial K_{ATP} channels is

critically important (Yao et al., 1999; McPherson and Yao, 2001). In turn, especially for anaesthetic preconditioning, a recent study of Novalija et al. (2003) demonstrated that these reactive oxygen species activate PKC. These data from anaesthetic preconditioning by volatile agents can be interpreted in parallel to our findings in xenon preconditioning: they suggest an upstream location of K_{ATP} channels to PKC in the signal transduction. Also a recent study of Ludwig and co-workers is in line with our results. They demonstrated that PKC-ε translocation is a downstream event of mitochondrial K_{ATP} channels in isoflurane-induced preconditioning in rats (Ludwig et al., 2004). In contrast to our results showing a causal relationship between PKC and mitochondrial K_{ATP} channels, the group of Nakae et al. (2003) suggested that isoflurane activates mitochondrial K_{ATP} channels via PKC independent pathways in an in vitro model of guinea pig cardiomyocytes.

The group of Bouwman et al. (2004) could not find a link between mitochondrial K_{ATP} channels and ROS stimulated PKC-δ activation in sevoflurane-induced preconditioning in vitro. These contradictory findings may be explained by the use of different preconditioning agents and discrepancies between in vivo and in vitro conditions.

Regarding in vitro implication of K_{ATP} channels in anaesthetic preconditioning, already in 1996 Han and co-workers not only demonstrated that isoflurane reduces the inhibitory effect of ATP on K_{ATP} channel-opening (Han et al., 1996), but also that the isoflurane metabolite trifluoroacetic acid directly activates K_{ATP} channels. This (in vitro) effect of isoflurane was not prevented by the PKC-blockers polymyxine B and staurosporine. Kohro et al. (2001) extended these investigations and demonstrated in isolated guinea pig cardiomyocytes that administration of isoflurane or sevoflurane increased the opening probability of mitochondrial K_{ATP} channels in a dose dependent manner. In contrast to this study, Zaugg et al. (2002) found in isolated rat cardiomyocytes that isoflurane or sevoflurane did not increase the open-state probability of mitochondrial K_{ATP} channels directly, but that this effect depended on activation of PKC.

As a limitation of our study, it should be taken into account that we focused on mitochondrial K_{ATP} channels since 5-HD is a specific blocker of this type of channels. We did not investigate the implication of the sarcolemmal K_{ATP} channels. There exists information from the literature concerning ischaemic and pharmacological preconditioning showing that the selective sarcolemmal K_{ATP} channel blocker HMR1098 does not affect ischaemic preconditioning (Sato et al., 2000c,b). In addition, for pharmacological preconditioning by nicorandil (activator of K_{ATP} channel with a nitrate-like action) and by bradykinin, it was demonstrated that mitochondrial and not sarcolemmal K_{ATP} channels mediate the cardioprotection (Sato et al., 2000b; Kita et al., 2000). The ischaemic preconditioning mimicking agent adenosine was shown to prime K_{ATP} channels in a PKC dependent manner in rabbit ventricular myocytes and this effect was found to be specific for mitochondrial K_{ATP} channels (Sato et al., 2000a). In contrast to all these studies, Tanno et al. (2001) suggest both the sarcolemmal and the mitochondrial K_{ATP} channels to be involved in the infarct size limiting effect of K_{ATP} channel opener in an isolated perfused rabbit heart model.

The PI_3K/PDK-1 pathway, the second putative activation pathway for PKC has yet not been investigated for xenon-induced preconditioning, and also for ischaemic preconditioning there exists only very few data. Only one very recent study by Raphael et al. (2005) showed that the cardioprotection afforded by isoflurane in the rabbit heart is mediated by activation of PI_3K. This study in part supports our results from the present study. However, the phosphorylation and translocation of PDK-1 or PKC-ε were not subject of this study. PDK-1 phosphorylates several kinases like protein kinases A (PKA), G (PKG) and C (PKC). Therefore, PDK-1 is an enzyme which triggers several important cellular processes including cellular survival, glucose transport and metabolism, tumor progression as well as protein translation. PDK-1 function itself is mediated in part by the phosphoinositide 3′-OH-kinase (PI_3K) pathway and the lipid products of PI_3K, phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4-5-trisphosphate stimulate the activation of PDK-1 signaling pathways (for review see (Storz and Toker, 2002)).

For late ischaemic preconditioning in the rabbit heart, it has been demonstrated that the PI_3K/PDK-1 pathway plays an essential role (Kis et al., 2003). Moreover, during reperfusion and the so called "postconditioning" there seems to exist a cross-talk between different survival kinases including PI_3K, the upstream kinase of PDK-1 (Hausenloy et al., 2004; Tsang et al., 2004). In contrast to our results for xenon preconditioning, the group of Baines found that insulin induced preconditioning of isolated perfused rabbit hearts is mediated via PI_3K but not via PKC or K_{ATP} channels (Baines et al., 1999). A very recent study of Penna et al. (2005) demonstrated that the endogenous release of platelet-activating factor during ischaemia induces cardioprotection via PI_3K and PKC dependent pathways.

The only study investigating an implication of the PI_3K/PDK-1/Akt pathway in context with anaesthetics is a "postconditioning" study from the group of Chiari et al. They show that isoflurane induces PI_3K when given during reperfusion. Moreover, by the use of Wortmannin they demonstrate that PI_3K is functionally involved in the cardioprotective effect of isoflurane (Chiari et al., 2005). From these data it is obvious that there exist mostly studies investigating the functional role of the upstream kinase of PDK-1, the PI_3K. Since to date there is a lack of specific inhibitors directly blocking the activation of PDK-1, it is not possible to block PDK-1 itself in vivo. However, there exists convincing evidence that PDK-1 is predominantly activated via PI_3K. As shown by the complete loss of PDK-1 activation after blockade of PI_3K with a low dose of Wortmannin (15 μg/kg, in comparison to other studies using 0.6 mg/kg (Chiari et al., 2005)) we can conclude that PDK-1 acts in fact as downstream target of PI_3K and its activation is functionally involved in xenon-induced preconditioning.

From the data presented we cannot demonstrate a relationship between K_{ATP} channels and PDK-1. However, our aim was to clarify which pathways lead to the activation of PKC-ε and which is the relationship between them and PKC. The role of ROS released by the mitochondrial K_{ATP} channels and their contribution to activation of PDK-1 and PKC has to be investigated in detail in future studies.

We used only one concentration of xenon and our results must be limited to this concentration. However, rather the cardioprotective effects with focus on the mechanistic insights than the anaesthetic properties of xenon were the main subject of the present study.

The present data are limited to the experimental in vivo animal model and allow only a limited transfer to the clinical situation. Nevertheless, detailed understanding of the underlying molecular mechanisms and the regulation of different key enzymes during the cardioprotection exerted by xenon may help to expand the knowledge on the way to xenon as a potential ideal anaesthetic gas for patients at high-risk for cardiac events during the perioperative period.

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