Original Research

Activation of peroxisome-proliferator-activated receptors α and γ mediates remote ischemic preconditioning against myocardial infarction *in vivo*

Christopher Lotz[1], Maria Lazariotto[2], Andreas Redel[1], Thorsten M Smul[1], Jan Stumpner[1], Christoph Blomeyer[1], Tobias Tischer-Zeitz[1], Johannes Schmidt[1], Joanna Pociej[1], Norbert Roewer[1], Franz Kehl[3] and Markus Lange[1]

[1]Department of Anesthesiology; [2]Department of Cardiac, Thoracic and Thoracic Vascular Surgery, Bayerische Julius-Maximilians-Universität, Würzburg; [3]Department of Anesthesiology, Staedtisches Klinikum Karlsruhe, Karlsruhe, Germany
Corresponding author: Christopher Lotz, Universität Würzburg, Klinik und Poliklinik für Anästhesiologie, Zentrum Operative Medizin, Oberdürrbacher Str. 6, 97080 Würzburg, Germany. Email: Lotz_C@klinik.uni-wuerzburg.de

Abstract

Remote ischemic preconditioning (remote IPC) elicits a protective cardiac phenotype against myocardial ischemic injury. The remote stimulus has been hypothesized to act on major signaling pathways; however, its molecular targets remain largely undefined. We hypothesized that remote IPC exerts its effects by activating the peroxisome-proliferator-activated receptors (PPARs) α and γ, which have been previously implicated in cardioprotective signaling. Male New Zealand white rabbits ($n = 78$) were subjected to a 30-min coronary artery occlusion followed by three hours of reperfusion. Three cycles of remote IPC consisting of 10-min renal ischemia/reperfusion were performed. The animals either received the PPARα-antagonist GW6471 or the PPARγ-antagonist GW9662 alone or combined with remote IPC. Infarct size was determined gravimetrically. Tissue levels of 15d-prostaglandin J_2 (15d-PGJ_2), as well as the PPAR DNA binding were measured using specific assays. Reverse transcriptase polymerase chain reaction was used to analyze changes in endothelial nitric oxide synthase or inducible nitric oxide synthase (iNOS) mRNA expression in relative quantity (RQ). Data are mean $\pm$ SD. As a result, remote IPC significantly reduced the myocardial infarct size (42.2 $\pm$ 4.9%* versus 61 $\pm$ 1.9%), accompanied by an increased PPAR DNA-binding (189.6 $\pm$ 19.8RLU* versus 44.4 $\pm$ 9RLU), increased iNOS expression (3.5 $\pm$ 1RQ* versus 1RQ), as well as 15d-PGJ_2 levels (179.7 $\pm$ 7.9 pg/mL* versus 127.9 $\pm$ 7.6 pg/mL). The protective response elicited by remote IPC, as well as the accompanying molecular changes were abolished by inhibiting PPARα (56.8 $\pm$ 4.7%; 61.1 $\pm$ 14.2RLU; and 1.91 $\pm$ 0.96RQ, respectively) or PPARγ (57.4 $\pm$ 3.3%; 52.7 $\pm$ 16.9RLU; and 1.54 $\pm$ 0.25RQ, respectively). (*Significantly different from control $P < 0.05$). In conclusion, the obtained results indicate that both PPARα and PPARγ play an essential role in remote IPC against myocardial infarction, impinging on the transcriptional control of iNOS expression.

Keywords: myocardial ischemia/reperfusion-injury, nitric oxide, cardioprotection

Experimental Biology and Medicine 2011; **236**: 113–122. DOI: 10.1258/ebm.2010.010210

Introduction

In 1993, Przyklenk *et al.*[1] extended the concept of preconditioning by showing that intramyocardial protection could be transferred from the area of one coronary artery to another, a phenomenon called remote ischemic preconditioning (remote IPC). Subsequent studies demonstrated that remote IPC is not restricted to the heart; and that furthermore, transient episodes of ischemia and reperfusion of one organ, such as the kidney, are able to protect another organ, such as the heart.[2] Since remote IPC seems to be much easier to apply in a clinical setting than IPC, e.g. by using repetitive blood-pressure cuff inflations and deflations, it has recently received increasing attention.[3,4] The mechanisms identified in this protection at a distance are similar to those of classic ischemic preconditioning (IPC) and include the generation of reactive oxygen species (ROS),[5] as well as the production of nitric oxide (NO).[6]

NO is known to hold a decisive role in cardioprotective signaling[7] and recent investigations further linked its production to pharmacological cardioprotection afforded by

Copyright © 2011 by the Society for Experimental Biology and Medicine

the activation of peroxisome-proliferator-activated receptors (PPARs),[8–10] as pharmacological inhibition of nitric oxide synthetase (NOS) abrogated both the cardioprotective effects of the PPARα agonist WY 14,463,[11] as well as the PPARγ agonist rosiglitazone.[12] PPARs are a family of nuclear receptors, consisting of three known isoforms (α, β, γ), of which PPARα, as well as PPARγ, are involved in lipid and lipoprotein metabolism and play a central role in the regulation of myocardial fatty acid oxidation.[13] The receptors are activated upon the action of multiple known ligands, most prominently 15d-prostaglandin J_2 (15d-PGJ_2)[14] and the administration thereof has been shown to process cardioprotective properties as well.[10]

However, the role of PPARα and PPARγ in remote IPC has not been investigated to date. Thus, we hypothesized that the activation of PPARα and PPARγ plays a pivotal role in mediating remote IPC against myocardial infarction via a mechanism involving the increased production of 15d-PGJ_2, as well as distinct changes of NOS function. To test our hypothesis we used a well-established *in vivo* infarction model of the rabbit heart. The obtained results indicate that both PPARα and PPARγ play an essential role in remote IPC against myocardial infarction, impinging on the transcriptional control of inducible nitric oxide synthase (iNOS) expression.

Material and methods

All experimental procedures and protocols carried out in this investigation were reviewed and approved by the Animal Care and Use Committee of the local authorities (Government of Lower Franconia, Würzburg, Germany). All procedures conformed to the *Guiding Principles in the Care and Use of Animals* of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.[15]

Materials

GW6471 [(2S)-2-[[(1Z)-1-methyl-3-oxo-3-[4-(trifluoromethyl) phenyl]-1-propenyl] amino]-3-[4-[2-(5-methyl-2-phenyl-4-oxazolyl)ethoxy]phenyl]propyl]-carbamic acid ethyl ester] was purchased from Tocris Bioscience (Bristol, UK). GW9662 [N-((2S)-2-(((1Z)-1-methyl-3-oxo-3-(4-(trifluoromethyl) phenyl)prop-1-enyl)amino)-3-(4-(2-(5-methyl-2-phenyl-1,3-oxazol-4-yl)ethoxy)phenyl)propyl) propanamide] was purchased from Sigma-Aldrich (Steinheim, Germany). TRIZOL reagent was obtained from Invitrogen (Carlsbad, NM, USA); an iSkript cDNA synthesis kit was purchased from Bio-Rad Laboratories (Munich, Germany) and a QuantiTech SYBR PCR kit was obtained from Qiagen (Valencia, CA, USA). Primers for the reverse transcriptase polymerase chain reactions (RT-PCR) were purchased from Biomers (Ulm, Germany). A PPAR transcription factor assay kit was obtained from Novagen (Madison, WI, USA). 15d-PGJ_2 was measured by an enzyme immunoassay kit obtained from Cayman Chemicals (Tallinn, Estonia). All other chemicals were obtained from Sigma-Aldrich.

