

**Changes in gene expression following cardiac pacing-induced delayed cardioprotection in
the canine heart**

PhD Thesis

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SUMMARY

Understanding the mechanism behind the cardioprotection induced by preconditioning might provide a new therapeutic tool against the life threatening ventricular arrhythmias that often lead to sudden cardiac death.

Therefore in Study I, we aimed to investigate gene expression changes following ischemia and reperfusion in dogs subjected to rapid right ventricular cardiac pacing 24 hours previously by a genome-wide approach using cDNA microarray analysis. We also aimed to examine the transcription rate of 45 additional genes which have already been suggested to be involved in ischemic PC but little is known about their role in the cardiac pacing induced antiarrhythmic protection. Furthermore, in Study II, 29 genes were selected to explore the time course changes of gene expressions over 24 hours, which elapses between rapid cardiac pacing and the ischemia/reperfusion period.

In Study I, we have identified 23 genes with altered expression; some of these, for example, MEF2A (involved in angiogenesis) or mAKAP (an anchoring protein), were firstly demonstrated to be significantly up-regulated in response to preconditioning.

We have proved that rapid right ventricular pacing alters the expression of genes involved in the delayed protection. In Study I, 6 out of 45 genes exhibited significant down-regulation and 16 showed up-regulations in response to ischemia and reperfusion, compared with the control dogs. Among these, genes encoding different proteins, such as NO producing enzymes, heat shock proteins, members of the survival signaling pathways or pro-apoptotic factors showed the most marked changes. In Study II, we have proved that pacing induces time-dependent alterations in the expression of the 29 genes examined. Immediately after the cessation of pacing, 8 genes showed significant up-regulation and 6 significant down-regulation. After these initial changes, some of these early up- or down-regulated genes either remained unchanged or showed a biphasic transcriptional pattern; i.e. after an initial change in their transcription these genes were normalized to the mRNA level of the unpaced hearts but their expression started to change again at later time points. There were also genes, such as those encode eNOS and iNOS, which exhibited significant up-regulation only 12h after the pacing stimulus. The resultant increase in eNOS protein content and activation certainly contributes to an increased NO production, to which we attribute an important role in late phase of the protection. We are aware that changes at gene expression level do not always reflect changes at protein level, nevertheless the present results provide evidence for gene expression changes following cardiac pacing, and when the antiarrhythmic protection is observed.

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LIST OF ABBREVIATIONS

AEST	S-(2-aminoethyl)-isothiourea
Akt	also known as Protein kinase B (or PKB)
ATF-3	Activating transcription factor 3
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
Ct	Threshold cycle
ECL Kit	Enhanced chemiluminescence kit
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
ERK	Extracellular-signal-regulated kinase
GPCR	G protein coupled receptor
GSK3β	Glycogen synthase kinase 3 beta
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
HSP22, 27, 28, 70	Heat shock protein 22, 27, 28, 70
L-NAME	L-NG-Nitroarginine Methyl Ester
LAD	Left anterior descending artery
LCX	Left circumflex artery
MAPKAPK-3	Mitogen-activated protein kinase-activated protein kinase-3
mitoK_{ATP}	Mitochondrial K _{ATP} channel
mPTP	Mitochondrial permeability transition pore
PDE	Phosphodiesterase
PC	Preconditioning
PI3K/ PI3 kinase	Phosphatidylinositol 3-kinase
PKCϵ	Protein kinase C epsilon type
PKG	cGMP-dependent protein kinase or Protein Kinase G
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RDA	Representational difference analysis
ROS	Reactive oxygen species
sGC	Soluble guanylyl cyclase
SDS	Sodium dodecyl sulfate
Src	also known as tyrosine-protein kinase Src (short for sarcoma)
SSC buffer	Saline-sodium citrate buffer
TRIS-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
VBP	Ventricular premature beats
VDAC	Voltage-dependent anion channel
VF	Ventricular fibrillation
VT	Ventricular tachycardia

1. INTRODUCTION

One of the most important consequences of acute myocardial ischemia, is the occurrence of the life threatening ventricular arrhythmias, which are responsible in most instances for sudden cardiac death. This is one of the major causes of mortality in the developed countries. Due to several disadvantages of the current antiarrhythmic drug therapy, resulting from a proarrhythmic effect, significant efforts have been made to explore new therapeutic strategies against ventricular arrhythmias. For example preconditioning is one of the endogenous cardioprotective phenomena, which may offer a new tool to attenuate ventricular arrhythmias.

1.1 The antiarrhythmic effect of ischemic preconditioning and rapid cardiac pacing

The cardioprotective phenomenon, termed ischemic or 'classic' preconditioning was first described by Murry et al. [1] in 1986. They have reported that multiple brief ischemic episodes protect the heart against the severe consequences of a more prolonged ischemic insult, such as ATP loss or myocardial cell death. Since then, it has been established that preconditioning not only reduces infarct size, but markedly attenuates the severity of the ischemia and reperfusion-induced ventricular arrhythmias [2], and enhances the recovery of contractile function following ischemia and reperfusion [3].

Apart from the 'classical' ischemic preconditioning, there are other stimuli which are able to induce cardioprotection. For example, in dogs subjected to two, 2 min periods of cardiac pacing at a rate of 300 beats min⁻¹, the number and the severity of arrhythmias, such as ventricular tachycardia and ventricular fibrillation, were markedly reduced compared with the unpaced controls [4].

The antiarrhythmic effect of preconditioning appears in two phases. For example, Kaszala et al showed when pacing the hearts four times for 5 min, at a rate 240 beats min⁻¹ there is an early protective phase, which occurs immediately after the stimulus, but it lasts for less than 1 h. The antiarrhythmic protection re-appears spontaneously approximately 20-24h later and, this phase is termed as delayed or 'second window' of protection [5]. Interestingly, if the pacing protocol is repeated at a time when the protection from the previous stimulus has already been lost (i.e. 48 h after the first pacing), the antiarrhythmic protection persists even for days [6].

1.2 Mechanism of the early protection induced by preconditioning

Since first described in 1986, the phenomenon of preconditioning stimulated considerable interest to explore the underlying mechanism with the hope of finding a new therapeutic strategy against myocardial ischemic damage and its serious consequences. Despite the extensive research the precise mechanism by which preconditioning induces cardioprotection is still not fully understood.

It is well accepted that endogenous substances generated and released from the myocardium in response to the preconditioning stimulus and play a role in the initiation and mediation of the protection, first published in [7]. These substances, such as adenosine, bradykinin, opioids, prostanoids, nitric oxide and ROS, bind to their receptors or other targets and activate different signal transduction pathways and end-effectors [8].

Studies identified numerous signaling pathways that might have important role in the early phase of the protection. For example, adenosine, bradykinin, prostanoids, opioids that are released by an ischemic stimulus bind to GPCR and activate PI3 kinase. This stimulates numerous downstream pathways, such as the PI3K-Akt-eNOS-NO-sGC-PKG, the PI3K-Akt-ERK, the PI3K-Akt-GSK3 β or the PI3K-Akt-BAD/caspase9 mediated signaling processes reviewed by [8]. PKC ϵ and ERK are also downstream parts of the PI3 cascade [8]. For example, we have demonstrated that the NO-sGC-PKG signaling pathway is important in the induction and mediation of the early protection. The experimental evidence comes from studies in which the inhibition of NO synthesis by L-NAME markedly attenuated the early antiarrhythmic protection [9]. Furthermore, the same result was observed if sGC was inhibited with methylene blue [10].

These PC-induced survival pathways act on numerous end effectors. One of these, for example, is the mitochondria which plays a central role in the ischemia and reperfusion injury. During ischemia but mainly upon reperfusion, the ROS and Ca²⁺ overload lead to the forming and opening of the mPTP at the mitochondrial level, resulting in the depletion of ATP, irreversible oxidation of proteins, lipids, and DNA within the myocardium, and finally lead to robust mitochondrial swelling, cytochrome c release and later to cell death [11].

The survival signaling, induced by PC, acts directly on the inhibition of mPTP or prevents the conditions which lead to the opening of mPTP. One of the end-effectors for this is the mitoK_{ATP} channels, which are involved in regulating mitochondrial matrix volume. Early studies showed that inhibitors of mitoK_{ATP} channels blocked [12] and the openers mimicked the protection

afforded by PC [13, 14]. The activated mitoK_{ATP} decreases mitochondrial membrane potential which reduces the Ca²⁺ uptake during ischemic damage [15]. This activation mechanism is conducted through the NO-mediated signaling and also by PKCε [16, 17]. The activation of mitoK_{ATP} also results in the release of small levels of ROS which is thought to play a trigger role in PC by further enhancing the activation of PKCε, thereby amplifying the survival signaling [18]. Another target of the survival signaling is to inhibit the formation of mPTP directly [19, 20]. The inhibition of GSK3β through the PI3K pathway reduces the formation of mPTP [21]. Furthermore, PKCε has also been shown to phosphorylate VDAC through which the mPTP formation is blocked [22].

Apoptotic processes have also been implicated in ischemia and reperfusion injury. Apoptosis occurs via the activation of the caspase cascade which was also discovered as one of the targets of the survival pathways [23]. For example, Akt, activated after a PC stimulus, binds and inhibits the proapoptotic members of the Bcl-2 family, such as BAD and casp9 by phosphorylation through which the apoptotic processes are inhibited following the ischemia and reperfusion injury [24]. Another example is ERK, which is activated by PKCε in response to a PC stimulus and phosphorylates therefore inhibits the proapoptotic factor BAD [25].

Apart from the changes in cell viability, the metabolic and electric communication between cardiomyocytes is also damaged during ischemia and reperfusion which is in close relation with the generation of arrhythmias [26]. There is evidence from *in vitro* experiments that PC preserves gap junction function by preventing the dephosphorylation of the main gap junction forming protein Cx43 resulting from myocardial ischemia [27]. In response to a PC stimulus, the activated survival kinases such as PKC isoforms (α and ε) as well as PKG phosphorylate Cx43 thereby contributing to the attenuation of the generation of arrhythmias after the coronary artery occlusion [27].

1.3 Mechanism of the late protection induced by preconditioning

There is abundant evidence that gene expression changes play an important role in the delayed phase of cardioprotection afforded by preconditioning, reviewed in [28]. The endogenous substances that are released by preconditioning serve as triggers for the development of the early phase through activation of the survival signaling and acting on various end-effectors. The activation of these pathways decays with time, resulting in the attenuation and then the loss of

protection. Nevertheless, these pathways also stimulate the transcription of cardioprotective genes that induce the late protection, which occurs approximately 20-24h after the initial PC stimulus [28].

