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Preconditioning and postconditioning:

The essential role of the mitochondrial permeability transition pore

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Abstract

Objective—The opening of the mitochondrial permeability transition pore (mPTP) at the time of myocardial reperfusion is a critical determinant of cell death. Emerging studies suggest that suppression of mPTP opening may underlie the cardioprotection elicited by both ischemic preconditioning (IPC) and postconditioning (IPost). To further evaluate the role of the mPTP in cardioprotection, we hypothesized that hearts deficient in cyclophilin-D (CYP-D-/-), a key component of the mPTP, will be resistant to cardioprotection conferred by ischemic and pharmacological preconditioning and postconditioning.

Methods and results—Male/female wild type or CYP-D-/- mice were subjected to 30 min of ischemia and 120 min of reperfusion. In wild type mice subjected to *in vivo* myocardial ischemia-reperfusion injury, a significant reduction in myocardial infarct size was observed with the following treatments ($n \geq 6$/group; $P < 0.05$): (1) IPC (28±4% vs. 46.2±4% in control); (2) Diazoxide (5 mg/kg) pre-treatment (26.4±3% vs. 54±10% in vehicle control); (3) IPost-1 or IPost-2, three or six 10-s cycles of ischemia-reperfusion (27.2±3% and 32±4%, respectively vs. 46.2±4% in control); (4) Bradykinin (40 μg/kg) (28.3±1% vs. 48±4% in vehicle control); (5) cyclosporin-A (10 mg/kg) (32.3±3% vs. 48±4% in vehicle control) (6) sanglifehrin-A (25 mg/kg) (29.3±3% vs. 48±4% in vehicle control). Interestingly, however, no infarct-limiting effects were demonstrated in CYP-D-/- mice with the same treatment protocols: (27.9±5% in control vs. 31.2±7% with IPC, 30.2±5% with IPost-1, 24.7±8% with IPost-2; 30.1±4% in vehicle control vs. 26.4±7% with diazoxide; 24.6±4% in vehicle control vs. 24.9±5% with bradykinin, 26.8±7% with cyclosporin-A, 32.5±6% with sanglifehrin-A: $n \geq 6$/group: $P > 0.05$).

Conclusion—This study demonstrates that the mPTP plays a critical role in the cardioprotection elicited by ischemic and pharmacological preconditioning and postconditioning.

Keywords

Ischemia; Mitochondria; Reperfusion; Preconditioning

1. Introduction

Ischemic preconditioning (IPC), first described by Murry and co-workers in 1986 [1] as an effective endogenous protective phenomenon whereby exposure to one or more brief episodes of sub-lethal myocardial ischemia and reperfusion increased the resistance of the myocardium to a subsequent sustained ischemic insult, has been extensively studied and yet the actual mechanism of protection remains unclear. Its clinical application has been restricted by the necessity to intervene before the onset of myocardial ischemia, which is not

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possible in the case of an acute myocardial infarction. A more amenable cardioprotective strategy, which can be applied at the time of myocardial reperfusion, is the recently described phenomenon of ischemic postconditioning (IPost) [2], in which the application of brief periods of myocardial ischemia and reperfusion at the immediate onset of reperfusion confers cardioprotection, has proven far more amenable to clinical application [3].

Emerging studies suggest that the cardioprotection elicited by both IPC and IPost may be mediated through the modulation of the mitochondrial permeability transition pore (mPTP), a non-specific channel of the inner mitochondrial membrane, whose opening in the first few minutes of myocardial reperfusion mediates cell death by uncoupling oxidative phosphorylation and inducing mitochondrial swelling [4,5]. Both IPC and pharmacological preconditioning have been demonstrated to confer cardioprotection through the inhibition of mPTP opening [6-8]. More recently, the cardioprotection elicited by IPost has also been linked to the suppression of mPTP opening [9,10].

Although the core components of the mPTP were believed to consist of the voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT) and cyclophilin-D (CYP-D), recent knock-out studies have cast doubt on the involvement of VDAC and ANT [11,12]. In contrast, recent studies have provided convincing evidence that cyclophilin-D is an important regulatory component of the mPTP, such that mice deficient in CYP-D are resistant to mPTP opening induced by calcium or oxidative stress and sustain both smaller myocardial and cerebral infarcts in response to ischemia-reperfusion injury [13-16].

