

Cardio-protective determinants are conserved in aged human myocardium after ischemic preconditioning

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Abstract Ischemic preconditioning (IPrec) improves post-ischemic dysfunctions of the myocardium along with activation of protein kinase C isoforms including PKC δ . Moreover, expression of cardio-protective determinants can reduce ischemic damages. Because IPrec is limited in aged hearts, we assessed in an experimental model the impact of aging on PKC δ and selected protective proteins in the preconditioned myocardium from adult (≤ 55) and older (≥ 70 years) humans. Adult myocardium showed PKC δ up-regulation after IPrec along with improved post-ischemic contractility. Although there was no functional benefit, PKC δ increased in older myocardium as well. Subsequent mRNA analyses demonstrated that IPrec stabilizes the mRNA expression of protective proteins (Hsp70, Bcl-2/xL, IAPs) in both aging groups. Moreover, older hearts revealed increase in post-ischemic Hsp90 β . Our study indicates, that IPrec conserves the expression of cardio-protective determinants in aged hearts despite limited functional recovery.

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Key words: Ischemic preconditioning; Myocardium; Aging; Protein kinase C δ ; Apoptosis; Heat shock protein

1. Introduction

The pre-treatment of the myocardium with brief intervals of cardiac ischemia and reperfusion diminishes the myocardial damage induced by prolonged ischemia. This phenomenon is called ischemic preconditioning (IPrec) [1]. Mechanisms underlying the protection by preconditioning include the activation of G-coupled surface receptors, protein kinase C (PKC) translocation and opening of mitochondrial K_{ATP} channels (review [2]). Moreover, the activation of several protective determinants after ischemic pre-treatment might contribute to a reduced cell death by necrosis, such as inducible heat shock proteins [3], or by apoptosis like some members of the Bcl-2 family [4]. However, there is no strict discrimination of apoptotic and necrotic cell loss, especially in ischemia. While heat shock proteins are involved in apoptosis pathways as well [5], Bcl-2 proteins can reduce necrosis by antagonizing mitochondrial dysfunctions [6].

Recently, we demonstrated that IPrec has no beneficial effect on the post-ischemic contractile function of aged human myocardium [7]. This limitation is a crucial determinant in the

surgical treatment of elderly with coronary artery disease and other heart failures. Because the underlying mechanisms are barely investigated, this study aimed to assess the impact of aging on cardio-protective factors involved in ischemia and preconditioning.

Using an in vitro model, the level of the inducible hsp70 isoforms Hsp70-1 and Hsp70-2 was analyzed in isolated myocardium from adult and older heart surgery patients after ischemia and reperfusion. Both gene variants encode for the identical Hsp70 protein but are sequentially regulated [8]. Their divergent 3'-untranslated region allows specific mRNA analyses demonstrating that Hsp70-2 is induced by short-term ischemia, whereas Hsp70-1 induction is related to long-term ischemic conditions [9]. In addition to Hsp70, the expression of Bcl-2 proteins (Bcl-2, Bcl-xL) and of members of the inhibitor of apoptosis (IAP) family (hIAP-1, XIAP) was of special interest. hIAP-1 and XIAP effectively block the activity of apoptosis-related caspases and promote the nuclear factor (NF)- κ B-mediated cell survival via NF- κ B stimulation, too [10]. Proteolytic activation of caspases is the central element of the apoptotic machinery that finally results in the processing of a large variety of cellular substrates thereby contributing to the characteristic morphological and biochemical changes during apoptosis (review [11]). Two of those substrates are PKC δ and Hsp90 β , which are directly involved in cellular stress situations. While PKC δ is one of the PKC isoforms activated in cardiomyocytes after IPrec, the Hsp90 β protein impairs together with Hsp70 the execution phase of apoptosis down-stream of caspase activation [12]. Therefore, we investigated the impact of aging on PKC δ , Hsp90 β and other protective proteins in the preconditioned myocardium.