Previous studies have been identified several proteins as possible mediators in the late phase of protection such as the iNOS [6, 29, 30, 31, 32], Cox2 [29], aldose reductase, antioxidant enzymes, particularly MnSOD, [33], and heat stress proteins. Among the various cellular kinases that are activated by various PC stimuli, PKC [34] and Src protein tyrosine kinases play an essential role in the development of late PC [35].

It has now become clear, that the late protection is a complex phenotype involving multiple genes and can be considered as a whole genomic response initiated by various preconditioning stimuli. With the different tools of genomics which aims to study changes in gene expression, attention has turned to the exploration of the mechanism behind such multigenic phenomenon. Using large scale gene profiling techniques such as cDNA microarrays differential display or subtractive hybridization techniques, it was found that ischemic changes activate a protective genetic program which enables cardiomyocytes to overcome the harmful consequences of an otherwise lethal ischemic insult. Depre et al showed [36] in a pig ischemia model that genes from different cell survival pathways, including anti-apoptotic (i.e. the serine protease inhibitors plasminogen activator 1 and 2), cytoprotective genes (e.g. heat shock proteins) as well as cell growth promoters were up-regulated using gene microarray technique. Furthermore, Simkhovich et al. [37, 38] has found that protective genetic program was activated in rabbit and rat hearts following a brief episode of ischemia. They have found that transcripts, encoding the cytoprotective MAPKAPK-3, HSP 22, 27, 28, 70 iNOS and alpha B-crystalline, cell growth promoters (ATF-3 and H11 kinase) were increased. Ónodi et al has identified numerous genes with different functions following preconditioning using cDNA microarray with 3200 rat specific genes [39]. These genes were involved in protein degradation, stress response (heat shock proteins), apoptosis, metabolic processes and various regulatory mechanisms.

These studies have demonstrated that preconditioning mediated by short durations of reversible ischemia could result in the reprogramming the expression pattern of gene with diverse functions. For example, genes involved in stress response, such as antioxidants and heat shock proteins, were the most common genes which showed an altered transcription pattern in response to IPC [40]. Furthermore, genes encoding mitochondrial proteins and members of the survival signaling

or anti-apoptotic factors are also dramatically altered after IPC [40]. In our recent study, we have shown that cardiac pacing results in time-dependent changes in Cx43 gene and protein expression, which may alter gap junction function and influence arrhythmia generation during a subsequent ischemia and reperfusion insult [41].

There is no doubt that gene expression changes are important for the development of the late protection induced by short ischemic periods, but the transcriptional alterations in the antiarrhythmic adaptation induced by rapid cardiac pacing are less understood.

1.4 Mechanisms involved in the delayed antiarrhythmic protection induced by rapid cardiac pacing

The experimental evidence that bradykinin has an important role in the antiarrhythmic effect of cardiac pacing comes from previous results in anesthetized dogs. These demonstrated that incatibant (HOE 140), an antagonist of bradykinin at B2 receptors, attenuates the pacing-induced early and delayed antiarrhythmic protection [42, 43]. In the presence of incatibant, pacing the heart through the right ventricle increased the severity of arrhythmias and ischemia during the pacing protocol, and abolished the antiarrhythmic protection during coronary artery occlusion and reperfusion [42].

The first evidence that NO is a key mediator not only in the early but also in the late protection came from studies in which the non-selective iNOS inhibitor, dexamethasone abolished the delayed antiarrhythmic protection resulted from rapid cardiac pacing [29]. Later this had been confirmed by studies using more selective inhibitors of iNOS [6, 30, 31, 44]. For example, when aminoguanidine [31] and AEST [6] was administered prior to the occlusion in dogs that were subjected to cardiac pacing 24h previously, the antiarrhythmic protection was abolished. Similarly, the repeated cardiac pacing-induced prolonged protection [6] as well as the heavy physical exercise-induced delayed antiarrhythmic effect [44] were both sensitive for selective (AEST) and non-selective (L-NAME) inhibitors of iNOS, indicating a role for iNOS activation in the development of the delayed cardioprotection.

Since these inhibitors are not entirely selective for iNOS, for example dexamethasone inhibits cox-2 as well [29], whereas L-NAME interferes NO formation resulting from activation of any NOS enzymes [44], the role for other isoforms of NO producing enzymes in the delayed phase of the protection can not be ruled out.

Using *in vitro* methods, other studies have reported that the activity of constitutive calcium-dependent NOS enzymes (eNOS and nNOS) was increased only immediately after preconditioning, but remained unaffected 24h later [45], whereas the mRNA of iNOS was up-regulated and showed marked enzymatic activation only during the late phase of the protection [32]. They have put forth a hypothesis that NO is generated from the activation of constitutive NOS enzymes and induces the transcription of iNOS thereby enhances further NO generation at later time points (NO induced NO formation). Since in our previous studies we had only pharmacological evidence for the role of NO in the delayed antiarrhythmic protection we have now aimed to investigate this by using molecular biology approach.

1.5 Aims of the study

The objectives of the present studies were to explore the mechanism of the antiarrhythmic protection induced by rapid cardiac pacing by gene expression profiling. Therefore,

I. In Study I, we have attempted to examine gene expression changes following ischemia and reperfusion in dogs subjected to rapid right ventricular pacing twenty-four hours previously. Two approaches were used:

- cDNA microarray analysis which enables to identify the transcription patterns of genes activated by a certain intervention such as cardiac pacing, and
- qRT-PCR by which we analyzed the transcription of 45 selected genes, which had already been proposed to play roles in the delayed phase of the preconditioning-induced protection.

II. In Study II, the time course changes of gene expression following rapid cardiac pacing have been analyzed.

2. MATERIALS AND METHODS

2.1 Experimental protocols and surgical interventions

Adult mongrel dogs of both sexes were used (mean body weight 20 ± 1 kg) in these studies. The origin and upkeep of these dogs were in accordance with the Hungarian law (XXVIII, chapter IV, paragraph 31) regarding large experimental animals, which conforms with the Guide for the Care

and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

2.1.1 Experimental protocols

In Study I and II, a total of thirty-three dogs were instrumented by introducing a pacing electrode into the right ventricle (Figure 1).

In Study I, three dogs served as sham paced and sham operated controls (C). In these animals, the pacing electrode was introduced into the right ventricle, but the dogs were not paced. Twenty-four hours later these dogs were re-anesthetized, thoracotomised and instrumented but were not subjected to coronary artery occlusion. Another three sham-paced dogs, 24h after the instrumentation, were subjected to a 25 min occlusion of the left anterior descending (LAD) coronary artery. These animals served as the ischemic controls (IC). Other three dogs were paced on day one and 24h later were subjected to a 25 min LAD occlusion (PO group). After euthanasia with an intravenous overdose of the anesthetic the hearts were sectioned at the time points indicated by arrows (Figure 1) and tissue samples were taken for further biochemical analysis. In Study II, twelve animals were subjected to rapid cardiac pacing (P), whereas another twelve dogs served as sham-paced controls (SP). Dogs of the paced and non-paced groups were divided into 4 further groups, each containing 3 animals. From these dogs after euthanasia with an intravenous overdose of the anesthetic the hearts were excised immediately (P-0h/SP-0h), six (P-6h/SP-6h), twelve (P-12h/SP-12h) or twenty-four (P-24h/SP-24h) hours after the pacing or sham-pacing procedures (Figure 1).

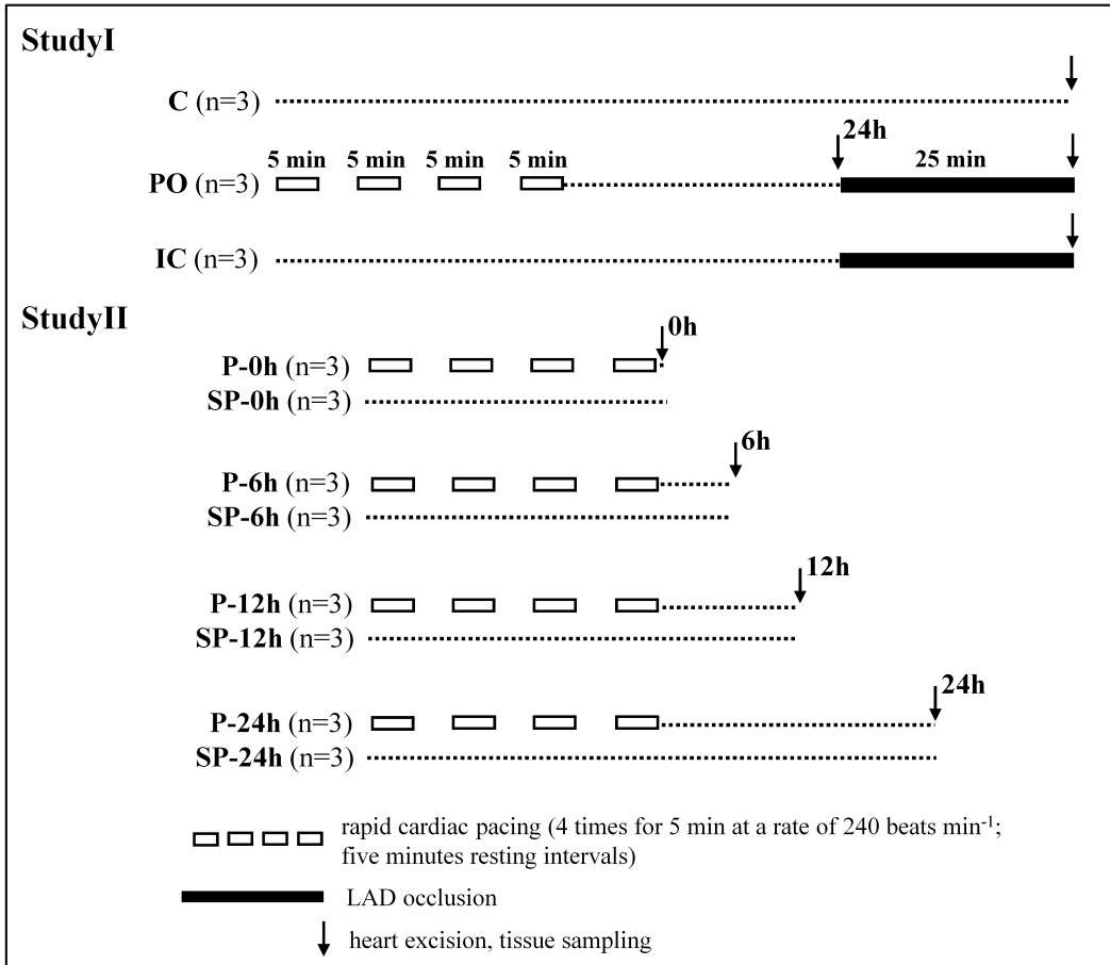


Figure 1. Experimental protocols. In Study I, relative gene expression changes were determined in three groups of dogs; controls were sham paced and sham operated dogs, without (C) and with ischemia (IC) and in paced dogs that were subjected to coronary artery occlusion and reperfusion, 24h after pacing (PO). In Study II, the time-course changes in gene expression occurring after rapid cardiac pacing were determined at four time points both in the unpaced and paced dogs (P-0h, P-6h, P-12h, P-24h).