General preparation

Male New Zealand White rabbits were anesthetized with sodium pentobarbital (30 mg/kg intravenous bolus, followed by an infusion of 20–30 mg/kg/h) via the left marginal auricular vein. Depth of anesthesia was verified by recurrent testing of palpebral reflexes and hind paw withdrawal throughout the experiment. After tracheotomy and tracheal cannulation, the animals were artificially ventilated (Cicero®; Draeger, Luebeck, Germany) using positive pressure with an air and oxygen mixture (70%/30%). Arterial blood drawn from the auricular artery was recurrently analyzed using an ABL 505 blood gas analyzer (Radiometer, Copenhagen, Denmark), and blood gases were maintained within a normal physiological range by adjusting the respiratory rate or tidal volume. Continuous measurement of the heart rate (HR) was performed using a saline-filled PE 50 polyethylene catheter inserted into the left ventricle (LV) *via* the right carotid artery. Mean arterial pressure was monitored by insertion of a saline-filled PE 10 polyethylene catheter *via* the right femoral artery into the descending aorta. Rectal body temperature was maintained at $38.5 \pm 0.5^{\circ}C$[16] by a servo-controlled heating pad (Foehr Instruments, Seeheim, Germany). After thoracotomy in the left fourth intercostal space and pericardiotomy, the left heart was exposed and suspended in a pericardial cradle. A silk ligature was placed halfway between the base and the apex of the heart around a prominent branch of the left anterior descending coronary artery to form a snare. By tightening the snare, a coronary artery occlusion (CAO) was produced, and reperfusion was instituted by loosening the snare. CAO was verified by epicardial cyanosis, regional dyskinesia in the ischemic zone and electrocardiographic changes. Adequate reperfusion was confirmed by an epicardial hyperemic response and reversion of electrocardiographic changes. For remote IPC the abdominal cavity was opened and the left renal artery was dissected free. An arterial vessel clamp was placed around the renal artery for renal artery occlusion (RAO). Renal reperfusion was achieved by removal of the clamp. RAO was verified by renal cyanosis; adequate reperfusion was accessed by renal hyperemia. Each rabbit received 300 U/kg heparin five minutes before RAO for anticoagulation.

Hemodynamic parameters, body temperature and electrocardiogram were recorded continuously and analyzed using a personal computer (Hewlett Packard, Palo Alto, CA, USA) and hemodynamic data acquisition and analysis software (Notocord® hem 3.5; Croissy sur Seine, France). Data were digitized at a sampling rate of 1000 Hz.

Experimental protocol

The experimental protocol used in this investigation is illustrated in Figure 1. Baseline systemic hemodynamic parameters were recorded following an one-hour equilibration period after completion of instrumentation and calibration. All rabbits were subjected to 30 min of CAO followed by three hours of reperfusion. Rabbits were randomly assigned to one of the six study groups ($n = 8$ per group) after completion of the preparation of each animal. Rabbits received

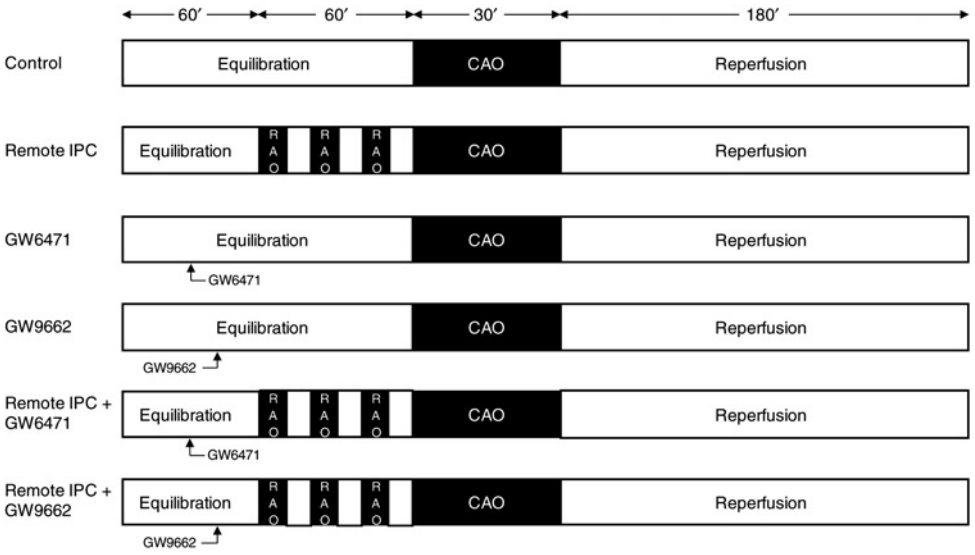

Figure 1 Experimental protocol. All animals were subjected to 30-min coronary artery occlusion (CAO) followed by three hours of reperfusion. The animals either received vehicle (Control) or three cycles of 10-min renal artery occlusion (RAO)/reperfusion as a remote ischemic stimulus (remote IPC). Additional groups received the peroxisome-proliferator-activated receptor (PPAR)α-antagonist GW6471 (GW6471) or the PPARγ-antagonist GW9662 (GW9662) alone or in combination with remote IPC (remote IPC + GW6471 and remote IPC + GW9662), respectively

either vehicle (25 μL dimethylsulfoxide [DMSO] in 2 mL 0.9% saline) (control), three cycles of renal ischemia/reperfusion (I/R) plus vehicle (remote IPC), GW6471 (4 mg/kg^{-1}) or GW9662 (1 mg/kg^{-1}). In two additional groups, remote IPC and GW6471 or GW9662, respectively, were co-administered. Each cycle of remote IPC consisted of 10 min of renal ischemia alternating with 10 min of renal reperfusion. The specific PPARα inhibitor GW6471 and the specific PPARγ inhibitor GW9662 were dissolved in DMSO and administered as an intravenous bolus injection (containing a final concentration of 0.0125% DMSO).

In an additional set of experiments ($n = 5$ per group), the heart and the left kidney were rapidly excised one hour after the last cycle of remote IPC or at the corresponding time point, respectively. Both organs were shock frozen in liquid nitrogen and stored at -80°C until further use.