2. Materials and methods

2.1. Experimental procedure

Right atrial appendages obtained from adult (≤ 55 years, $n=15$) and older patients (≥ 70 years, $n=15$) undergoing coronary bypass surgery were used in this study as previously described [7]. All patients suffered from coronary artery disease. Four trabeculae from each appendage were placed in an organ bath with continuously oxygenated Tyrode's solution. Specimens were electrically stimulated at 1 Hz and the force of contraction was registered (Hugo Sachs Electronics, Germany). Trabeculae were subjected to 30 min of experimental ischemia (hypoxic substrate-free Tyrode's solution) in combination with rapid pacing at 3 Hz, followed by 90 min of reoxygenation (normoxic solution periodically exchanged, stimulation at 1 Hz). In each experiment, two trabeculae were ischemically preconditioned for 5 min, followed by 10 min of reoxygenation. The isometric force was expressed as the percentage force of pre-ischemic baseline values. Trabeculae of additional patients (55–70 years, $n=10$), which were superfused in the same time course without ischemia (non-ischemic) and

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with ischemia (ischemic), served as independent control group. The use of human material was approved by the local ethics committee. For characteristics of patients and functional data of trabeculae see Table 1.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

At the end of each reoxygenation, both ischemic controls as well as both preconditioned trabeculae from the same patient were pooled. Total RNA was extracted using the TRIzol[®] reagent (Invitrogen, The Netherlands) and cleaned up with RNeasy columns (Qiagen, Chatsworth, CA, USA). RNA quality was controlled by agarose gel electrophoresis and quantity was determined by UV/Vis spectrophotometry. In a RT reaction, cDNA was synthesized from 200 ng of RNA with 100 U Superscript II[®] reverse transcriptase (Invitrogen). 2.5 µl cDNA reaction was used for PCR containing 1.5 mM MgCl₂, 5 pmol of each gene-specific primer (Table 2), 10 µM of each dNTP, and 1 U rTaqDNA polymerase (Promega, USA). PCR amplification was performed in a thermocycler (Biometra, Germany). After gel electrophoretic separation, intensity of PCR products was densitometrically evaluated using the AIDA 2.0 software (Raytest, Germany).

2.3. Western blot analysis

Total protein was extracted from the protein phase of the TRIzol[®] preparation according to manufacturer's instruction (Invitrogen), and concentration was measured by the BCA protein assay (Sigma-Aldrich, Germany). Samples containing 20 µg of protein were separated by SDS-PAGE and blotted onto nitrocellulose membrane. Non-transferred protein was visualized by staining of the analytic gel with Coomassie blue solution. Proteins bound to the blotting membrane were blocked with non-fat dry milk in TBST buffer (200 mM Tris-HCl, pH 7.5; 300 mM NaCl; 0.1% Tween 20) and incubated with the respective anti-human antibody: goat polyclonal Hsp90β and rabbit polyclonal PKCδ (both are from Santa Cruz Biotech, Santa Cruz, CA, USA), p20 Caspase-3 (Cell Signaling, USA), Caspase-9 (BD Pharmingen) and mouse monoclonal actin (Sigma, St. Louis, MO, USA). Bound antibodies were detected by horseradish peroxidase-conjugated secondary antibodies (Dianova, Germany). Chemiluminescence detection was performed with ECL^{plus} solution and Hyperfilm ECL (Amersham Bioscience, UK). Visualized signals for antibody detection and for total protein bands of the Coomassie blue-stained gel were densitometrically quantified. All analytic gels contained loading samples for the internal signal correction.

2.4. Data analysis

Data are means ± S.E.M. Paired sample sets were always analyzed side by side. Densitometrically determined values are expressed as optical density units (opt. density U). For comparison of preconditioned myocardium with the ischemic control of the same patient, Student's paired *t*-test was used (SigmaStat software, Jandel Corp., USA). For evaluation of independent data, Student's unpaired *t*-test was applied. *P* ≤ 0.05 indicates a significant difference of mean values.