2.1.2. Cardiac pacing procedure

The pacing procedure was performed as described previously [5]. In brief, the dogs were lightly anesthetized by the intravenous administration of sodium pentobarbitone (0.5 mg kg^{-1} i.v., Euthanyl[®] Bimeda-MTC Animal Health Inc.) and were allowed to breathe spontaneously. Arterial blood pressure was monitored from the left carotid artery using methods previously described in

detail (Kaszala et al 1996, Vegh et al 1992). Through the right jugular vein, a pacing electrode (CordisF4, Johnson & Johnson Company, USA) was inserted into the right ventricle such as that it made contact with the ventricular wall. The correct placing of the electrode was confirmed by recording the endocardial electrocardiogram. The dogs were then paced at a rate of 240 beats min^{-1} for four 5 min periods with 5 min rest intervals between the pacing stimuli. The sham-paced controls were those in which the pacing electrode was positioned into the right ventricle, as described above but the dogs were not paced. The effect of pacing was followed by measuring changes in subendocardial ST-segment elevation immediately after cessation of pacing using the pacing electrode.

2.1.3. Coronary artery occlusion

Twenty-four hours later, those dogs that had been selected for coronary artery occlusion (in the IC and PO groups) were re-anesthetized with a mixture of α -chloralose and urethane (60 and 200 mg kg^{-1} , respectively Sigma, USA), intubated and ventilated with room air using a Harvard respirator (Harvard Apparatus, USA) at a rate and volume sufficient to maintain arterial blood gases and pH within the normal limits [2]. Body temperature was measured from the mid-esophagus, and maintained at 37 ± 0.5 °C by means of a heating pad. Polyethylene catheters were inserted into the right femoral vein and into the femoral artery for further administration of the anesthetic and to measure arterial blood pressure. The catheter inserted in the left carotid artery previously was then led to the left ventricle to measure changes in left ventricular pressure and dP/dt .

A thoracotomy was performed at the fifth intercostal space and the anterior descending branch of the LAD was prepared for occlusion just proximal to the first main diagonal branch. Myocardial ischemia was induced by a 25 min occlusion of this artery as described previously (Vegh et al 1992). The severity of myocardial ischemia was assessed by the measurement of changes in the degree of inhomogeneity of electrical activation and in epicardial ST-segment by means of a composite electrode described previously [2]. The depth of anesthesia was monitored by the examination of the cornea and pain reflexes as well as by the measurement of blood pressure, and when it was necessary a further bolus injection of the anesthetic was given.

2.1.4 Evaluation of ventricular arrhythmias

Ventricular arrhythmias were assessed according to the Lambeth Conventions [46] with that modification as has been previously outlined [2]. In brief, the total number of VBPs, the incidence and number of episodes of ventricular tachycardia (defined as a run of four or more VBPs at a rate faster than the resting heart rate), and the incidence of VF were assessed during the occlusion period.

2.2 Myocardial tissue sampling

At the end of the experimental protocols the animals were given an overdose of the anesthetic, and the hearts were rapidly excised and placed in ice cold saline. Tissue samples were taken from the ischemic area (supplied by the LAD) and from the non-ischemic area (supplied by the LCX) for further analysis.

2.3 Isolation of RNA

Tissue samples from both the ischemic and non-ischemic myocardial regions were powdered in liquid nitrogen and 25 mg was used for RNA isolation. Then total RNA was purified with the RNeasy Fibrous Tissue Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. The integrity of RNA was assessed by gel electrophoresis and the concentration of total RNA was determined by UV-photometry. DNaseI digestion was also performed to avoid the potential genomic DNA contamination. The RNA preparations from 3 dogs in each group were pooled together. The total RNA preparations were used for microarray analysis and qRT-PCR measurements.

2.4 Microarray analysis

The microarray analysis was performed as described by [47]. In brief, human cDNA microarray containing 3200 human cDNA clones was used for the hybridization. For probe preparation, 2 µg of total RNA was reverse transcribed using poly-dT-primed Genisphere Expression Array 350 Detection Kit system (Genisphere, USA) according to the manufacturer's instructions. Both cDNA hybridization and capture reagent hybridization were carried out in a Ventana hybridization station (Ventana discovery, USA) using the "antibody" protocol. The first hybridization was performed at 40 °C for 6 hours in "FGL2" hybridization buffer (10xDenhart

solution, 0.25 M sodium phosphate buffer pH 7.0, 1mM EDTA, 1x SSC, 0.5% SDS), then 2.5 μ l each of Cy5 and Cy3 capture reagents in 200 μ l “Chiphycb” hybridization buffer (Ventana) were added to the slides and incubated at 42 °C for 2 hours. After hybridization, the slides were washed twice in 0.2 X SSC at RT for 10min, then dried and scanned.

Each array was scanned under a green laser (532 nm) (for Cy3 labeling) and under a red laser (660 nm) (for Cy5 labeling) using a ScanArray Lite (GSI Lumonics, Billerica, MA, USA) confocal fluorescent scanner with 10 μ m resolution. Image analysis was performed by ScanAlyze2 software (<http://www.microarrays.org/software.html>). Each spot was defined by manual positioning of a grid of circles over the image. The average pixel intensity and the local background of each spot were determined. To measure the ‘expression ratio’, MRAT (denotes the median of the set of background-corrected single pixel intensity ratios of the two channels within the spot) was determined. This average expression ratio for all genes on the array was normalized to 1.0. For background corrections those data were calculated as negatives where the average intensity of the spot was smaller than two times the average background of the same area. Significant spots have more than 0.55 CHGTB2 values (denotes fraction of pixels in the spot greater than background) in both Cy3 and Cy5 channels. To reduce the number of false positive or false negative ratios, two probes were generated from each RNA pool: a Cy5-labeled and a Cy3-labeled one in order to perform replicate experiments. Replica spots (on the same array) and replica experiments (two different arrays) resulted in four data points for every gene. Those spots were excluded from further analysis when ratios of the replica spots had a more than two-fold difference. The same restriction was applied for the average ratios of the replica experiments.

2.5 Quantitative Real-Time PCR (qRT-PCR)

Quantitative RT-PCR was performed by ABI7000 instrument (Applied Biosystems, Carlsbad, USA) with gene-specific primers to monitor gene expression changes. Two μ g of total RNA from each sample was reverse transcribed using ImPromII reverse transcriptase (Promega, USA) in the presence of poly(dT) primers at 42 °C for 1h. After this, cDNAs were diluted in 50 μ l water, and 1 μ l of this reaction mix was used as template for qRT-PCR. This was performed using the SYBRGreen protocol with SYBR Green PCR Master Mix with passive reference dye (Applied Biosystems, Carlsbad, USA) according to the manufacturer’s instructions at a final primer concentration of 500nM. The primers were designed using the PrimerExpress 2.0 (Applied

Biosystems, Carlsbad, USA) and verified by NCBI-BLAST. Relative expression ratios were calculated as normalized ratios to HPRT and to tubulin. A non-template control sample was applied for each run to control the genomic DNA contaminations of the template. Melting temperature analysis has been performed after each reaction to assess the quality of the reaction. The final relative gene expression changes were calculated as delta-delta Ct values. Genes with expression values lower than 0.5 or higher than 1.5 were considered to be down or up-regulated (the values correspond to an interval of $\log_2=-1$ and $\log_2=0.6$). All the PCRs were performed in triplicate with two parallel runs for each reaction. All data were expressed as mean \pm SD.

2.6 Immunoblot analysis

Transmural tissue samples from both the ischemic and non-ischemic regions of the left ventricle were powdered in liquid nitrogen and stored at -80°C . The samples were then homogenized in ice cold lysis buffer (20 mM Tris-HCl pH 7.4, 250 mM sucrose, 0.1% protease inhibitor cocktail, Sigma) and centrifuged at 2000g for 15 min. The supernatant was used either as a total protein fraction or centrifuged at 50000 g for 45 min and the pellet was re-suspended in ice cold lysis buffer and used as the membrane fraction. Protein concentrations were determined by the method of Lowry. Total protein samples of 100ug and membrane protein extracts of 80ug were separated on 8% polyacrylamide gels and transferred to PVDF membranes (Millipore). After blocking in 5% non-fat milk (for one hour at room temperature), the membranes were incubated with two different primary antibodies. Anti-eNOS (BD Transduction LaboratoriesTM) was used to measure the protein expression of eNOS (in a dilution of 1:3000) and anti-phospho-eNOS (peNOS, Ser1177, BD Transduction LaboratoriesTM) to detect the activation of eNOS (in a dilution of 1:1000) by the Akt/PI3 kinase pathway. This was followed by one hour incubation of HRP-conjugated goat anti-rabbit secondary antibody at room temperature (in a dilution of 1:8000). Blots were developed with ECL plus kit (GE Healthcare) and exposed to X-ray films (Amersham Biosciences). Band densities were measured using ImageJ software (NIH). For the precise comparison of the three parallel western blots, each pixel intensity was normalized to the highest value on each blot. For verification of equal loading, Coomassie staining was performed. Values are mean \pm S.E.M. * $P<0.05$ vs. SP group (Bonferroni correction).