Measurement of myocardial infarct size

Infarct size and area at risk (AAR) were gravimetrically determined according to standard procedures.[17] In brief, at the end of each experiment, the coronary artery was re-occluded and the AAR was determined by infusion of 2 mL patent blue (0.1 g/mL; Sigma-Aldrich, Taufkirchen, Germany). The rabbits were then euthanized with a lethal dose of pentobarbital, and the heart was rapidly excised. The heart was cut into five slices from apex to base, and the non-stained red myocardium was separated from the non-ischemic blue-stained normal area. The samples of ischemic and non-ischemic regions were incubated at 37°C for 20 min in 1% 2,3,5-triphenyltetrazoliumchloride in 0.1 mol/L phosphate buffer adjusted to pH 7.4. After overnight storage in 10% formaldehyde, infarcted (pale) and non-infarcted (brick-red) myocardium within the AAR was carefully separated and weighted. Infarct size (as % of left ventricular [LV] weight) was expressed as a function of the AAR (as % of LV weight). Rabbits with an AAR less than 15% of LV mass and those with intractable ventricular fibrillation or LV pump failure were excluded from the study.

RNA extraction and real-time PCR

Total RNA was isolated with TRIZOL Reagent (Invitrogen). First-strand cDNA synthesis was performed with 1 μg of total RNA in a 20-μL reaction mixture according to the recommended conditions in the Bio-Rad iSkript cDNA Synthesis kit (Bio-Rad Laboratories). For subsequent real-time PCR, the cDNA was amplified using an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided by the QuantiTech SYBR PCR kit (Qiagen), allowing quantitative detection of the PCR product in a 25-μL reaction volume. The primer sequences (Biomers) for endothelial nitric oxide synthase (eNOS) were 5′ CCA-CCA-GAG-AAC-GGA-G 3′ (sense, 00082327_5) and 5′ CCT-CTT-CCG-CCG-CCT 3′ (antisense), for iNOS 5′ GGC-CGG-CGG-CCT-CGA-GTT 3′ (sense, 00082327-3) and 5′ TTC-TGG-AAG-CTG-TGG-AGC 3′ (antisense, 00082327-4). The primer sequences used for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5′ GGG-CAA-GGT-CAT-CCC-CGA-GCT-GAA 3′ (sense, 00082327-1) and 5′ GAG-GTC-CAC-CAC-CCT-GTT-GCT-GTA 3′ (antisense, 00082327-2). The following temperature profile was used: 95°C for 10 min, 50 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. GAPDH was used to normalize differences in RNA isolation, RNA degradation and the efficiencies of the reverse transcription. Values are expressed in relative quantities (RQ) to the mRNA expression in the control group (set to 1).

Measurement of 15d-PGJ_2

Left ventricular and renal contents of 15d-PGJ_2 were measured using an enzyme immunoassay kit as described by the manufacturer.

Preparation of nuclear extracts

Preparation of nuclear extracts from left ventricular and renal tissue was carried out to determine the DNA-binding activity of PPAR. The frozen tissue was homogenized in ice-cold buffer A (10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA) with a glass grinder. After homogenation, 1% Triton X-100 was added and the samples were incubated on ice for 15 min, followed by centrifugation at 10,000*g* for five minutes at 4°C. The resulting supernatants were collected as the cytosolic fraction. The resulting pellets were resuspended in buffer A and again centrifuged at 10,000*g* for five minutes at 4°C. The resulting supernatants were discarded. The resulting pellets were suspended in ice-cold buffer B (20 mmol/L HEPES pH 7.9, 0.4 mmol/L NaCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 20% glycerol, 1% Triton X-100) and rocked for one hour at 4°C, followed by centrifugation at 20,000*g* for 10 min at 4°C. Resulting supernatants containing the nuclear proteins were collected and stored at −80°C until further use. Protein concentrations of the nuclear and cytosolic extracts were determined using a Bradford assay (Bio-Rad Laboratories).[18]

Determination of PPAR transcription factor activity

PPAR DNA-binding activity was accessed in left ventricular and renal tissue using a commercially available non-radioactive transcription factor assay.

In brief, nuclear extracts were incubated with a biotin-labeled DNA probe containing a PPAR-specific double-stranded consensus sequence and a single-stranded capture region. Activated PPARs bind specifically to the consensus sequence utilized in the assay. The samples were digested with a double-stranded DNA-specific nuclease, whereas probes with bound transcription factor are protected from digestion. Samples were subsequently transferred to a 96-well plate coated with single-stranded DNA complementary to the capture region. A chemiluminescent alkine phosphatase substrate was added and the output signal was measured using a microplate luminometer (Tecan, Maennedorf, Switzerland). Results are expressed as relative light units (RLUs).

Statistical analysis

Statistical analysis of data within and between groups was performed with one-way and two-way analysis of variance followed by *post hoc* Duncan testing (SPSS 16.0 software, The Apache Software Foundation, Forest Hill, MD, USA). Changes were considered statistically significant when the P value was less than 0.05. All data are expressed as mean ± SD.

Results

A total of 82 rabbits were instrumented to obtain 78 successful experiments. Three rabbits had to be excluded due to intractable ventricular fibrillation; one rabbit was excluded from analysis due to an AAR under 15% of LV mass.

Hemodynamics

Hemodynamic parameters of all experimental groups are shown in Table 1.

Myocardial infarct size

LV AAR was not significantly different between experimental groups (Table 2). Myocardial infarct size (% AAR) was 60.2 ± 1.9% ($n = 8$) in the control group. Remote IPC significantly ($P < 0.05$) reduced infarct size to 42.2 ± 4.9%* ($n = 8$). GW6471 and GW9662 alone had no influence on myocardial infarct size compared with control (56.4 ± 3.2%, $n = 8$ and 55.7 ± 3.3%, $n = 8$ respectively), but abolished infarct size reduction by remote IPC (56.7 ± 4.7%, $n = 8$ and 57.4 ± 3.3%, $n = 8$, respectively) (Figure 2).

PPAR DNA-binding activity

Remote IPC lead to a significant ($P < 0.05$) increase in myocardial PPAR DNA-binding activity (189.5 ± 19RLU*, $n = 5$) compared with control (44.4 ± 9RLU, $n = 5$). GW6471 (67.8 ± 25.6RLU, $n = 5$) and GW9662 (86.7 ± 24.8RLU, $n = 5$) alone did not alter PPAR DNA-binding in the heart, but abrogated the increase in PPAR DNA-binding by remote IPC (61.1 ± 34.2RLU, $n = 5$ and 52.7 ± 16.8RLU, $n = 5$, respectively) (Figure 3a).

In renal tissue no significant differences in PPAR DNA-binding activity could be observed between the study groups (Figure 3b).