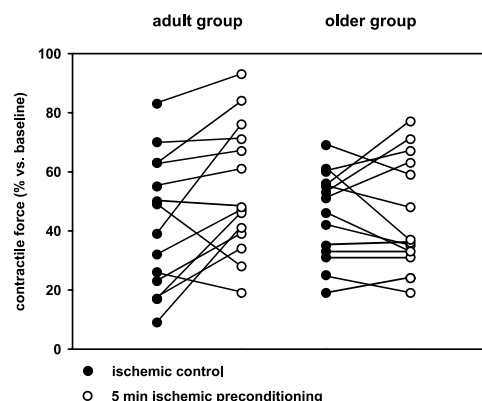


Fig. 1. Post-ischemic recovery of the contractile force of atrial trabeculae from individual adult patients (≤ 55 years) and older patients (≥ 70 years). Data are calculated 90 min after reoxygenation as percentage of pre-ischemic baseline values.

3. Results

This study demonstrated that atrial trabeculae from adult heart surgery patients show a higher baseline contractility (Table 1) and a significantly better preservation of the post-ischemic function by IPrec than trabeculae from the elderly (Fig. 1, Table 3). The contractile force recovery was determined by $55 \pm 5.5\%$ after reoxygenation compared to $44 \pm 6.0\%$ in non-preconditioned ischemic controls (*P* = 0.05). In contrast, preconditioning had no protective effect on trabeculae from patients older than 70 years (Table 3).

To assess the molecular basis of our observation, total RNA and protein were extracted from atrial trabeculae after reoxygenation and analyzed in age-dependent manner. As summarized in Table 3, total mRNA expressions of Hsp70-1 and Hsp70-2 were significantly increased by preconditioning in the myocardium from adult patients. Although older patients had no improved contractile function in response to preconditioning, both Hsp70 mRNAs were increased to similar extent. The same tendency was observed for Bcl-2 mRNA in the adult patient group and for Bcl-xL mRNA in the older patients. When studying IAP proteins, we found higher levels of hIAP-1 and XIAP mRNA in the preconditioned myocardium as well.

Table 1
Data of coronary artery patients included in this experimental study

Group	Adult patients		Older patients	
Clinical characteristics of patients				
Age (years)	49 ± 1		74 ± 1	
Angina pectoris stage	II		II	
Left ventricular EF (%)	59 ± 4		61 ± 3	
Mean aortic pressure (mm Hg)	83 ± 3		83 ± 5	
Drug therapy				
β-blockers	11		10	
ACE inhibitors	9		8	
Nitrates	8		7	
Baseline data of atrial trabeculae				
	ICo	IPrec	ICo	IPrec
Force of contraction (Nm)	4.9 ± 0.6 [#]	5.2 ± 0.5 [#]	3.6 ± 0.4	3.9 ± 0.3
Pulse amplitude (V)	10.4 ± 0.4	9.3 ± 0.3	10.3 ± 0.3	9.6 ± 0.3
Weight (mg)	5.9 ± 0.7	6.1 ± 0.5	5.1 ± 0.6	4.9 ± 0.5

Data are means ± S.E.M. (*n* = 15 each study group); [#]*P* < 0.05 vs. respective data of the older patient group (unpaired *t*-test). ACE, angiotensin-converting enzyme; EF, ejection fraction; ICo, ischemic control samples; IPrec, trabeculae pretreated with 5 min IPrec.

Table 2
Primers for RT-PCR analysis

mRNA	Accession number	5'–3' sequence primer	
		Sense	Antisense
Bcl-2	M14745	GACTTCTTCCGCCGCTACCG	GACAGCCAGGAGAAATGAAAC
Bcl-xL	Z23115	GGTGGTTGACTTCTCTCTAC	GAAAAGTATCCAGCCGCC
β -actin	XM037235	GAAGTGTGAGTGGACATCCG	AGCATTTCGGGTGGACGAT
hIAP-1	AF070674	GTTTCATCCGTCAAGTTCAAG	GTTCTTTCTCTGTTAGTCTCC
Hsp70-1	XM004188	CCGAGAAGGACGAGTTTGAG	GGAAATGCAAAGTCTTGAAGC
Hsp70-2	XM004189	CCGAGAAGGACGAGTTTGAG	TCGTACAGAAGGTGGCAGTG
XIAP	U32974	GATGCTGTGAGTTCTGATAGG	CTTAATGTCTTGAAACTGAAC
18S rRNA	M10098	GTTGGTGGAGCGATTTGTCTGG	AGGGCAGGGACTTAATCAACGC