3. RESULTS

3.1 Gene expression changes in control and paced dogs subjected to coronary artery occlusion and reperfusion twenty-four hours later

cDNA microarray analysis was performed in order to identify gene expression changes in the pacing-induced delayed antiarrhythmic protection. The expression values of the ischemic and preconditioned groups were compared to those obtained from the sham paced, sham operated control dogs. Their relative expression ratios are shown in Table 1. The data obtained from cDNA microarrays were confirmed by qRT-PCR analysis.

Out of the studied 3200 cDNAs four genes were down- and five genes were up-regulated in the ischemic group compared to the control dogs. Compared to the IC group, cardiac pacing resulted in an enhanced expression of 19 genes and lower expression of 4 genes. These genes with altered transcription rate encode transcription factors, ion channels and members of signal transduction pathways. Some of these, for example MEF2A and PDE4D9 were not significantly changed in response to myocardial ischemia, but were up-regulated following rapid cardiac pacing. In contrast, three genes (14-3-3zeta, AVPR1, AVPR2), that showed higher expression in the ischemic controls, were either not changed (14-3-3zeta) or down-regulated (AVPR1, AVPR2) in dogs paced 24h previously. The abbreviations of this section can be found in the Table 1.

Categories	Gene name	Accession	IC/C	PO/IC
ion channel/transcription factors	chloride intracellular channel protein 1 (NCC27)	XM_532079	0.37	2.36
	rearranged L-myc fusion (ZN-15 related zinc finger protein)	XM_532543	1.17	0.54
	myocyte enhancer-binding factor 2 (MEF2)	XM_536298	1.14	4.28
metabolic enzyme	alcohol dehydrogenase 1C, gamma polypeptide (ADH1C)	XM_535667	0.65	3.83
signal transduction	prolactin receptor (PRLR)	XM_536502	1.20	1.88
	arginine vasopressin receptor 1 (VR1)	XM_538263	2.16	0.43
	arginine vasopressin receptor 2 (VR2)	NM_001003177	2.12	0.45
	protein tyrosine phosphatase, receptor type, J precursor (PTPRJ)	XM_540737	0.52	0.38
	tumor necrosis factor receptor member 10A precursor (TRAIL-R1)	XM_543245	2.84	1.45
	interleukin-1 receptor-associated kinase 1 (IRAK-1)	XM_549367	0.54	2.69
	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (14-3-3 zeta)	XM_533072	1.89	0.53
	cAMP-specific phosphodiesterase variant (PDE4D9)	XM_535243	0.65	3.83
	transforming growth factor, beta 2 (TGFB2)	XM_545713	0.97	1.88
	phosphorylase b kinase gamma catalytic chain, skeletal muscle isoform (PHKG1)	XM_546902	0.53	2.24
others	ST3 beta-galactoside alpha-2,3-sialyltransferase 1 (ST3GAL1)	XM_539171	1.40	1.85
	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11	XM_543872	0.83	0.50
	granzyme K precursor	XM_546318	1.07	1.88
	zinc finger protein, X-linked (ZFX)	AF027765	1.01	1.91
	translocation associated membrane protein 1 (TRAM1)	NM_001003267	0.43	2.02
	phosphatidylinositol glycan, class Q isoform 2	XM_547216	0.30	3.34
	A-kinase anchor protein 6 (mAKAP)	XM_547766	0.81	2.28
	midline 1 isoform alpha	XM_548857	0.36	4.10
	necdin	XM_545810	0.45	2.49
	gastric inhibitory polypeptide receptor precursor (GIPR)	XM_541554	1.61	1.80
	WD-repeat protein 26 (WDR26)	XM_537237	1.05	1.88
	GPATCH8 G patch domain containing 8 (GPATCH8)	XM_537619	0.72	1.93

Table 1. Gene expression changes following cardiac pacing by cDNA microarray. The expression values were calculated from the intensity ratios obtained from the scanning analysis of the cDNA microarray as described in the section of Materials and Methods. (The values labeled with red indicate up-regulation while the data labeled with blue indicate down-regulation). Each experiment was performed twice.

3.2 Gene expression changes following cardiac pacing determined by qRT-PCR analysis

QRT-PCR was performed to study 45 genes which have previously been identified as potential mediators or important members of the preconditioning-induced cardioprotection. Thus, genes encoding gap junction proteins (Cx40; Cx43; Cx45) NO producing enzymes (iNOS, eNOS, nNOS), cyclooxygenases (COX1, COX2), antioxidants (CAT, Mn-SOD, Cu/Zn-SOD), anti-apoptotic and apoptotic proteins (BAX, BAK, Casp3, Casp9), heat shock proteins (HSP27, HSPA8), cytokines (IL-6, TNF α); growth factors (TGF β , TGFBR2), transcription factors (c-fos), members of different signal transduction pathways, such as different isoforms of the protein kinase C (PKC α ; PKC δ , PKC ϵ), of the JNKs (JNK1,2,3) and of the sGCs (sGC α 1/ β 1, sGC α 2/ β 2) were examined (Table 2).

Out of the 45 studied genes, six exhibited significant down-regulation and 16 showed up-regulation in response to myocardial ischemia when compared to the control dogs. Their expression ratios changed either to the opposite (11) or showed no alteration (10) in the PO group (Table 2). Among these nNOS, HSP70, PKC α , JNK1, GSK3 α , GYS1, BDKRB1 showed up-regulation, and BAX, BAK and casp3 down-regulation after rapid cardiac pacing.

Figure 2, 3 and 4 illustrate some of these genes from different functional categories, which showed expression changes in response to ischemia and/or preconditioning. The abbreviations of this section are listed in the Table 2.

Categories	Gene name	Accession	IC/C	PO/IC
gap junction	gap junction protein, alpha 5, 40kDa	NM_005266	0.29 (0.06)	1.17 (0.21)
	gap junction protein connexin 43 (Cx43)	AY462223	1.08 (0.08)	0.83 (0.11)
	gap junction protein, alpha 7, 45kDa	NM_005497	1.10 (0.41)	3.64 (2.79)
NO production	endothelial nitric oxide synthase (eNOS)	AF400594	2.34 (0.68)	0.62 (0.05)
	inducible nitric oxide synthase (iNOS)	NM_001003186	1.79 (0.23)	0.58 (0.03)
	neuronal nitric oxide synthase (nNOS)	HSU17327	0.80 (0.07)	2.16 (0.46)
antioxidants	catalase (CAT)	NM_001002984	1.06 (0.08)	0.29 (0.12)
	Cu/Zn superoxide dismutase (Cu/Zn-SOD)	AF346417	0.83 (0.09)	0.70 (0.07)
	Mn superoxide dismutase (Mn-SOD)	Y00985	2.80 (1.71)	1.08 (0.50)
	cytochrome C oxydase	T19137	1.54 (0.11)	0.35 (0.08)
miscellaneous	cyclooxygenase-1 (COX1)	NM_000962	1.02 (0.16)	0.95 (0.12)
	cyclooxygenase-2 (COX2)	NM_001003354	1.04 (0.41)	1.23 (0.43)
	matrix metalloproteinase 2 (MMP2)	BC002576	0.30 (0.11)	1.96 (0.19)
apoptotic genes	BCL2-associated X protein (BAX)	NM_001003011	1.85 (0.17)	0.28 (0.06)
	Bcl-2 antagonist/killer protein (BAK)	DQ002813	1.50 (0.02)	0.24 (0.04)
	caspase 3 apoptosis-related cysteine peptidase (CASP3)	NM_001003042	2.23 (0.50)	0.37 (0.09)
	caspase 9 apoptosis-related cysteine peptidase (CASP9)	DQ116956	2.51 (0.36)	0.98 (0.07)
heat shock proteins	heat shock 27kDa protein 1 (HSP27)	NM_001540	1.88 (0.05)	0.45 (0.05)
	heat shock 70kDa protein 8 (HSP70)	NM_006597	1.82 (0.18)	2.77 (0.65)
	heat shock 70kDa protein 1A (HSPA1A)	NM_005345	2.68 (0.56)	0.56 (0.09)
cytokines	tumor necrosis factor alpha (TNF α)	NM_001003244	0.60 (0.08)	0.46 (0.04)
	interleukin-6 (IL-6)	U12234	0.49 (0.04)	4.09 (0.94)
growth factors	transforming growth factor beta 1 (TGF β 1)	NM_000660	3.43 (0.98)	0.20 (0.02)
	transforming growth factor, beta receptor II	NM_003242	0.89 (0.16)	0.50 (0.02)
transcription	nuclear factor of kappa light polypeptide cellular oncogene c-fos	NM_003998	1.11 (0.22)	1.14 (0.30)
		V01512	0.30 (0.11)	1.45 (0.24)
signal transduction	protein kinase C, delta (PKC δ)	NM_001008716	2.65 (0.55)	0.37 (0.07)
	protein kinase C, alpha (PKC α)	NM_002737	0.95 (0.15)	2.57 (0.61)
	protein kinase C, epsilon (PKC ϵ)	NM_005400	1.66 (0.20)	0.53 (0.07)
	mitogen-activated protein kinase 8 (JNK1)	NM_139049	0.83 (0.10)	3.31 (0.07)
	mitogen-activated protein kinase 9 (JNK2)	NM_139070	0.76 (0.10)	0.93 (0.03)
	mitogen-activated protein kinase 10 (JNK3)	BC035057	1.26 (0.37)	1.19 (0.39)
	glycogen synthase kinase 3 alpha (GSK3 α)	BC027984	0.45 (0.08)	1.94 (0.32)
	glycogen synthase kinase 3 beta (GSK3 β)	BC000251	10.14 (1.03)	0.73 (0.06)
	glycogen synthase 1 (muscle) (GYS1)	NM_002103	2.07 (0.13)	3.35 (1.09)
	muscle glycogen phosphorylase (PYGM)	AF066859	1.30 (0.29)	1.14 (0.15)
	protein phosphatase 2 (PP2A)	BC032954	1.04 (0.08)	0.54 (0.05)
	adenylate cyclase 6 (AD6)	BC064923	1.29 (0.16)	0.80 (0.11)
	guanylate cyclase soluble, alpha1 (GC α 1)	NM_000856	1.38 (0.33)	1.03 (0.21)
	guanylate cyclase, soluble, beta1 (sGC β 1)	NM_000857	0.98 (0.11)	0.57 (0.02)
	guanylate cyclase soluble, alpha2 (sGC α 2)	NM_000855	2.36 (0.27)	0.35 (0.07)
	guanylyl cyclase soluble, beta 2 (sGC β 2)	AF038499	1.19 (0.19)	0.50 (0.07)
	natriuretic peptide receptor A (NPR1)	NM_000906	1.20 (0.11)	0.79 (0.09)
	bradykinin receptor B1 (BDKRB1)	NM_000710	0.43 (0.08)	4.91 (2.80)
	bradykinin receptor B2 (BDKRB2)	AY275465	9.08 (9.36)	0.25 (0.12)

Table 2. Gene activity changes 24h after cardiac pacing in dogs subjected to coronary artery occlusion and reperfusion. The 45 genes examined were divided into different groups according to the function of proteins they encode. Genes with expression values lower than 0.5 or higher than 1.5 were considered to be down or up-regulated. Values are means \pm SD (indicated in brackets) of three parallel qRT-PCR reactions. (Values labeled with red indicate up-regulation while the data labeled with blue indicate down-regulation).