RT-PCR analysis of eNOS and iNOS mRNA expression

RT-PCR was used for the analysis of iNOS and eNOS expression in left ventricular tissue. Remote IPC significantly ($P < 0.05$) increased iNOS mRNA levels in the heart (3.5 ± 1RQ*, $n = 5$) compared with the control group. In contrary, no changes of eNOS mRNA expression could be observed in response to the remote ischemic stimulus. The application of either the PPARα inhibitor GW6471 (1.91 ± 0.96RQ) or the PPARγ inhibitor GW9662 (1.54 ± 0.25RQ), respectively, abolished the increase in iNOS mRNA expression, while showing no influence on eNOS mRNA expression compared with the control group (Figure 4).

15d-PGJ_2 levels

Remote IPC (179.7 ± 7.9 pg/mL*, $n = 5$), as well as the combined application of remote IPC and GW6471 (186.7 ± 13.2 pg/mL*, $n = 5$) or remote IPC and GW9662 (164.9 ± 9.4 pg/mL*, $n = 5$) significantly ($P < 0.05$) increased levels of 15d-PGJ_2 in the heart compared with the control group (127.9 ± 7.6 pg/mL, $n = 5$). GW6471 or GW9662 alone did not alter myocardial tissue levels of 15d-PGJ_2 (139.5 ±

Table 1 Systemic hemodynamic parameters

				Reperfusion		
	Base	**Remote IPC**	**CAO**	**60 min**	**120 min**	**180 min**
HR (min^{-1})						
Control	275 ± 12	269 ± 10	250 ± 9	244 ± 9	245 ± 8	252 ± 11
Remote IPC	256 ± 12	245 ± 10	246 ± 10	255 ± 9	253 ± 8	257 ± 11
GW6471	245 ± 12	253 ± 10	255 ± 10	254 ± 9	240 ± 9	243 ± 11
GW9662	258 ± 12	255 ± 10	264 ± 9	251 ± 9	240 ± 8	224 ± 11*
Remote IPC + GW6471	243 ± 13	242 ± 11	237 ± 9	238 ± 9	237 ± 9	240 ± 12
Remote IPC + GW9662	246 ± 12	249 ± 10	255 ± 10	251 ± 9	250 ± 8	249 ± 11
MAP (mmHg)						
Control	73 ± 5	75 ± 4	73 ± 4	68 ± 4	63 ± 4	67 ± 3
Remote IPC	86 ± 5	65 ± 4	69 ± 4	66 ± 4	65 ± 5	66 ± 4
GW6471	68 ± 5	69 ± 4	66 ± 4	62 ± 3	70 ± 5	73 ± 4
GW9662	79 ± 5	87 ± 6	78 ± 4	75 ± 4	67 ± 4	63 ± 5
Remote IPC + GW6471	78 ± 5	74 ± 4	73 ± 4	68 ± 4	63 ± 4	62 ± 5
Remote IPC + GW9662	90 ± 5	74 ± 4	75 ± 4	72 ± 4	71 ± 4	66 ± 5
RPP (HR × MAP/1000)						
Control	19 ± 2	19 ± 1	20 ± 1	17 ± 1	16 ± 1	18 ± 1
Remote IPC	19 ± 2	17 ± 1	16 ± 1	17 ± 1	17 ± 1	17 ± 1
GW6471	17 ± 2	16 ± 1	15 ± 1	17 ± 1	15 ± 1	16 ± 1
GW9662	20 ± 2	21 ± 1	20 ± 1	22 ± 2	19 ± 2	17 ± 1
Remote IPC + GW6471	18 ± 2	17 ± 1	16 ± 1	16 ± 1	15 ± 1	14 ± 1
Remote IPC + GW9662	22 ± 2	18 ± 1	17 ± 1	18 ± 1	17 ± 1	16 ± 1

HR, heart rate; MAP, mean arterial pressure; RPP, rate pressure product; IPC, ischemic preconditioning
Data were analyzed at the end of the equilibration period (=Base), after the last cycle of remote IPC (remote IPC), at the end of the coronary artery occlusion (=CAO) and 60, 120 and 180 min after the onset of coronary reperfusion, respectively. Data are mean ± SD
*Significantly different from baseline ($P < 0.05$)

16 pg/mL, $n = 5$ and 138.7 ± 2.4 pg/mL, $n = 5$, respectively) (Figure 5a). Renal tissue levels of 15d-PGJ_2 did not alter significantly differently between the experimental groups (Figure 5b).

Discussion

The results of the current study demonstrate that the activation of myocardial PPARα and PPARγ comprises an essential role in mediating remote IPC against myocardial infarction *in vivo*. Pharmacological inhibition of PPARα with GW6471 or PPARγ with GW9662 both abolished the myocardial infarct size reduction elicited by the remote ischemic stimulus, as well as the accompanying increase in iNOS mRNA expression, indicating an impaired NO production in these groups.

Przyklenk *et al.* were the first to demonstrate protection against myocardial I/R injury through remote IPC in the canine heart. Four cycles of five minutes I/R of the left circumflex coronary artery were able to protect local and remote myocardium against ischemic injury.[1] Subsequent studies confirmed and extended these findings by showing cardioprotective effects, utilizing different remote IPC protocols in various species, including humans.[4,19] In the current study, we used a protocol of three cycles of RAO, each cycle consisting of 10-min renal ischemia and 10-min renal reperfusion. The magnitude of infarct size reduction achieved with this protocol was comparable with that described in other studies in the rabbit heart during remote IPC,[19] as well as with classical IPC.[20,21]

Myocardial infarct size reduction by the remote IPC stimulus was accompanied by a significantly increased myocardial PPAR DNA-binding activity, indicating a substantial activation of the PPARs in this setting. The magnitude of myocardial PPAR DNA-binding increased approximately four-fold, corresponding well to the data obtained by McLeod *et al.*,[22] showing a 2.5-fold increase in the transcript levels of peroxisome-proliferator-activated receptor gamma coactivator 1α (PGC-1α) two hours after

Table 2 Myocardial infarct size analysis *in vivo*

Experimental group	*n*	IS/AAR (%)	AAR (%)	AAR (g)	LV (g)	Body weight (kg)
Control	8	60.2 ± 1.9	44.5 ± 3.5	1.54 ± 0.15	3.31 ± 0.14	2.6 ± 0.1
Remote IPC	8	42.2 ± 4.9*	37.3 ± 4.3	1.35 ± 0.20	3.26 ± 0.16	2.5 ± 0.1
GW6471	8	56.4 ± 3.2	34.2 ± 5.0	0.97 ± 0.21	2.82 ± 0.29	2.4 ± 0.1
GW9662	8	56.7 ± 4.7	39.5 ± 4.2	1.25 ± 0.12	3.18 ± 0.12	2.6 ± 0.1
Remote IPC + GW6471	8	56.7 ± 4.7	36.9 ± 4.1	1.05 ± 0.29	2.86 ± 0.14	2.5 ± 0.1
Remote IPC + GW9662	8	57.4 ± 3.3	35.7 ± 4.1	1.15 ± 0.15	3.10 ± 0.13	2.6 ± 0.1