Detailed analyses of constitutively expressed ('house-keeping') genes, which are frequently used for data correction of inter-sample variations, revealed that the total expression of β -actin mRNA but also of the ribosomal 18S rRNA is improved in the preconditioned myocardium compared to non-preconditioned controls (Fig. 2). Interestingly, the myocardial level of 18S rRNA and of other target mRNAs directly correlated with the post-ischemic contractile force. This was obvious for the total mRNA expression of both IAP proteins (hIAP-1, XIAP) and Bcl-2 proteins (Bcl-2, Bcl-xL) in the entire study group ($r > 0.39$, $n = 30$, $P \leq 0.05$ in all cases). For Hsp70-1, but not for Hsp70-2, we also found positive correlations using the differential evaluation mode (i.e. contractile force recovery of preconditioned trabeculae minus internal non-preconditioned control; $r = 0.38$, $P = 0.05$).

Based on the fact that data calculations in relation to 'house-keeping' genes may result in misleading interpretations of the gene expression, all mRNA data are given per total RNA amount used in each analysis. Instead, a separate patient group was created ($n = 10$). Trabeculae of this group were superfused in the same time course but without ischemia. The direct comparison of mRNA levels in the preconditioned ischemic myocardium with those in non-ischemic controls clearly showed that the ischemic pre-treatment does not induce but stabilizes the mRNA expression which was indeed decreased in most non-preconditioned specimens (Table 3). Age-dependent differences have not been determined.

The strong impairment of ischemia on the mRNA expression machinery was not identified for the protein expression. Neither the protein levels of β -actin nor of Hsp-90 β and PKC δ were significantly changed in the ischemic myocardium

compared with non-ischemic controls (Figs. 2 and 3). Hsp90 β was stably expressed after ischemia with and without ischemic preconditioning, too. On the other hand, specimens from the older myocardium were characterized by a two-fold increase in Hsp90 β protein. In contrast, the post-ischemic expression of PKC δ was induced after preconditioning which can be determined in both aging groups (Fig. 4). Simultaneous analyses of the proteolysis of PKC δ and Hsp90 β by caspases revealed cleavage products for PKC δ but not for Hsp90 β . The PKC δ fragment p43 was processed in trabeculae after ischemic intervention (Fig. 3). Preconditioning had no influence on the formation of p43 per entire PKC δ which was determined with $19 \pm 3.6\%$ in the preconditioned trabeculae and $18 \pm 3.8\%$ in ischemic controls ($n = 30$). Although there were no age-dependent differences in the percentage cleavage of PKC δ , older patients tended to a higher total level of p43 PKC δ in the post-ischemic myocardium (Fig. 3).

Ischemia-reperfusion occurs along with mitochondrial dysfunctions and the release of cytochrome c into the cytosol that triggers the caspase cascade via activation of caspase-9 [13]. Therefore, caspase-9 and its down-stream target caspase-3 were additionally investigated. These analyses revealed that neither caspase-9 nor caspase-3 are processed into active p35 caspase-9 or active p20 caspase-3 on detectable level (data not shown).