3.3 Time-course changes in gene expression following cardiac pacing in dogs

In order to assess whether cardiac pacing induces time-dependent changes in gene expression occurring over a period of 24h which elapses between the pacing stimulus and the appearance of the delayed effect, the transcription of 29 genes were analyzed immediately, 6, 12 and 24 hours after cardiac pacing. Table 3 summarizes the gene expression changes observed at various time intervals after cardiac pacing. The results are expressed as ratios of gene expression data obtained from the time-matched sham-paced control hearts.

There were eight genes which were immediately up-regulated after the cessation of pacing. These genes encode HSP90, MnSOD, Bcl-2, PKC ϵ , ERK1, sGC α 1, sGC β 1, sGC α 2. There were six genes which exhibited early down-regulation following the pacing stimulus, such as CAT, casp3, casp9, GSK3 β and MMP9. Of these early up- or down-regulated genes, MnSOD, PKC ϵ , casp3 and casp9, GSK3 β and MMP9 remained unchanged (i.e. up and down-regulated) at almost all time-points, whereas the others, like HSP90, ERK1, Bcl-2 and sGC isoforms, showed biphasic transcriptional changes; i.e. after the initial up-regulation these genes were down-regulated (mostly at 6h) but up-regulated again around 12 and 24h (Table 3). There were also genes, such as those encode NO producing enzymes eNOS, iNOS and the gap junction forming protein Cx43, which exhibited significant up-regulation only 12h after the cessation of pacing (Table 3). Surprisingly, the expressions of genes responsible for the production of mitochondrial proteins, as well as Cox2, were not significantly altered.

Categories	Gene name	Accession	P-0h/SP	P-6h/SP	P-12h/SP	P-24h/SP
heat shock proteins	heat shock 27kDa protein (HSP27)	NM_001003295	0.50 (0.01)	0.36 (0.07)	0.92 (0.21)	1.08 (0.37)
	heat shock 70kDa protein (HSP70)	NM_006597	0.99 (0.72)	0.19 (0.13)	0.69 (0.48)	0.32 (0.16)
	heat shock 90kDa protein (HSP90)	NM_001017963.2	1.78 (0.19)	0.85 (0.17)	1.50 (0.25)	1.11 (0.31)
gap junctions	connexin 43 (Cx43)	NM_001002951	1.43 (0.18)	0.40 (0.10)	2.20 (0.44)	1.15 (0.28)
antioxidants	catalase (CAT)	NM_001002984	0.17 (0.13)	0.25 (0.12)	0.89 (0.70)	0.87 (0.46)
	Mn superoxide dismutase (MnSOD)	NM_000636	2.30 (0.46)	1.80 (0.26)	1.61 (0.15)	2.02 (0.50)
NO production	endothelial nitric oxide synthase (eNOS)	NM_001003158	0.67 (0.07)	1.09 (0.06)	2.19 (0.53)	0.59 (0.11)
	inducible nitric oxide synthase (iNOS)	NM_001003186	1.47 (0.45)	1.15 (0.22)	2.43 (0.27)	0.99 (0.21)
apoptosis	apoptosis-related cysteine peptidase 3 (casp3)	NM_001003042	0.36 (0.09)	0.33 (0.13)	0.32 (0.12)	1.28 (0.08)
	apoptosis-related cysteine peptidase 9 (casp9)	XM_847070	0.18 (0.16)	0.15 (0.11)	0.32 (0.05)	1.23 (0.22)
	BCL2-associated X protein (BAX)	NM_001003011	0.72 (0.28)	0.32 (0.02)	0.74 (0.38)	1.15 (0.22)
	B-cell CLL/lymphoma 2 (Bcl2)	NM_001002949	2.78 (0.99)	0.45 (0.15)	1.62 (0.54)	2.66 (1.02)
signal transduction	protein kinase C, alpha (PKC α)	NM_002737	1.42 (0.39)	0.87 (0.36)	2.15 (0.40)	1.90 (0.23)
	protein kinase C, delta (PKC δ)	NM_001008716	1.05 (0.01)	0.57 (0.14)	1.44 (0.32)	1.31 (0.23)
	protein kinase C, epsilon (PKC ϵ)	NM_005400	6.73 (2.43)	1.77 (0.32)	2.27 (0.31)	2.08 (0.37)
	glycogen synthase kinase 3 beta (GSK3 β)	NM_002093	0.37 (0.10)	0.34 (0.08)	0.71 (0.30)	0.44 (0.09)
	extracellular signal-regulated kinase 1 (ERK1)	NM_002746	2.32 (0.37)	0.49 (0.24)	0.72 (0.42)	2.47 (0.78)
	extracellular signal-regulated kinase 2 (ERK2)	NM_002745	0.98 (0.35)	0.95 (0.36)	1.26 (0.66)	0.59 (0.34)
	guanylate cyclase soluble, alpha 1 (sGC α 1)	NM_001018035	1.71 (0.14)	1.44 (0.26)	1.12 (0.21)	2.44 (0.16)
	guanylate cyclase soluble, beta 1 (sGC β 1)	NM_001018034	2.97 (0.53)	2.51 (0.64)	1.27 (0.19)	2.60 (0.51)
guanylate cyclase soluble, alpha 2 (sGC α 2)	NM_000855	2.21 (0.81)	1.18 (0.07)	1.62 (0.11)	2.41 (0.90)	
mitochondria	cytochrome c (CytC)	NM_001197045	1.05 (0.25)	0.85 (0.21)	0.79 (0.22)	1.00 (0.54)
	ATP synthase, Fo complex, subunit F6	NM_001003703	1.48 (0.14)	0.93 (0.30)	0.64 (0.05)	0.51 (0.09)
	cytochrome c oxidase subunit Va (COX5A)	NM_004255	1.38 (0.15)	0.82 (0.05)	0.02 (0.28)	1.16 (0.07)
	NADH dehydrogenase Fe-S protein2, 49kDa	NM_001166159	1.33 (0.19)	0.59 (0.06)	0.80 (0.07)	0.76 (0.09)
	NADH dehydrogenase Fe-S protein1, 75kDa	NM_005006	1.05 (0.18)	0.65 (0.16)	0.46 (0.08)	0.52 (0.07)
others	matrix metalloproteinase 2 (MMP2)	NM_004530	0.63 (0.19)	0.65 (0.09)	1.04 (0.18)	0.73 (0.02)
	matrix metalloproteinase 9 (MMP9)	NM_001003219	0.06 (0.08)	0.17 (0.04)	1.08 (0.38)	0.59 (0.31)
	cyclooxygenase-2 (Cox2)	NM_001003354	0.19 (0.09)	0.28 (0.14)	0.87 (0.41)	0.27 (0.15)

Table 3. Time-course changes in gene expression following cardiac pacing in dogs. The 29 genes examined were divided into different groups according to their functions. Genes with expression values lower than 0.5 or higher than 1.5 were considered to be down or up-regulated. Values are means \pm SD (indicated in brackets) of three parallel qRT-PCR reactions. (The values labeled with red indicate up-regulation while the data labeled with blue indicate down-regulation.)

Some of the results summarized in Table 2 and Table 3 are also illustrated in figures (Figure 2, 3 and 4) by selecting those genes which have shown significant transcription alterations following cardiac pacing, and also after the 25 min of coronary artery occlusion and reperfusion may have essential functional role in the delayed cardioprotection afforded by preconditioning.

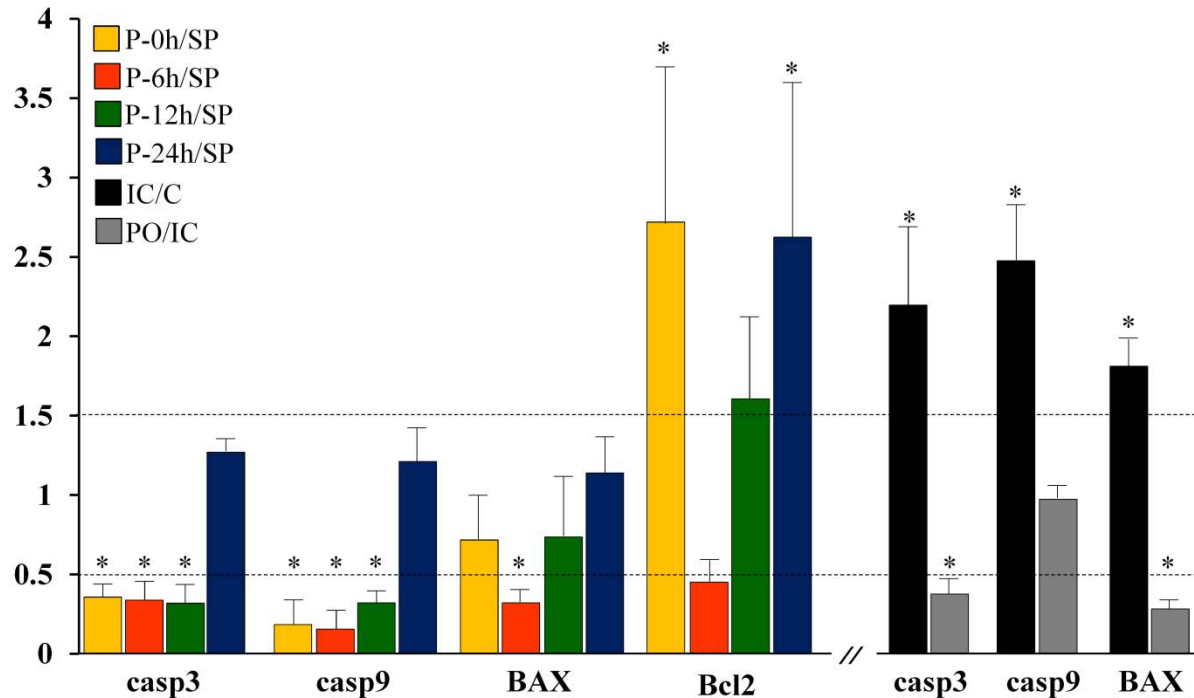


Figure 2. The effect of rapid cardiac pacing and ischemia/reperfusion on the expression of genes involved in apoptosis. Values are means \pm SD. The lines indicate the interval between 0.5 and 1.5 fold of regulation.