To demonstrate the pivotal role of the mPTP in cardioprotection, we hypothesized that CYP-D-deficient mice will be resistant to the cardioprotection elicited by both ischemic and pharmacological preconditioning and postconditioning.

2. Methods

2.1. Animals

Experiments using animals were carried out in accordance with the United Kingdom Home Office Guide on the Operation of Animal (Scientific Procedures) Act of 1986. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. B6Sv129F1 mice were obtained from Harlan (UK) and CYP-D-/- mice were bred from pairs provided by Baines et al [14].

2.2. In vivo model of acute myocardial ischemia-reperfusion injury

Wild type (B6129SvF1) and CYP-D-/- mice (male or female, 8-10 weeks, 20-30 g) were anesthetized by intraperitoneal injection with a combination of ketamine, xylazine and atropine (0.01 ml/g, final concentration of ketamine, xylazine and atropine were 10 mg/ml, 2 mg/ml and 0.06 mg/ml, respectively) and body temperature was maintained at 37 °C. The external jugular vein and carotid artery were isolated and cannulated for drug administration and mean arterial blood pressure (MABP) measurement, respectively. A tracheotomy was performed for artificial respiration at 120 strokes/min and 220 μl stroke volume using a rodent Minivent (type 845, Harvard Apparatus, Kent, UK) and supplemental oxygen was supplied. A limb lead I electrocardiogram (ECG) was recorded. A left anterior thoracotomy and a chest retractor were used to expose the heart. Ligation of the left anterior descending (LAD) coronary artery was performed ~2 mm below the tip of the left auricle using a 8/0 prolene monofilament polypropylene suture. Successful LAD coronary artery occlusion was confirmed by the presence of ST elevation and decreased in arterial blood pressure. At the end of the reperfusion, the heart was isolated and the aortic root was cannulated and used to inject 2,3,5-triphenyltetrazolium chloride (TTC, 5 ml of 1%) in order to demarcate the infarcted tissue. The LAD coronary artery was then re-ligated and Evans blue dye (2 ml of

0.5%) was perfused to delineate the area at risk (AAR). The heart was frozen and sectioned perpendicularly to the long axis (1-2 mm thick). The slices were then transferred to 10% neutral buffer formalin for 2 hours at room temperature to stabilize the staining. AAR and infarct size were determined by computerized planimetry using the NIH software Image, following incubation with 10% formaldehyde for 2 hours at room temperature. AAR was expressed as a percentage of the left ventricle and infarct size was expressed as a percentage of the AAR.

2.3. Treatment protocols for myocardial infarction studies

Wild type and Cyp-D-/- mice were assigned to one of the 10 groups (see Fig. 1). All hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion.

1. Control: there was no intervention either before or after coronary artery occlusion;
2. Vehicle control-1: 0.01 M NaOH given 10 min before the index ischemic period;
3. Vehicle control-2: saline or mixture of Cremophor EL (polyethoxylated castor oil) with ethanol-94% given immediately upon reperfusion;
4. IPC: the coronary artery was occluded for 5 min followed by 5 min of reperfusion before the prolonged coronary artery occlusion;
5. IPost-1: immediately at the onset of reperfusion, reflow was initiated for 10 s followed by 10 s of reocclusion, repeated for a total of three cycles; and
6. IPost-2: the reflow-occlusion algorithm describe above was repeated for a total of six cycles;
7. Dzx: pharmacological preconditioning with diazoxide (5 mg/kg) given 10 min before the index ischemic period;
8. Brady: pharmacological postconditioning using bradykinin (40 μg/kg) given immediately upon reperfusion;
9. CsA: Cyclosporin-A (10 mg/kg) was given immediately upon reperfusion;
10. SfA: Sanglifehrin-A (25 mg/kg) was given immediately upon reperfusion.

In a separate set of experiments, wild type and CYP-D-/- mice were subjected to 45 min of ischemia followed by 120 min of reperfusion with or without IPC in which the coronary artery was occluded for 5 min followed by 5 min of reperfusion before the prolonged coronary artery occlusion.