4. Discussion

The hallmark of IPrec is the preservation of myocardial structure and cardiac function [14]. However, animal studies showed that aging is a limiting determinant for the myocardial

Table 3
Post-ischemic functional data of atrial trabeculae and expression of cardio-protective determinants

	Non-ischemic controls	Adult group		Older group	
		ICo	IPrec	ICo	IPrec
Developed force of contraction (% vs. baseline)					
Force (%)	67.1 $\pm$ 7.8 [#]	44 $\pm$ 6.0	55 $\pm$ 5.5*	44 $\pm$ 3.9	46 $\pm$ 5.1
Total mRNA level (opt. density U)					
18S rRNA	181 $\pm$ 16.1 [#]	123 $\pm$ 20.6	168 $\pm$ 11.3*	118 $\pm$ 18.1	159 $\pm$ 15.6*
β -actin	176 $\pm$ 5.9	147 $\pm$ 14.4	180 $\pm$ 10.0*	143 $\pm$ 12.4	168 $\pm$ 11.7*
Hsp70-1	425 $\pm$ 29.5 [#]	251 $\pm$ 29.5	332 $\pm$ 27.1*	218 $\pm$ 30.1	279 $\pm$ 30.7*
Hsp70-2	191 $\pm$ 22.4	143 $\pm$ 30.4	182 $\pm$ 27.2*	99.2 $\pm$ 14.5	141 $\pm$ 16.9*
Bcl-2	208 $\pm$ 12.2	159 $\pm$ 19.8	204 $\pm$ 14.7*	142 $\pm$ 16.4	175 $\pm$ 16.0
Bcl-xL	176 $\pm$ 31.5 [#]	105 $\pm$ 24.1	145 $\pm$ 19.5	92.3 $\pm$ 18.2	145 $\pm$ 16.3*
hIAP-1	116 $\pm$ 20.5 [#]	72.1 $\pm$ 17.1	105 $\pm$ 10.9*	60.1 $\pm$ 12.5	96.3 $\pm$ 13.6*
XIAP	194 $\pm$ 68.4	158 $\pm$ 54.6	258 $\pm$ 52.7	154 $\pm$ 42.8	228 $\pm$ 47.5*

Mean data $\pm$ S.E.M. ($n = 10$, non-ischemic control group; $n = 15$, group of adult and older patients); * $P < 0.05$ vs. ischemic control (paired t test); [#] $P < 0.05$ vs. ischemic control values of the entire study group (unpaired t -test). ICo, non-preconditioned ischemic control trabeculae; IPrec, trabeculae subjected to 5 min ischemic preconditioning.