AAR, area at risk; LV, left ventricle; IS, myocardial infarct size; IPC, ischemic preconditioning; PPAR, peroxisome-proliferator-activated receptor
Animals subjected to remote IPC exhibited a significantly decreased myocardial infarct size ($P < 0.05$ versus Control). This protective effect was abolished by the prior administration of either the PPARα-antagonist GW6471 or the PPARγ-antagonist GW9662. No significant differences in the AAR, LV weight or body weight of the animals could be observed between the experimental groups. Data are mean ± SD

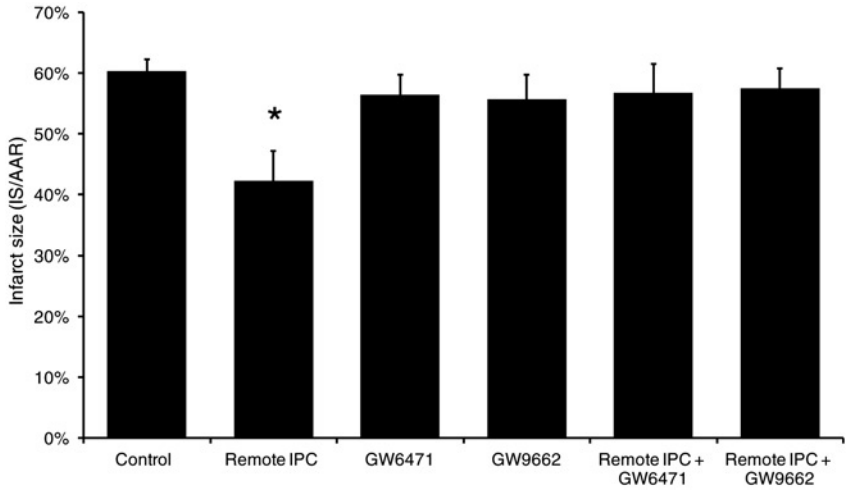

Figure 2 Myocardial infarct size (IS) (as % of left ventricle [LV] weight) expressed as a function of the left ventricular area at risk (AAR) (as % of LV weight) in rabbits subjected to myocardial ischemic injury. The administration of three repetitive cycles of remote ischemic preconditioning (remote IPC) significantly reduced the resulting infarct size, a cardioprotective effect, that was abolished by the preceding blockade of peroxisome-proliferator-activated receptor (PPAR)γ or PPARα, respectively. All data are mean $\pm$ SD. $N = 8$ in all groups. *Significantly ($P < 0.05$) different from control

an IPC stimulus. The application of either a specific blocker of PPARα or PPARγ decreased the myocardial PPAR DNA-binding activity back to control levels, allowing the conclusion that both isoforms were activated by the remote ischemic stimulus. However, the assay used in this study only allows an overall assessment of PPAR activity and does not distinguish between the different isoforms. Therefore, we are not able to decipher any differences in the exact magnitude of transcriptional activation of either PPARα or PPARγ in the heart. Furthermore, in contrary to the myocardial data obtained, PPAR DNA-binding activity was not altered in the renal tissue investigated.

Participation of PPARα and PPARγ in cardioprotection has recently been increasingly recognized. In the murine heart, myocardial I/R injury downregulated PPARα mRNA expression, while the PPARα agonist GW7646 not only reversed this finding, but also improved myocardial dysfunction and attenuated myocardial I/R injury.[9] Similar results were obtained in rats using clofibrate and WY-14643 as PPARα ligands.[8] Multiple studies were also

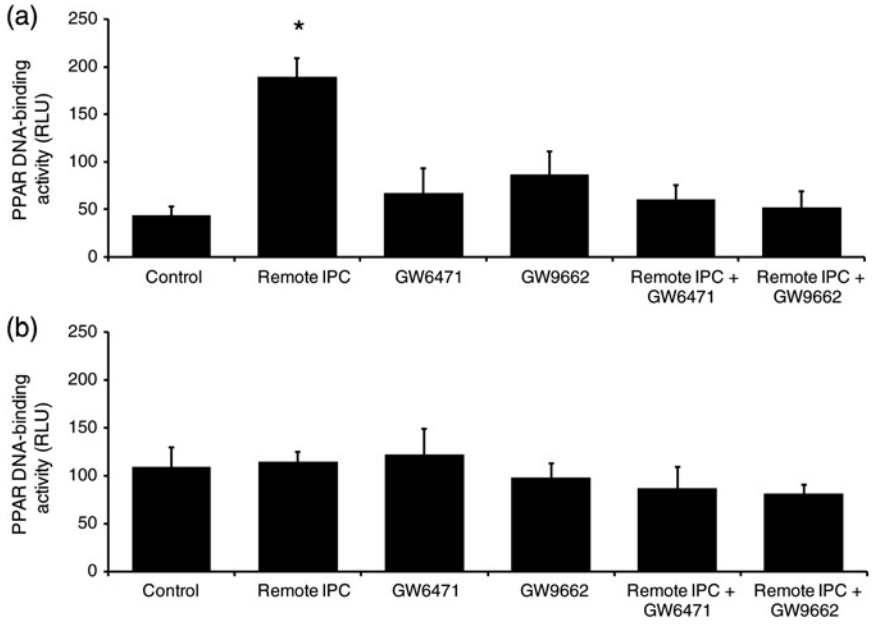

Figure 3 PPAR DNA-binding activity in left ventricular (a), as well as renal (b) tissue expressed in relative light units (RLUs). Three cycles of remote ischemic preconditioning (remote IPC) were accompanied by a significantly increased peroxisome-proliferator-activated receptor (PPAR) DNA-binding activity in the heart, indicating a robust activation of these receptors by the remote stimulus. The administration of either the PPARα-antagonist GW6471 or the PPARγ-antagonist GW9662 (GW9662) abrogated this finding, consistent with the activation of both isoforms during the protective response. No significant differences from the control group were observed in the renal samples, indicating a distinct response between the remote and the target organ, respectively. All data are mean $\pm$ SD. $N = 5$ in all groups. *Significantly ($P < 0.05$) different from control