Figure 2 shows that cardiac pacing significantly down-regulated the transcription of genes which are involved in apoptosis (casp3, casp9 and Bax) immediately (0h), 6 and 12h after cardiac pacing, but at 24h the expression of these genes was not significantly different from the non-paced controls. In contrast, pacing enhanced, at each time point, except 6h, the expression of gene encoding the anti-apoptotic Bcl-2 (Figure 2). Myocardial ischemia also altered the expression of casp3, casp9 and Bax. These exhibited significant up-regulation compared to the sham group and their expression ratios changed either to the opposite (casp3 and Bax) or showed no alteration (casp9) after cardiac pacing (Figure 2).

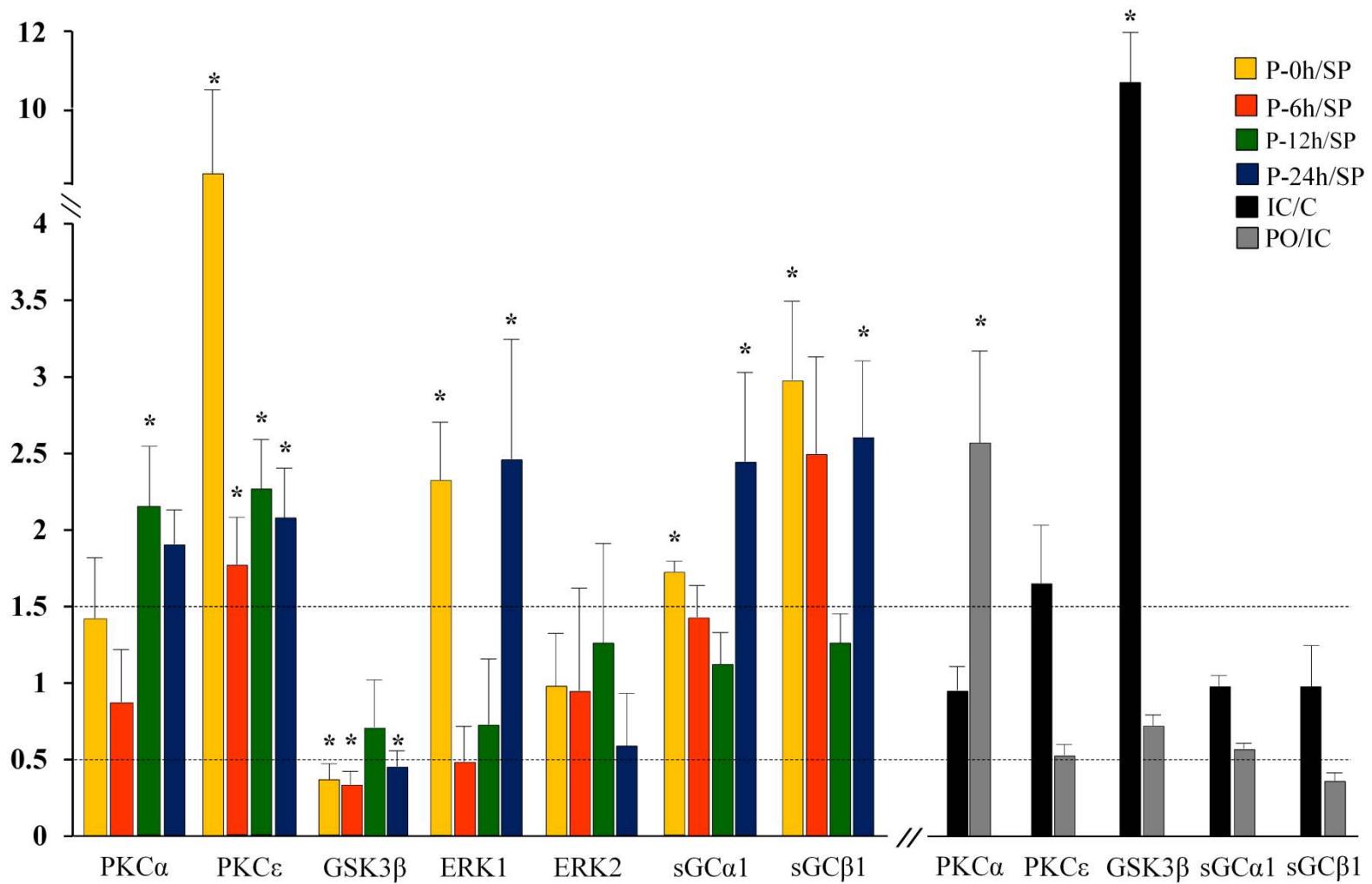


Figure 3. The effect of rapid cardiac pacing and ischemia/reperfusion on the expression of genes encoding proteins involved in signal transduction pathways. Values are means \pm SD. The lines indicate the interval between 0.5 and 1.5 fold of regulation. (Note: ERK1 and ERK2 were not involved in Study II.)

The time-course pattern of genes encoding proteins involved in signal transduction showed large variation. Of these genes only PKC ϵ exhibited enhanced transcription in response to rapid cardiac pacing at each time point. PKC α was only up-regulated at 12 and 24h whereas, the expression of PKC δ remained virtually unchanged (Figure 3). ERK1 showed an early (0h) and late (24h) up-regulation, while GSK3 β was down-regulated at almost all the measured time points. Genes encoding the isoforms of sGC were significantly up-regulated immediately, and 24h after the cessation of the pacing stimulus (Figure 3). In response to ischemia, GSK3 β showed a significant down-regulation while the other genes were not altered. These genes in the PO group showed no significant changes except for PKC α which was up-regulated when compared to the ischemic controls.

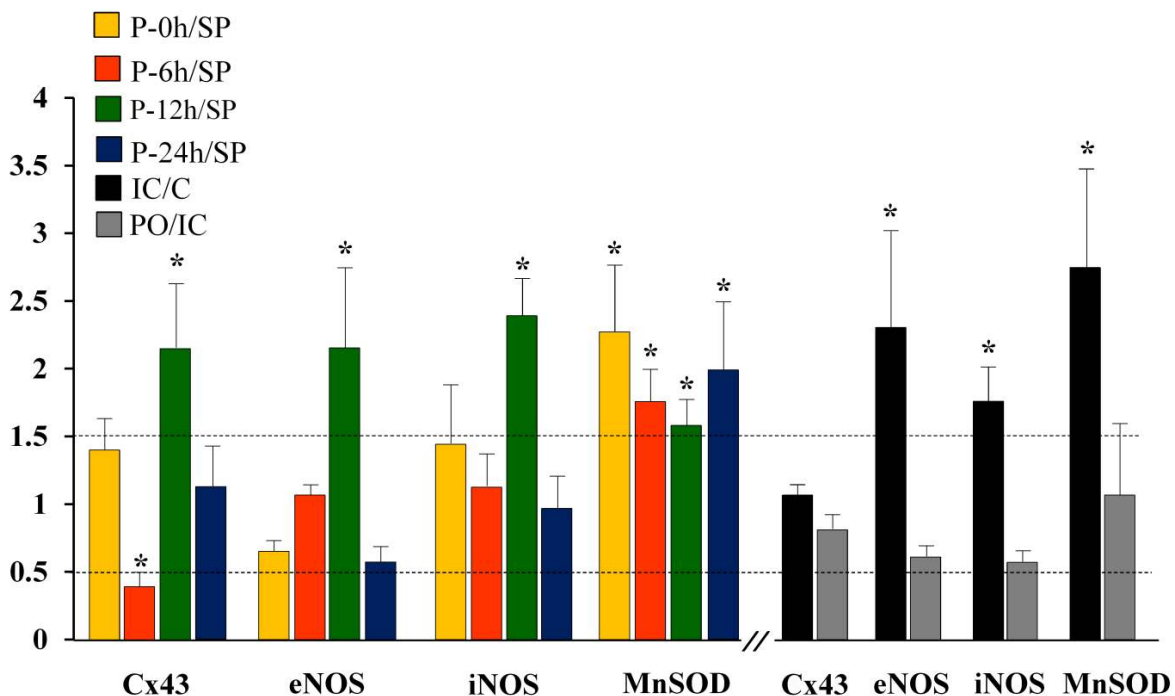


Figure 4. The time-course changes in expression of genes which encode eNOS, iNOS, MnSOD enzymes as well as Cx43 and their alterations at the time of delayed PC. Values are means \pm SD. The lines indicate the interval between 0.5 and 1.5 fold of regulation. Myocardial ischemia up-regulated the expression of MnSOD, iNOS and eNOS but the relative expression ratios of these genes were not altered when rapid cardiac pacing was performed 24h earlier.

The genes responsible for the transcription of eNOS, iNOS were significantly up-regulated only 12h after pacing. (Figure 4). In contrast, the mRNA expression of MnSOD was up-regulated at all time points. The transcription level of Cx43 down-regulated at 6h but up-regulated 12h after pacing and, as we have described previously, these changes in Cx43 gene expression were in association with arrhythmia generation resulting from ischemia and reperfusion [41].

3.4 Time-course changes in eNOS protein content and activation

We have performed experiments in which, using the sham-paced control and paced heart samples, the time course of eNOS protein content and activity were determined by Western blot analysis (Figure 6). The results clearly show that compared with the non-paced controls, in the paced dogs eNOS protein content was significantly increased only 24h after the pacing stimulus (Figure 5a), whereas the activity of this enzyme (assessed by measuring the phosphorylated form) was increased twice; i.e. immediately and 24h after pacing (Figure 5b).