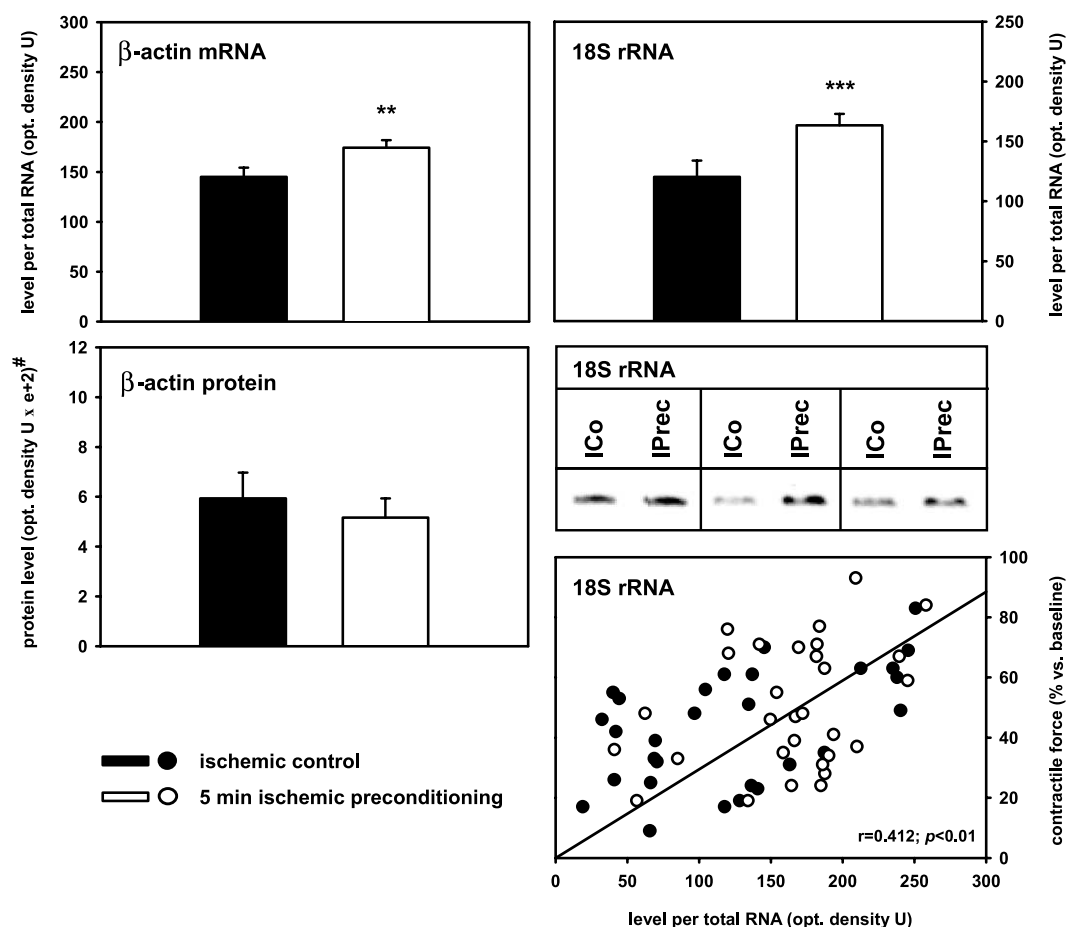


Fig. 2. Post-ischemic mRNA and protein level for β -actin in preconditioned atrial trabeculae (IPrec) and ischemic controls (ICo) for all patients of study ($n=30$). Data are shown in comparison with the 18S rRNA expression which positively correlates with the functional recovery.
[#]Correction of inter-sample variations per total protein staining.

protection achieved with IPrec [15]. For ethical reason the impact of age in human myocardium is more indirectly drawn from clinical reports (review [16]). Therefore, we established an experimental model studying isolated myocardium of heart surgery patients [7]. In this study we clearly demonstrated that ischemic preconditioning has beneficial effect on adult human myocardium, whereas it fails to preserve the contractile function from a sustained ischemia in older patients.

Several cellular mechanisms are associated with IPrec including the PKC-dependent activation of mitochondrial K_{ATP} channels that finally preserves the mitochondrial function (review [17]). PKC appears to be the first element of a complex kinase cascade that is activated during ischemia in the preconditioned heart. Cardiac myocytes contain at least six different PKC isoforms [18] but preconditioning results in the activation of only two, namely PKC ϵ and PKC δ [19]. Depending on the experimental model and species used, the induction of PKC α and PKC η by simulated ischemia [20] or pharmacological agents that lead to cardio-protection from ischemia [21] is described as well. Although the inability of most antagonists to discriminate among multiple PKC isoforms does limit specific investigations of the PKC signalling, PKC δ might play a critical role in modulating the mitochondrial function because PKC δ directly translocates to mitochondria after its activation [21]. In our study, we observed an increased level of PKC δ in the preconditioned myocardium

of adult and older patients to similar extent. Although the PKC δ up-regulation does not describe its activation by sub-cellular translocation, this observation can not directly confirm data of Tani et al. suggesting a reduced PKC δ activity as a limiting factor in the preconditioning response of aged hearts [22]. While the protective role of PKC ϵ was clearly shown [19], it is still controversial whether PKC δ does contribute to myocardial protection. PKC δ activation was found to play an essential role in the opioid-mediated cardio-protection against ischemia [23] but other studies using PKC-selective antagonists demonstrated that PKC δ enhances the ischemia-induced damage of cardiomyocytes [24]. Moreover, PKC δ is required for apoptotic cell death and the caspase-specific cleavage of PKC δ into p43 PKC δ further amplifies this process by generating an active kinase domain that translocates to mitochondria and rapidly induces mitochondrial dysfunction [25]. The tendency of aged myocardium to process higher levels of post-ischemic p43 PKC δ is a possible explanation why older patients do less respond to cardio-protection after ischemic pre-treatment. However, we did not find a relation of PKC δ level and p43 generation with the less effective preconditioning response in older hearts suggesting a more complex network of the entire PKC action in the preconditioned myocardium. In this context, it has to keep in mind that other PKC isozymes are also targets of the caspase proteolysis including the cardio-protective PKC ϵ [25].