2.4. Materials

All chemicals were purchase from Sigma-Aldrich, Dorset, UK unless otherwise stated. CsA (Calbiochem, La Jolla, USA), heparin (CP Pharmaceuticals Ltd., Wrexham, UK), ketamine (Vetalar®; Pharmacia Animal Health Ltd, Northamptonshire, UK), SfA (Novartis Pharma, Basel), xylazine (Rompun®; Bayer Plc., Berkshire, UK). CsA and SfA were dissolved in a mixture of Cremophor EL (polyethoxylated castor oil) with ethanol-94%. Diazoxide and bradykinin were dissolved in 0.01 M NaOH and saline, respectively.

2.5. Statistical analysis

All values are expressed as mean±S.E.M. Data were analyzed by unpaired *t*-test or one-way ANOVA followed by a Dunnett's multiple comparison post-hoc test where appropriate. A $P<0.05$ was considered to be statistically significant.

3. Results

In general, apart from the slight increase in heart rate in the wild type IPC at a single time point, there were no significant differences between groups in heart rate and mean arterial blood pressure (Table 1).

3.1. CYP-D-deficient mice are resistant to ischemic preconditioning and postconditioning

The area at risk (AAR) was comparable among the treatment groups. In wild type mice, IPC, IPost-1 and IPost-2 reduced infarct size (expressed as infarct size as a % of AAR) from 46±4% in control to 28±4%, 27±3% and 32±4%, respectively ($P<0.05$; Fig. 2A). As expected, hearts from CYP-D-/- mice sustained a smaller infarct size (46±4% in wild type control vs. 28±5% in CYP-D-/- control; $P<0.05$; Fig. 2). Interestingly, however, IPC, IPost-1 and IPost-2 did not reduce infarct size in the CYP-D-/- mice (31±7% with IPC, 30±5% with IPost-1, 25±8% with IPost-2 vs. 28±5% in control; $P>0.05$; Fig. 2B).

In mice subjected to 45 min of ischemia, IPC significantly reduced infarct size in wild type mice (59±2% to 31±5%, $P<0.05$) but not in the CYP-D-/- mice (38±7% and 28±7%, $P>0.05$; Fig. 3).

3.2. CYP-D-deficient mice are resistant to pharmacological cardioprotection

The AAR was comparable among the treatment groups. In wild type mice, diazoxide-mediated preconditioning reduced infarct size significantly (53±7% versus 26±3%; $P<0.05$). Pharmacological inhibition of mPTP opening at time of reperfusion using CsA and SfA also reduced infarct size (48±4% with control vs. 32±3% with CsA and 29±3% with SfA; $P<0.05$; Fig. 4A), as did bradykinin given at reperfusion (28±1%; $P<0.05$; Fig. 4A). However, treatment with these pharmacological agents elicited no reduction in infarct size in CYP-D-/- mice (30±4% in control vs. 26±7% with diazoxide; 25±4% in control vs. 25±5% with bradykinin, 27±7% with CsA, 33±6% with SfA; $P>0.05$; Fig. 4B).

4. Discussion

This study demonstrates for the first time that hearts deficient in CYP-D, an essential regulatory component of the mPTP, are resistant to the cardioprotection elicited by both IPC and IPost. In addition, these hearts were not amenable to cardioprotection mediated by the pharmacological preconditioning mimetic, diazoxide, or the pharmacological postconditioning agent, bradykinin. Furthermore, as expected no reduction in myocardial infarct size was observed in hearts treated with the standard pharmacological mPTP inhibitors, CsA and SfA, confirming CYP-D as the target through which these drugs act to inhibit mPTP opening. Although we demonstrated clear cardioprotection in terms of infarct size reduction, we saw no changes in mean arterial blood pressure and heart rate. These in situ studies confirm the essential role of the mPTP in the setting of cardioprotection elicited by preconditioning and postconditioning.

The role for the mPTP as a mediator of lethal ischemia-reperfusion injury was first proposed by Crompton's laboratory in the late 1980s in pivotal studies demonstrating: (1) that mPTP opening occurred in response to calcium, oxidative stress, inorganic phosphate and relative ATP depletion, conditions that are recreated during ischemia-reperfusion [17] and (2) that CsA could inhibit mPTP opening and confer cardioprotection [18]. Subsequent studies by Halestrap's laboratory demonstrated that the mPTP remains closed during myocardial ischemia and only opens at time of myocardial reperfusion [4], positioning itself as a target for cardioprotection as demonstrated by a reduction in myocardial infarct size with the pharmacological mPTP inhibitors, given at time of reperfusion [5].