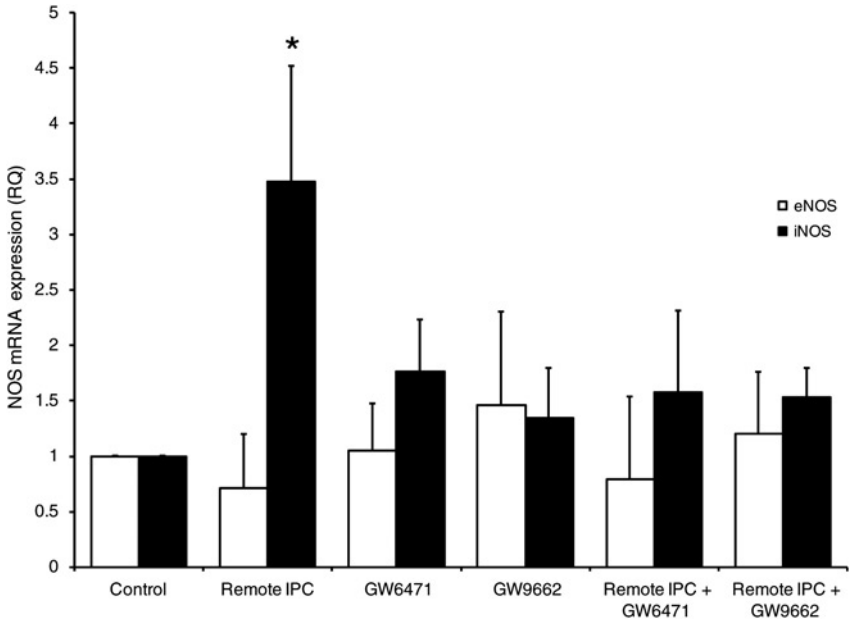

Figure 4 Left ventricular mRNA expression of iNOS and eNOS. RT-PCR was utilized to examine changes in iNOS and eNOS expression elicited by remote IPC, as well as their inter-relation to PPARα and PPARγ signaling. The remote ischemic stimulus elicited a differentiated response as a significantly enhanced iNOS expression, dependent on both PPARα and PPARγ activation was found, while no alterations in eNOS mRNA expression could be observed. These data indicate that both PPARα and PPARγ impinge on the transcriptional control of iNOS expression during remote IPC. GAPDH was used to normalize differences in RNA isolation, RNA degradation and the efficiencies of the reverse transcription. Values are expressed relative to the mRNA expression in the control group (set to 1). All data are mean $\pm$ SD. $N = 5$ in all groups. *Significantly ($P < 0.05$) different from control. iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; PPAR, peroxisome-proliferator-activated receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RQ, relative quantity

able to demonstrate cardioprotective effects afforded by ligand activation of PPARγ using rosiglitazone treatment,[23] as well as ciglitazone, showing reduced myocardial I/R injury accompanied by enhanced PPAR DNA binding.[10] Nevertheless, some studies also failed to depict any beneficial effects by PPARα or PPARγ;[24,25] however, the experimental models used in these studies differed significantly from ours, not only by utilizing different animal species,

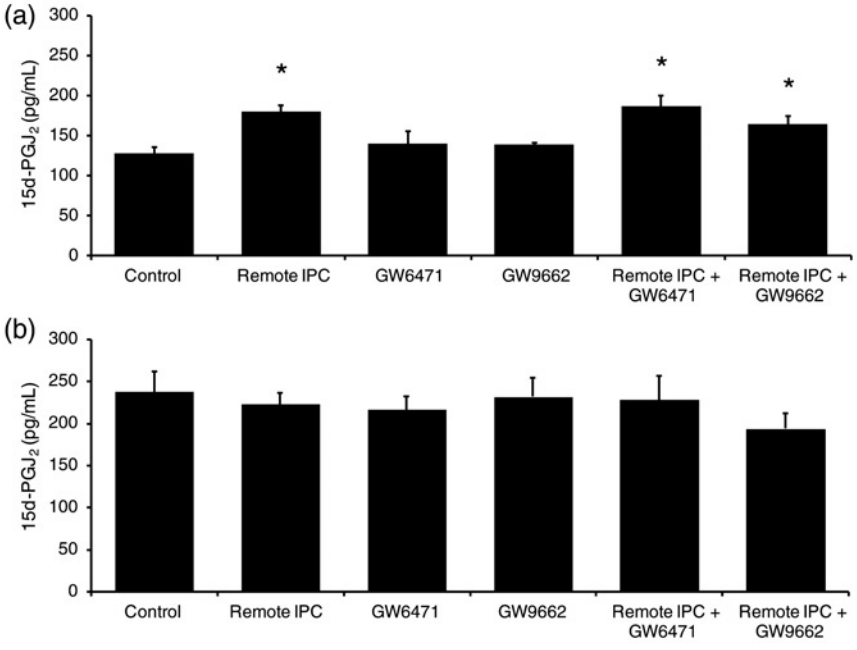

Figure 5 Myocardial (a), as well as renal (b) tissue levels of 15d-prostaglandin J_2 (15d-PGJ_2) expressed in pg/mL. 15d-PGJ_2 acts as a known activator of PPARγ, as well as PPARα, respectively. The obtained data indicate that the remote ischemic stimulus elicited an increase in myocardial 15d-PGJ_2, suggesting 15d-PGJ_2 as an upstream activator of the receptors in this setting. In contrary, no significant changes were observed in the renal tissue investigated, corroborating the data obtained in the PPAR-DNA-binding experiments. All data are mean $\pm$ SD. $N = 5$ in all groups. *Significantly ($P < 0.05$) different from control

but by orally treating the animals for 12 weeks with a PPARα agonist[24] or eight weeks with different PPARγ agonists,[25] respectively.

An additional finding of our study were significantly higher myocardial tissue levels of 15d-PGJ_2 subsequent to the remote ischemic stimulus, while no such increase was present in the renal tissue investigated. 15d-PGJ_2 is known to modulate the transcriptional activities of various nuclear transcription factors and is a well-known endogenous ligand of PPARγ,[26] moreover efficiently activating PPARα.[27] The exogenous administration of 15d-PGJ_2 has previously been shown to elicit infarct size reducing effects, which were associated both with the activation of PPARα and PPARγ.[8] Our finding of an increased myocardial 15d-PGJ_2 after the remote stimulus, which was not abrogated by the blockade of either PPARα or PPARγ complies with its role as an upstream activator of the PPARs, and indicates that 15d-PGJ_2 acted as an endogenous activator of the PPARs during the remote IPC stimulus. Furthermore, the current finding complements the well-established notion of prostaglandins holding up an essential role in cardioprotective signaling.[28] However, due to its molecular structure, 15d-PGJ_2 is known to display multiple cellular functions. It is a cyclopentone prostaglandin acting as a Michael reaction acceptor, reacting with cysteine thiol groups in proteins. Therefore we cannot completely rule out that 15d-PGJ_2 acted on signaling pathways other than PPARα and PPARγ during the protective response. Nevertheless, the majority of the cellular events mediated by 15d-PGJ_2 are actually known to be due to PPAR activation.[26]