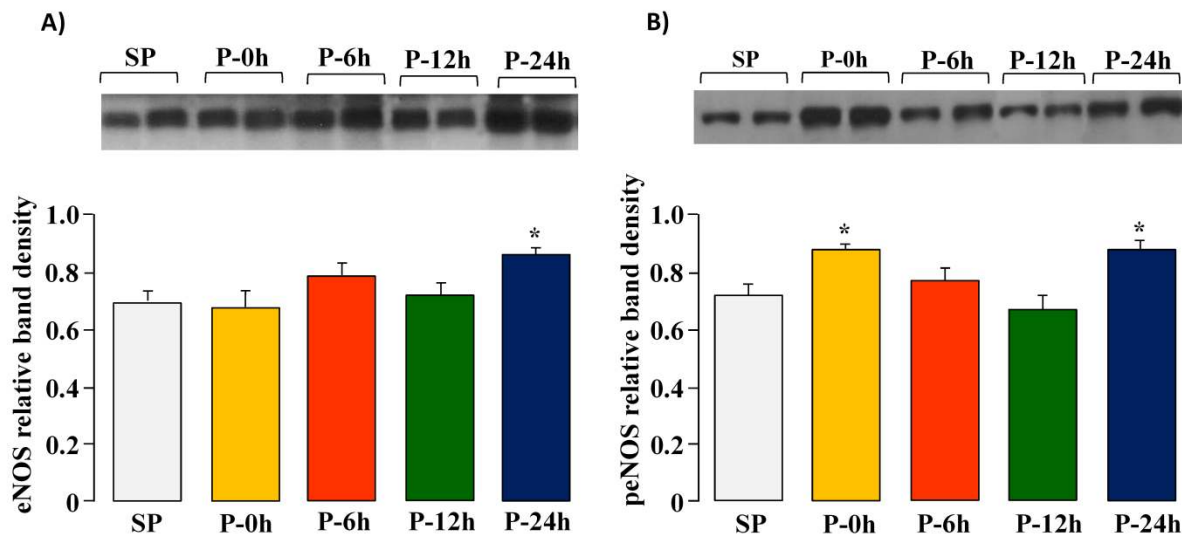


Figure 5. Time-course changes in eNOS protein content (A) and activation (B) determined at various time intervals after rapid cardiac pacing by Western blot. Values are mean \pm S.E.M. *P<0.05 vs. SP group (Bonferroni correction).

4. DISCUSSION

4.1 New findings

I. We have identified 23 genes with altered expression induced by cardiac pacing in a large animal model of ischemia and reperfusion. Cardiac pacing resulted in an enhanced expression of 19 genes and lower expression of 4 genes. The genes with altered transcription rate encode transcription factors, ion channels and members of signal transduction pathways. Some of these, for example MEF2A or mAKAP, has been firstly demonstrated to be significantly up-regulated during the pacing induced protection.

II. We have proved that rapid right ventricular pacing is associated with altered expression of genes involved in the delayed protection. Forty-five genes were selected which have previously identified as mediators or important members of the PC induced cardioprotection. Out of these genes, six exhibited significant down-regulation and 16 showed up-regulation in response to myocardial ischemia when compared to the control dogs. Their expression ratios changed either to the opposite (11) or showed no alteration (10) in the PO group. Among these nNOS, HSP70, PKC α , JNK1, GSK3 α , GYS1, BDKRB1 showed up-regulation, and BAX, BAK and casp3 down-regulation after rapid cardiac pacing.

III. We are the first to show that cardiac pacing induces time course changes in gene expression during the time interval which elapses between the PC stimulus and the appearance of the delayed protection. There were 8 genes which were up-regulated immediately after the cessation of pacing (HSP90, MnSOD, Bcl-2, PKC ϵ , ERK1, sGC α 1, sGC β 1, sGC α 2) and there were 6 genes which exhibited an early down-regulation following the pacing stimulus (CAT, casp3, casp9, GSK3 β , MMP9 and Cox2). Some of these early up- or down-regulated genes remained unchanged (i.e. up and down-regulated, MnSOD, PKC ϵ , casp3 and casp9, GSK3 β and MMP9) at almost each observed time-points, whereas others, like ERK1, Bcl-2 and sGC isoforms, showed biphasic transcriptional changes; i.e. after the initial up-regulation these genes were down-regulated (mostly at 6h) but were up-regulated again around 12 and 24h. There were also genes, eNOS, iNOS and Cx43, which exhibited significant up-regulation only 12h after the pacing stimulus.

IV. We propose that an increase in eNOS protein expression and activity, besides the increased iNOS activation, may also play a role in NO production which mediates the late phase of the protection.

4.2. Changes in transcription levels of different genes after delayed PC by cDNA microarray.

In the last few years, cDNA microarrays [39, 48, reviewed by 40] or other large-scale expression profiling technologies, such as subtractive hybridization [36] or RDA [49] are increasingly used to determine transcriptional profiles induced by PC in different animal models. In order to understand the different genomic responses, the knowledge of cDNA sequences is required. Since commercial microarrays, printed with thousands of known cDNAs, are primarily available for rodents (rats or mice) large scale genomic analysis have been performed in these animal models to investigate gene expression profiles occurring during ischemia and reperfusion or after preconditioning [37, 38, 39.]. Only few studies aimed to examine the preconditioning-induced genomic responses in large animals such as pigs and dogs. For example, in dogs, using cDNA RDA analysis, Zubakov et al. have found that genes involved in inflammatory response were highly up-regulated during the late phase of preconditioning [49]. In a porcine model, induction of genes encoding transcription factors, heat shock proteins, calcium-handling proteins and growth factors have been found in response to preconditioning [50].

Thus, one of the aims of these studies was to find a relationship between the antiarrhythmic effect and the gene expression changes that occur in dogs subjected to coronary artery occlusion and reperfusion 24h after rapid cardiac pacing. To achieve this, microarray analysis was performed with 3200 cDNAs and the results were confirmed by qRT-PCR.

Among the genes examined, MEF2A showed significant up-regulation during the late phase of the protection when compared to the ischemic controls (Table1). The transcription factor encoded by this gene belongs to the MEF2 family which controls vascular development [51, 52]. The enhanced transcription of this gene supports those previous findings that ischemic PC induces angiogenesis through VEGF mediated signaling and that the target gene of this pathway is a transcription factor from the MEF2 family [53, 54]. Another gene, TGF β 2 also showed a higher expression at the time of the appearance of the late phase of the protection when compared to the ischemic controls (Table1). This is in accordance with a previous study in which four repetitive cycles of coronary artery occlusion have been used to stimulate vascular growth [55] and found

that TGF β was significantly up-regulated 48 h after this preconditioning stimulus. These findings might indicate that cardiac pacing induces processes which are involved in vascular growth.

Another gene which was significantly up-regulated following pacing was PDE4D9. This encodes an isoform of a phosphodiesterase enzyme responsible for cAMP degradation in cardiac myocytes [56] (Table1). In rat isolated and perfused hearts, ischemic PC increased tissue cAMP level, which was normalized upon reperfusion, whereas PDE activity showed opposite changes; i.e. reduced during ischemia and increased following reperfusion [57]. Furthermore, the cAMP-dependent PDE activities were higher throughout a 25 min sustained ischemia in the preconditioned than in control hearts and this was accompanied by a significantly less accumulation of cAMP in the PC hearts. The reduction in cAMP may play a role in the antiarrhythmic effect by decreasing the activity of the PKA and thereby by reducing calcium influx through L-type calcium channels. The role of cGMPs in the regulation of PDE4 phosphodiesterases is not clear either in the early or the delayed phase of protection. In rat isolated hearts, using cDNA microarray, a considerable induction in the expression of a cGMP phosphodiesterase (PDE9A1) has been found following preconditioning [39], suggesting that PDEs may play an important trigger and mediator role in the early and in the delayed cardioprotection.

Another gene that showed enhanced transcription during the delayed phase of the pacing-induced protection is mAKAP (Table1). This protein is an anchoring protein which connects cAMP-binding proteins, such as, PKA, EPAC1 and the phosphodiesterase PDE4D3 to form macromolecular complexes [58]. This complex plays an important role in the regulation of the cAMP-PKA signaling pathway by anchoring these proteins to different cell organelles. This of our finding is the first to demonstrate that the expression of an anchoring protein is altered after preconditioning. Similarly, genes encoding V1 and V2 receptors have not been examined in relation to PC. We have found that these genes were down-regulated after cardiac pacing and showed an enhanced expression in response to ischemia.

In summary, among the 3200 cDNAs that have been examined, only 10 genes showed altered expression in response to the acute ischemic and reperfusion insult, whereas 19 genes to rapid cardiac pacing. One of the reasons for these limited results might be that at the time of the experiments genome-wide chips were not available for large animals, such as dogs or pigs. Thus, we used the human cDNA library for the analysis of microarrays which certainly limits the number of positive signals in the gene expression analysis, despite the high homology between

the human and canine genome. However, using a human cDNA microarray with 3200 genes we were able to follow the expression changes of more genes than those of the previous studies.

Another limitation of the study is that the cDNA microarray analysis provides information only about the mRNA level of genes but it does not reflect the expression or the function of proteins that have certainly produced and possibly acted at the time of sampling. This might also explain that only a limited number of genes have been found with altered expression during the late phase of the protection induced by cardiac pacing.

The results of these previous studies have prompted us to perform further investigations, in which the time course changes of gene expression were analyzed during a 24h period, which elapses between the preconditioning stimulus and the test ischemia. Also, some proteins for example Cx43 in a previous and eNOS in this current study were also examined to obtain information on their expression changes at the protein level due to cardiac pacing.

4.3 Effects of rapid cardiac pacing on gene expression changes

In this study, numerous genes were selected for RT-PCR analysis from various functional categories that had been previously shown to relate to the delayed protection (Table 2 and Table 3). Furthermore, this is the first study which has attempted to examine the sequence of gene expression changes following a PC stimulus.

Previous results from various animal models confirmed the involvement of NOS enzymes (iNOS; eNOS; nNOS), antioxidant enzymes (Cu/Zn-SOD; MnSOD; CAT), cyclooxygenases (COX1; COX2), and heat shock proteins (HSPs) in the delayed cardioprotective effect of preconditioning. Previous results suggested that the antioxidant factor MnSOD has a particular role both in the early and the delayed phase of protection [33, 59, 60]. The transcription of this gene was significantly enhanced in response to cardiac pacing at all the observed time points (Figure 4). Compared to the sham-paced and sham-operated controls, the expression of this gene was also significantly up-regulated after myocardial ischemia and it was not significantly altered by cardiac pacing (Figure 4). Das et al also found that repeated ischemia and reperfusion caused an immediate up-regulation in the expression of MnSOD in isolated buffer-perfused rat hearts [59]. MnSOD content was also increased with a peak at 24h after four 5 min episodes of regional ischemia and reperfusion in canine myocardium [33]. This finding supports the proposed parallelism between the induction of MnSOD and the protection, although a direct evidence for a

cause-and effect relationship between the up-regulation of MnSOD and the pacing-induced protection has not yet been established.