The crucial role of the mPTP as mediator of myocardial ischemia-reperfusion injury has now been confirmed in studies demonstrating that mice lacking CYP-D, a presumed component of the mPTP, demonstrated increased resistance to mPTP opening and sustained smaller myocardial infarcts [14,15] and reduced cerebral infarcts [16]. Our study has confirmed these findings, demonstrating that CYP-D-/- mice exhibited a smaller myocardial infarct size compared to the wild type mice. The availability of these CYP-D-/- mice provides more specific evidence for the involvement of the mPTP in ischemia-reperfusion injury, than the use of pharmacological mPTP inhibitors with their attendant non-specific effects.

Of importance, we demonstrated that CYP-D-/- mice were resistant to the cardioprotective benefits elicited by both IPC and IPost, suggesting a fundamental role for the mPTP in these settings. Interestingly, in contrast to the wild type mice, CYP-D-/- mice remain resistant to the cardioprotective effect of IPC when the ischemic period was increased to 45 min. These results demonstrate that the threshold for protection is unchanged in the CYP-D-/- mice which further supports a role for the mPTP in cardioprotection.

Our results provide strong support for the emerging studies implicating the suppression of mPTP opening as a potential end-effector of cardioprotection in IPC [6-8] and IPost [9,10]. In these settings, it is the pathological opening of the mPTP which occurs on reperfusing ischemic myocardium that is believed to be suppressed by the cardioprotective interventions of IPC and IPost, and therefore in mice lacking CYP-D, a essential regulatory component of the mPTP, there is no target for cardioprotection, and therefore no infarct size reduction is observed with IPC and IPost. We appreciate that this study provides confirmatory evidence implicating the role of the mPTP in the settings of ischemic and pharmacological preconditioning and postconditioning, but the actual mechanism through which preconditioning and postconditioning inhibit the mPTP opening remains to be investigated. However, we postulate that mPTP inhibition may be through the activation of protein kinases such as Akt [10,19], GSK-3β [8] or PKC-ε [20].

The above scenario places the mPTP as the potential common end-effector of IPC and IPost. However, there is preliminary evidence to suggest that the mPTP may act as a mediator of cardioprotection in the setting of IPC, perhaps by undergoing non-pathological opening during the preconditioning phase, thereby inducing a mild form of mitochondrial stress [21]. In this latter study, it was demonstrated that the pharmacological inhibition of mPTP opening using either CsA or SfA, prior to the sustained ischemic episode, abrogated the infarct-limiting effects elicited by IPC, diazoxide, adenosine and dinitrophenol in isolated perfused rat hearts [21]. An intriguing alternative explanation therefore as to why CYP-D-/- mice are not amenable to cardioprotection induced by ischemic and pharmacological preconditioning may be that the mPTP may be acting as a ‘mediator’ of cardioprotection during the pre-ischemic phase, although further studies are required to confirm this.

In conclusion, we demonstrate for the first time that hearts deficient in CYP-D, an essential component of the mPTP, are resistant to the infarct-limiting effects of ischemic and pharmacological preconditioning and postconditioning, confirming the essential role of the mPTP in mediating cardioprotection in these settings.

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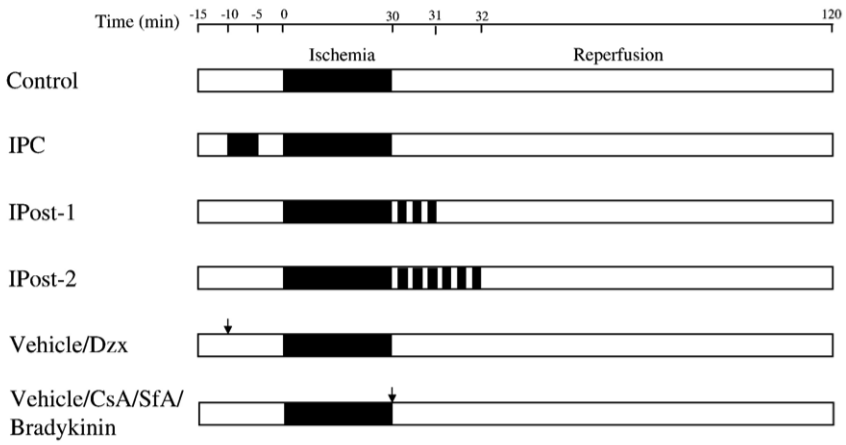

Fig. 1.