As a subsequent step, we focused our investigation on possible changes in NO signaling elicited by the remote stimulus, as well as its interaction with the respective PPAR activation in this setting. Studies not only support a role for NO in remote IPC, but also recently established an important link between PPAR-mediated cardioprotection and NO signaling.[11,12] Protection against lung, intestine and pancreatic I/R injury through remote IPC was associated with an increase in NO levels.[29] Furthermore, bilateral occlusion of the internal carotid arteries leads to a reduced myocardial infarct size in the murine heart, an effect not reproducible in iNOS knockout animals.[6] These findings were further corroborated by Li *et al.*,[30] showing increased iNOS mRNA in the heart after preconditioning of the hind limbs, while no cardioprotection was found in mice with targeted deletions of the iNOS gene. Our study corroborates these findings and adds new insights by showing that the remote ischemic stimulus augmented an increase in myocardial iNOS expression, dependent on the activation of PPARα and PPARγ. Using RT-PCR measurements, we were able to decipher a differential response in NOS signaling subsequent to the remote stimulus. It is known that tissue ischemia triggers rapid induction of specific transcription factors, setting the stage for subsequent tissue injury during reperfusion,[31] and that changes in iNOS transcription can already be observed after 20 min.[32] While the eNOS mRNA expression did not differ significantly throughout the experimental groups, remote IPC significantly increased the iNOS expression in the heart. Furthermore, pharmacological inhibition of either PPARα or PPARγ abrogated the observed effect, indicating the activation of a PPARα/PPARγ-NOS pathway in the heart. This pathway seems to be exclusive to the heart further considering the distinct response between heart and kidney observed in the PPAR transcription factor activity assay, as well as the absence of increased renal 15d-PGJ2 levels in our study. However, one has to consider that the observed increase in iNOS mRNA only provides limited information about the actual change in iNOS activity, as well as the absolute values of NO in the heart, as the enzyme is explicitly known to be regulated by post-translational modifications. Nevertheless, taking into account that the remote IPC stimulus lead to a 3.5-fold increase in iNOS mRNA, we believe that it is safe to conclude that the protective response was indeed governed by an increased availability of NO in a PPARα/PPARγ dependent fashion.

Moreover, the results imply the presence of a yet unknown, mechanistic pathway linking the remote organ to the heart. Neural pathways, as well as humoral mediators, like opioids or endocannabinoids, have been suggested to connect the remote organ to the heart during remote IPC. Hajrasouliha *et al.*[33] demonstrated cannabinoid receptor 2 (CB2) receptor activation during remote IPC and proposed that endocannabinoids generated during ischemia of the remote organ may enter the bloodstream and activate CB2 receptors. Since enhanced endocannabinoid levels are known to activate PPARα[34] and PPARγ agonists[35] are capable of interacting directly with the endocannabinoid system, most likely endocannabinoids may have been generated during renal I/R. In turn, activation of myocardial CB receptors may have subsequently led to the increase in PPARα and PPARγ activity. Nevertheless, future studies are required to test this hypothesis.

The current results must be interpreted within the constraints of several potential limitations. Myocardial infarct size is determined primarily by the size of the AAR and extent of coronary collateral perfusion. In the present investigation, the AAR expressed as a percentage of total left ventricular mass was similar between groups and rabbits have been shown to exhibit little if any coronary collateral blood flow.[36] Therefore, it seems unlikely that differences in collateral perfusion between groups account for the observed results. Furthermore, the possibility that GW6471 and GW9662 may have affected other signaling cascades or proteins involved in myocardial protection cannot be completely excluded from the analysis. Nonetheless, GW6471 and GW9666 have proven to be highly selective inhibitors of PPARα[37] and PPARγ,[38] respectively.

In summary, the results of the current study demonstrate an essential role for PPARα, as well as PPARγ in mediating the cardioprotective response elicited by remote IPC by impinging on the downstream production of NO.

Author contributions: CL, CB and TT-Z conducted the *in vivo* myocardial infarct size experiments; J Schmidt and J Stumpner measured the PPAR-DNA-binding activity, as well as the 15d-PGJ_2 tissue levels; ML and JP conducted the RT-PCR experiments; AR, TMS, NR and FK were

involved in the experimental design and reviewed/edited the manuscript. CL and ML designed the studies, analyzed the data and wrote the manuscript.

Funding: This work was supported by an intramural grant of the Interdisciplinary Centre for Clinical Research (IZKF) to Christopher Lotz, Wuerzburg, Germany.

REFERENCES

1 Przyklenk K, Bauer B, Ovize M, Kloner RA, Whittaker P. Regional ischemic 'preconditioning' protects remote virgin myocardium from subsequent sustained coronary occlusion. *Circulation* 1993;**87**:893–9

2 Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD. Myocardial protection by brief ischemia in noncardiac tissue. *Circulation* 1996;**94**:2193–200

3 Botker HE, Kharbanda R, Schmidt MR, Bottcher M, Kaltoft AK, Terkelsen CJ, Munk K, Andersen NH, Hansen TM, Trautner S, Lassen JF, Christiansen EH, Krusell LR, Kristensen SD, Thuesen L, Nielsen SS, Rehling M, Sorensen HT, Redington AN, Nielsen TT. Remote ischaemic conditioning before hospital admission, as a complement to angioplasty, and effect on myocardial salvage in patients with acute myocardial infarction: a randomised trial. *Lancet* 2010;**375**:727–34

4 Hausenloy DJ, Mwamure PK, Venugopal V, Harris J, Barnard M, Grundy E, Ashley E, Vichare S, Di C, Kolvekar S, Hayward M, Keogh B, MacAllister RJ, Yellon DM. Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. *Lancet* 2007;**370**:575–9

5 Weinbrenner C, Schulze F, Sarvary L, Strasser RH. Remote preconditioning by infrarenal aortic occlusion is operative via delta1-opioid receptors and free radicals *in vivo* in the rat heart. *Cardiovasc Res* 2004;**61**:591–9

6 Tokuno S, Hinokiyama K, Tokuno K, Lowbeer C, Hansson LO, Valen G. Spontaneous ischemic events in the brain and heart adapt the hearts of severely atherosclerotic mice to ischemia. *Arterioscler Thromb Vasc Biol* 2002;**22**:995–1001

7 Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia–reperfusion injury. *Physiol Rev* 2008;**88**:581–609

8 Wayman NS, Hattori Y, McDonald MC, Mota-Filipe H, Cuzzocrea S, Pisano B, Chatterjee PK, Thiemermann C. Ligands of the peroxisome proliferator-activated receptors (PPAR-gamma and PPAR-alpha) reduce myocardial infarct size. *FASEB J* 2002;**16**:1027–40

9 Yue TL, Bao W, Jucker BM, Gu JL, Romanic AM, Brown PJ, Cui J, Thudium DT, Boyce R, Burns-Kurtis CL, Mirabile RC, Aravindhan K, Ohlstein EH. Activation of peroxisome proliferator-activated receptor-alpha protects the heart from ischemia/reperfusion injury. *Circulation* 2003;**108**:2393–9

10 Zingarelli B, Hake PW, Mangeshkar P, O'Connor M, Burroughs TJ, Piraino G, Denenberg A, Wong HR. Diverse cardioprotective signaling mechanisms of peroxisome proliferator-activated receptor-gamma ligands, 15-deoxy-Delta12,14-prostaglandin J2 and ciglitazone, in reperfusion injury: role of nuclear factor-kappaB, heat shock factor 1, and Akt. *Shock* 2007;**28**:554–63