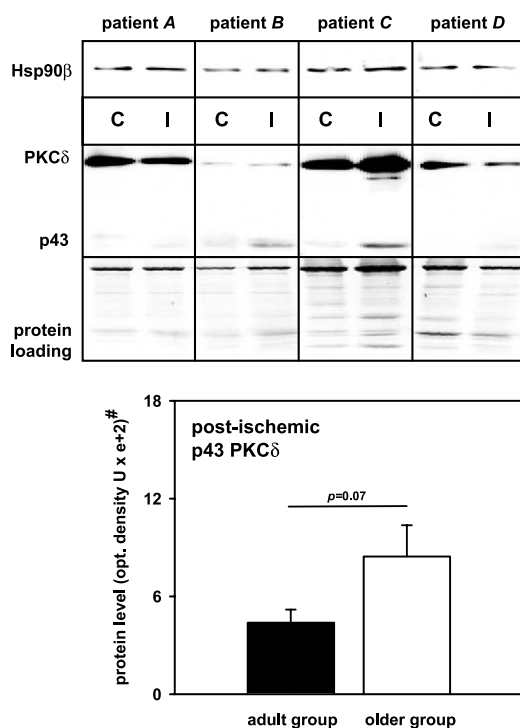


Fig. 3. Western blot analysis of Hsp90β, PKCδ and its cleavage product p43 PKCδ in the post-ischemic myocardium (I) in comparison with the internal non-ischemic control (C) from individual patients (A to D) of intermediate age (55–70 years). Data of the age-dependent protein level of the active p43 PKCδ subunit in non-preconditioned ischemic controls are shown below. #Inter-sample correction per β-actin and total protein staining.

Although caspase-specific cleavage products have been identified for PKCδ, it is unknown to what extent apoptotic caspases are still active during the post-ischemic period because we did not identify the generation of active caspase-3 and -9 after 90 min of reoxygenation. This might base on the

fact that oxidized conditions can modify the caspase activity. Caspases are redox-sensitive enzymes and prolonged oxidative stress, as happening in post-ischemic reperfusion, can convert active caspases to an inactive state [26]. Unfortunately, the amount of trabeculae material was limited to analyze the enzymatic activity of caspases in addition. Moreover, the expression of heat shock proteins can mediate caspase inhibition. Hsp90β and Hsp70 block the activity of caspase-9 via sequestration of its cofactor Apaf-1 [12]. An age-dependent impact on this process is not yet described.

In contrast to PKCδ, for Hsp90β we neither identified the cleavage at caspase-specific site in the ischemic myocardium nor an altered expression after IPrec. Although Hsp90 up-regulation is shown in preconditioned cardiomyocytes [27], there are also opposite reports [28] confirming our findings. Hsp90β was elevated in the ischemic myocardium of older patients suggesting a physiological role of Hsp90β in the protection of aged hearts. In this context, an increase in Hsp90β might counteract the reduced tolerance of the aged myocardium to ischemia frequently reported [29], but the association with ischemia-independent aging processes is possible as well. In contrast to Hsp90β, the Hsp70 expression was not significantly changed in older myocardium after ischemia and preconditioning. However, IPrec stabilized the total mRNA level of Hsp70-1/-2 but also of Bcl-2, Bcl-xL, hIAP-1 and XIAP against prolonged ischemia in adult but also in aged myocardium. This seems to base on a general protection of the transcription machinery and/or mRNA stability, that frequently correlated with the recovery of the post-ischemic cardiac function. As indicated by β-actin mRNA, the myocardial level was reduced after ischemia-reoxygenation compared to non-ischemic controls but to less extent in the preconditioned myocardium. Because the protein level of β-actin was not affected by ischemia, this seems to be a mRNA-related mechanism.