Interestingly, one of the other examined antioxidant factors CAT, was down-regulated at the early time points after cardiac pacing, and also after a 25 min period of ischemia preceded by overdrive pacing 24h before (Table 2 and 3). This result is in contrast with other studies using isolated Langendorff-perfused hearts in which an enhanced transcription for this gene has been found [59, 60]. However, these results were not confirmed by *in vivo* studies; i.e. neither catalase in dogs [33] nor other antioxidant enzymes in conscious pigs [61] were up-regulated in the time of the delayed protection.

It is known that activation of signaling pathways is involved in the development of the early protection [8]. For example, adenosine, bradykinin, and opioids that are generated and released by brief preconditioning ischemic periods bind to GPCRs and lead to activation of the PI3K pathway and of its downstream targets, such as Akt or ERK [8]. Akt (also known as PKB) has a central role in the regulation of other proteins by phosphorylation [24]. Akt phosphorylates and thereby activates eNOS which thus produces nitric oxide, which is one of the key messenger molecules both in the early and the late survival pathways [62]. Furthermore, Akt can also inhibit target proteins through phosphorylation such as GSK3 β and the proapoptotic BAD or casp3 and 9 which, result in the inhibition of apoptotic processes during ischemia and reperfusion [24]. It has been suggested that PKC ϵ is also activated by the PI3kinase, possibly through the Akt-eNOS-sGC mediated pathways [17]. ERK is also activated by PKC ϵ [25] in response to a preconditioning stimulus and phosphorylates the proapoptotic factor BAD.

In our present studies, several members of the signaling pathways were examined to determine their alterations following cardiac pacing (time-dependent study) and also during ischemia and reperfusion in the time of the delayed cardioprotection. As mentioned above, several members of signaling pathways have been proposed to be activated by a PC stimulus, but little is known how the expression of these genes is changed before and during the late phase of the protection. There is a lack of information about the sequence of gene expression changes during that time interval, which elapses between the PC stimulus and the appearance of the delayed protection.

We have found that genes encoding PKC ϵ , ERK1 and different isoforms of sGC were early up-regulated after cardiac pacing (Figure 3). For example, the gene encoding PKC ϵ was markedly expressed at all the observed time points and remained up-regulated during ischemia. The transcription of this gene was also increased (not significant) after the myocardial ischemia but it

was not significantly altered at the time of the delayed phase of protection induced by pacing. Even so, the marked up-regulation of PKC ϵ immediately and 24h after the pacing stimulus might indicate a role in both phases of the antiarrhythmic protection. There is pharmacological evidence that the activation of and the following translocation of PKC ϵ is necessary for the development of late protection [63, 64].

The expression of genes encoding sGC α 1 and sGC β 1 was not significantly altered either by ischemia in dogs subjected to a 25 min ischemia/reperfusion insult (PO vs. IC). However, these genes were changed following cardiac pacing in a biphasic pattern. They were immediately up-regulated after the pacing stimulus (P-0h vs. SP) after which they were either normalized or down-regulated. However, these genes were up-regulated again 12 or 24h after pacing (Figure 3). The importance of sGC-mediated signaling pathway in the early protection was suggested in our previous study in which the administration of methylene blue completely abolished the early antiarrhythmic effect of ischemic preconditioning [10]. More recent studies, using ODQ a selective inhibitor of sGC, have confirmed the role of NO-sGC pathway in the early [65] and also in the late [66] phase of the protection. The present results which clearly show a biphasic transcriptional activation of sGC α 1, sGC α 2 and sGC β 1 following cardiac pacing, provide a further support for the role of this pathway in both phases of the protection.

Another gene GSK3 β which was markedly altered after cardiac pacing is one of the key members of the preconditioning-induced survival pathway [8]. The enzymatic activity of this kinase is inhibited by phosphorylation and by interaction with other proteins. Under normal conditions, GSK3 β is active and usually inactivates its primed substrates. As a key kinase it integrates multiple signaling pathways, and regulates many cellular processes, such as it inhibits transcription factors and induces apoptosis [67]. It has been found that preconditioning by inhibiting GSK3 β through phosphorylation, results in cardioprotection [68]. Signaling through Akt, PKC, PKA and ERK phosphorylates and therefore inhibits GSK3 β , which in turn reduces the phosphorylation and activation of different proteins, such as the pro-apoptotic BAX [69], or inhibits the opening and formation of the mPTPs [21]. In our study, the expression of GSK3 β was markedly down-regulated at almost each time point in the paced dogs. Furthermore, pacing reduced GSK3 β expression during ischemia compared with the unpaced controls in which the transcription of this gene was considerably increased (Figure 3). This and the previous results indicate that cardiac pacing keeps the expression of the cardioprotective kinases, such as PKC ϵ , PKCa, ERK1, sGC α 1/ β 1, up-regulated over a longer period of time whereas it down-regulates

the transcription of GSK3 β . These results suggest that pacing facilitates the survival pathways during the both phases of the protection and suppresses the expression of genes which are involved in the harmful consequences of ischemia/reperfusion.

It is well established that NO, released during a preconditioning stimulus, plays a trigger and mediator role in both the early and delayed phase of the protection [9, 30]. We have now provided further evidence for this by demonstrating a marked increase in the transcription of both eNOS and iNOS 12h after cardiac pacing. Furthermore, this was the only time point when the expression of these genes was significantly up-regulated (Figure 4). This finding might have a particular importance, since previous studies in conscious rabbits have reported that the activity of eNOS and nNOS was increased only immediately after preconditioning, but it was not affected 24h later [45], whereas the transcription of iNOS was up-regulated only at the time of the late phase of the protection [32].

There is no doubt that iNOS expression and the resultant increase in NO generation is essential for the development of the delayed antiarrhythmic protection. We have pharmacological evidence for this using various, more or less selective inhibitors of iNOS [6, 29, 31, 44], which are all markedly attenuated or even abolished the delayed protection against arrhythmias resulted from cardiac pacing [6, 29, 31] or heavy physical exercise [44, 70]. Since, these inhibitors are not entirely selective for iNOS the role of eNOS-derived NO formation in the delayed phase of the protection can not be ruled out. To support this hypothesis we have performed additional experiments to measure eNOS protein content and activation (Figure 5). The results show that eNOS protein content was significantly increased in the paced dogs, whereas the activity of this enzyme was increased twice; i.e. immediately and 24h after pacing (Figure 5b). Thus we suggest, like the others [32, 45] that the release of NO immediately after the preconditioning stimulus most likely results from the increased activation of eNOS. This NO by stimulating the transcript of iNOS and eNOS, that occurs in dogs 12h after pacing, results in further NO generation and availability at the time of the delayed protection. However, in contrast with others [32, 45], we propose that an increase in eNOS protein expression and activity, besides the increased iNOS activation, may also play a role in NO production which mediates the late phase of the protection. There are studies that confirm that anti-apoptotic processes, such as deletion of apoptotic proteins [71] or increased expression of anti-apoptotic factors [72, 73] reduce the ischemia-reperfusion mediated cell death. Other results also demonstrate that PC attenuates apoptotic cell death by decreasing the transcription of the pro-apoptotic BAX [74] and by increasing the expression of

the anti-apoptotic Bcl-2 [75]. The present results demonstrate that the anti-apoptotic Bcl-2 was mostly up-regulated (immediately, 12 and 24 after cardiac pacing), whereas genes encoding the pro-apoptotic BAX and members of the apoptotic caspase cascade were down-regulated at various time points. In the ischemic controls the pro-apoptotic genes were enhanced while in the paced dogs, subjected to coronary artery occlusion and reperfusion 24h later, the pro-apoptotic genes were markedly down-regulated. These results demonstrate that cardiac pacing protects the heart against apoptotic cell death.

Rapid cardiac pacing also modified the expression of the gene encoding the main gap junction forming protein Cx43. It was down-regulated 6 h after rapid cardiac pacing and was up-regulated again at 12 h. In our previous study we have found that pacing influenced the protein expression and the subcellular location of Cx43 [41]. According to these results, twelve hours after the pacing stimulus the protein content of Cx43 was decreased and this was preceded by a down-regulation of mRNA at 6 h. Also, the signals obtained from the Cx43-labeled immunofluorescence images showed that the ischemia-induced structural impairment of the intercalated discs was preserved after the pacing stimulus. Preconditioning also preserved gap junction permeability and Cx43 phosphorylation compared to the ischemic controls.

In summary, these results provide evidence for the first time that cardiac pacing induces time-course dependent gene expression changes, which contribute to the appearance of the early and the late phase of the protection. We have found genes which were immediately altered after rapid cardiac pacing. These encode proteins that are already known to have a role in the early phase of protection. Some of these genes preserved their altered transcription over a 24h time interval, whereas others showed a biphasic (early and late) activation pattern, indicating a role of these genes in the development of delayed phase of the antiarrhythmic protection. There were a few genes which were altered at later time points. For example, iNOS or eNOS were activated 12 h after the PC stimulus and these changes resulted in enhanced protein production at 24h, when the late phase usually appears. We are aware that changes in gene expression does not provide information about the protein content, but these present results provide evidence for a time-dependent change in gene expression which occur between the PC stimulus and the appearance of the delayed protection.

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“SCIENCE: a way of finding things out and then making them work. Science explains what is happening around us the whole time. So does RELIGION, but science is better because it comes up with more understandable excuses when it's wrong.”-Terry Pratchett, *Wings* (1997)

7. ANNEX

Reprints of full Papers

1. **Kovács M**, Papp R, Varga-Orvos Z, Ménesi D, Puskás LG, Végh A. Changes in gene expression following cardiac pacing-induced delayed cardioprotection in the canine heart. *Acta Biol Hung.* 2010 Dec;61(4):434-48.
2. **Mária Kovács**, Márton Gönczi, Edina Kovács, Ágnes Végh. Time course analysis of cardiac pacing-induced gene expression changes in the canine heart. *Mol Cell Biochem.* 2013 Jan;372(1-2):257-66. doi: 10.1007/s11010-012-1467-8.