11 Bulhak AA, Sjoquist PO, Xu CB, Edvinsson L, Pernow J. Protection against myocardial ischaemia/reperfusion injury by PPAR-alpha activation is related to production of nitric oxide and endothelin-1. *Basic Res Cardiol* 2006;**101**:244–52

12 Gonon AT, Bulhak A, Labruto F, Sjoquist PO, Pernow J. Cardioprotection mediated by rosiglitazone, a peroxisome proliferator-activated receptor gamma ligand, in relation to nitric oxide. *Basic Res Cardiol* 2007;**102**:80–9

13 Huss JM, Kelly DP. Nuclear receptor signaling and cardiac energetics. *Circ Res* 2004;**95**:568–78

14 Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr Rev* 1999;**20**:649–88

15 Bayne K. Revised guide for the care and use of laboratory animals available. American Physiological Society. *Physiologist* 1996;**39**:199, 208–11

16 Walther OE, Riedel W, Iriki M, Simon E. Differentiation of sympathetic activity at the spinal level in response to central cold stimulation. *Pflugers Arch* 1971;**329**:220–30

17 Lange M, Smul TM, Blomeyer CA, Redel A, Klotz KN, Roewer N, Kehl F. Role of the beta1-adrenergic pathway in anesthetic and ischemic preconditioning against myocardial infarction in the rabbit heart *in vivo*. *Anesthesiology* 2006;**105**:503–10

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

19 Birnbaum Y, Hale SL, Kloner RA. Ischemic preconditioning at a distance: reduction of myocardial infarct size by partial reduction of blood supply combined with rapid stimulation of the gastrocnemius muscle in the rabbit. *Circulation* 1997;**96**:1641–6

20 Liu GS, Thornton J, Van Winkle DM, Stanley AW, Olsson RA, Downey JM. Protection against infarction afforded by preconditioning is mediated by A1 adenosine receptors in rabbit heart. *Circulation* 1991;**84**:350–6

21 Qiu Y, Rizvi A, Tang XL, Manchikalapudi S, Takano H, Jadoon AK, Wu WJ, Bolli R. Nitric oxide triggers late preconditioning against myocardial infarction in conscious rabbits. *Am J Physiol* 1997;**273**:H2931–6

22 McLeod CJ, Jeyabalan AP, Minners JO, Clevenger R, Hoyt RF Jr., Sack MN. Delayed ischemic preconditioning activates nuclear-encoded electron-transfer-chain gene expression in parallel with enhanced postanoxic mitochondrial respiratory recovery. *Circulation* 2004;**110**:534–9

23 Abdelrahman M, Sivarajah A, Thiemermann C. Beneficial effects of PPAR-gamma ligands in ischemia-reperfusion injury, inflammation and shock. *Cardiovasc Res* 2005;**65**:772–81

24 Morgan EE, Rennison JH, Young ME, McElfresh TA, Kung TA, Tserng KY, Hoit BD, Stanley WC, Chandler MP. Effects of chronic activation of peroxisome proliferator-activated receptor-alpha or high-fat feeding in a rat infarct model of heart failure. *Am J Physiol Heart Circ Physiol* 2006;**290**:H1899–904

25 Xu Y, Gen M, Lu L, Fox J, Weiss SO, Brown RD, Perlov D, Ahmad H, Zhu P, Greyson C, Long CS, Schwartz GG. PPAR-gamma activation fails to provide myocardial protection in ischemia and reperfusion in pigs. *Am J Physiol Heart Circ Physiol* 2005;**288**:H1314–23

26 Kim EH, Surh YJ. 15-deoxy-Delta12,14-prostaglandin J2 as a potential endogenous regulator of redox-sensitive transcription factors. *Biochem Pharmacol* 2006;**72**:1516–28

27 Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM. A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor gamma and promotes adipocyte differentiation. *Cell* 1995;**83**:813–19

28 Hide EJ, Ney P, Piper J, Thiemermann C, Vane JR. Reduction by prostaglandin E1 or prostaglandin E0 of myocardial infarct size in the rabbit by activation of ATP-sensitive potassium channels. *Br J Pharmacol* 1995;**116**:2435–40

29 Peralta C, Fernandez L, Panes J, Prats N, Sans M, Pique JM, Gelpi E, Rosello-Catafau J. Preconditioning protects against systemic disorders associated with hepatic ischemia-reperfusion through blockade of tumor necrosis factor-induced P-selectin up-regulation in the rat. *Hepatology* 2001;**33**:100–13

30 Li G, Labruto F, Sirsjo A, Chen F, Vaage J, Valen G. Myocardial protection by remote preconditioning: the role of nuclear factor kappa-B p105 and inducible nitric oxide synthase. *Eur J Cardiothorac Surg* 2004;**26**:968–73

31 Yan SF, Fujita T, Lu J, Okada K, Shan Y, Mackman N, Pinsky DJ, Stern DM. Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. *Nat Med* 2000;**6**:1355–61

32 Liu SF, Barnes PJ, Evans TW. Time course and cellular localization of lipopolysaccharide-induced inducible nitric oxide synthase messenger RNA expression in the rat *in vivo*. *Crit Care Med* 1997;**25**:512–18

33 Hajrasouliha AR, Tavakoli S, Ghasemi M, Jabehdar-Maralani P, Sadeghipour H, Ebrahimi F, Dehpour AR. Endogenous cannabinoids contribute to remote ischemic preconditioning via cannabinoid CB2 receptors in the rat heart. *Eur J Pharmacol* 2008;**579**:246–52

34 Sagar DR, Kendall DA, Chapman V. Inhibition of fatty acid amide hydrolase produces PPAR-alpha-mediated

analgesia in a rat model of inflammatory pain. *Br J Pharmacol* 2008;**155**:1297–306

35 Lenman A, Fowler CJ. Interaction of ligands for the peroxisome proliferator-activated receptor gamma with the endocannabinoid system. *Br J Pharmacol* 2007;**151**:1343–51

36 Maxwell MP, Hearse DJ, Yellon DM. Species variation in the coronary collateral circulation during regional myocardial ischaemia: a critical determinant of the rate of evolution and extent of myocardial infarction. *Cardiovasc Res* 1987;**21**:737–46

37 Xu HE, Stanley TB, Montana VG, Lambert MH, Shearer BG, Cobb JE, McKee DD, Galardi CM, Plunket KD, Nolte RT, Parks DJ, Moore JT, Kliewer SA, Willson TM, Stimmel JB. Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPARalpha. *Nature* 2002;**415**:813–17

38 Leesnitzer LM, Parks DJ, Bledsoe RK, Cobb JE, Collins JL, Consler TG, Davis RG, Hull-Ryde EA, Lenhard JM, Patel L, Plunket KD, Shenk JL, Stimmel JB, Therapontos C, Willson TM, Blanchard SG. Functional consequences of cysteine modification in the ligand binding sites of peroxisome proliferator activated receptors by GW9662. *Biochemistry* 2002;**41**:6640–50

(Received July 8, 2010, Accepted September 23, 2010)