The most common approach to correct inter-sample variations of transcription analyses is the normalization per constitutively expressed control genes like β-actin. Because the

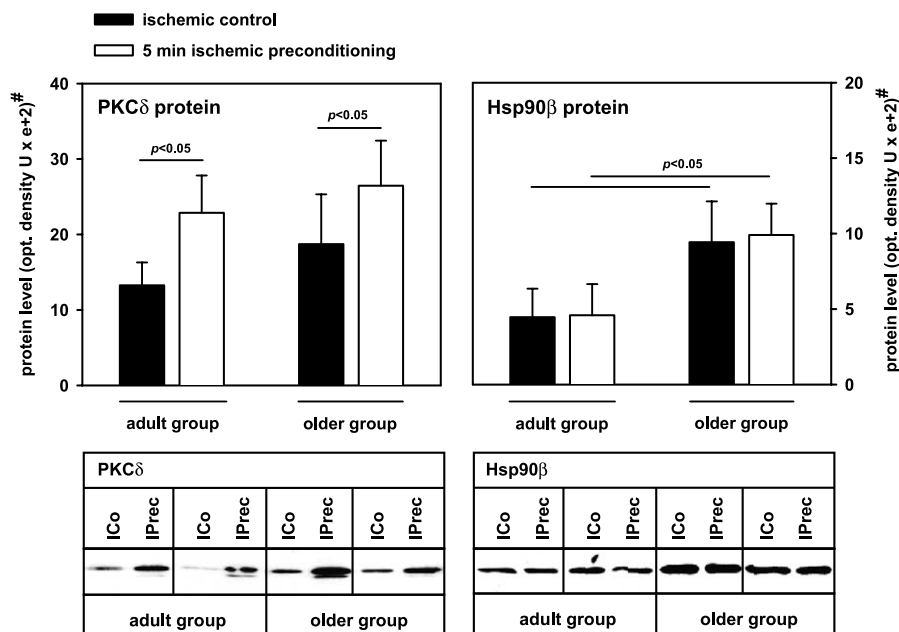


Fig. 4. Post-ischemic level of PKCδ and Hsp90β protein in preconditioned atrial trabeculae (IPrec) and ischemic controls (ICo) from adult and older patients ($n = 15$ each study group). #Correction of inter-sample variations per β-actin and total protein staining.

steady-state levels of such ‘house-keeping’ genes cannot always be assumed under hypoxic and other stress conditions [30], we included the quantification of ribosomal RNA (18S rRNA). However, even ribosomal RNA can be impaired as a consequence of sub-lethal stress insults [31] as demonstrated in our study as well. For this obvious impact by ischemia, we represented our data per analyzed amount of total RNA as usually done in standard-calibrated competitive PCR or in real-time PCR analysis. Nevertheless, the stress-altered mRNA expression of ‘house-keeping’ genes is critical and can result in faulty data calculations, especially for slightly changed expressions. This might have led to controversial reports in the past. Based on the fact that the post-ischemic mRNA level of genes including several cardio-protective determinants was improved after preconditioning, our transcription analyses demonstrate a protecting effect on molecular level in addition to the functional recovery of the myocardium. Age-dependent differences in the expression of protective determinants, including Hsp70 and Bcl-2 proteins, have not been determined. These observations support data of Loubani et al. revealing that age does not influence the benefit of preconditioning on structural basis of human trabeculae [32], furthermore suggesting differences between the functional and morphological preservation after preconditioning.

Taken together, our study indicates that the post-ischemic expression of determinants involved in IPrec is still preserved in aged hearts despite less functional benefit in response to preconditioning. This has been shown for PKC δ induction in the preconditioned myocardium but also for the stable expression of selected cardio-protective determinants suggesting a more complex network of factors responsible for the age-dependent functional decline in the preconditioning response.

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