Experimental protocols for wild type and CYP-D-/- mice subjected to 30 min LAD coronary artery occlusion followed by 120 min of reperfusion. The arrow indicates the administration of treatment. Dzx, diazoxide; CsA, cyclosporin-A; SfA, sanglifehrin-A.

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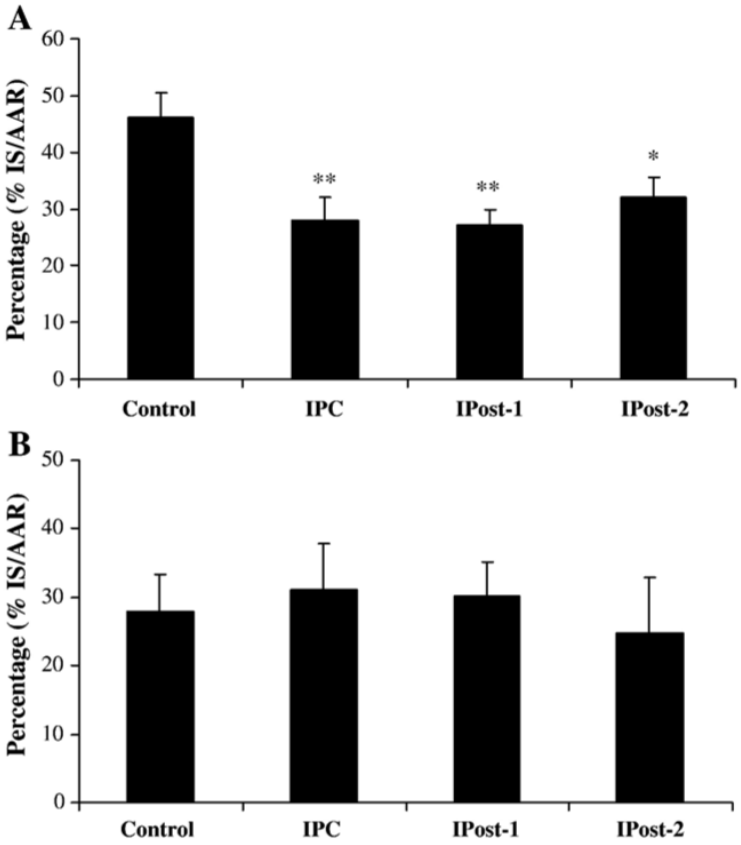

Fig. 2.

CYP-D-/- mice are resistant to IPC and IPost. Infarct size (IS) expressed as a % of the area at risk (AAR) in wild-type (A) or CYP-D-/- (B) mice subjected to control, IPC, IPost-1, or IPost-2 (n=6-9/group). *P<0.05 and **P<0.01 vs. control.

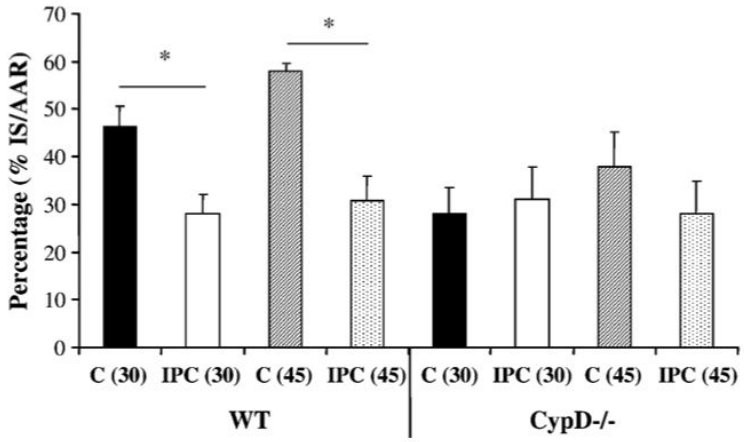

Fig. 3.

CYP-D-/- mice are resistant to IPC with 45 min of ischemia. Infarct size (IS) expressed as a % of the area at risk (AAR) in wild-type or CYP-D-/- mice subjected to control or IPC with 30 min or 45 min of ischemia (n=6-9/group). *P<0.05.

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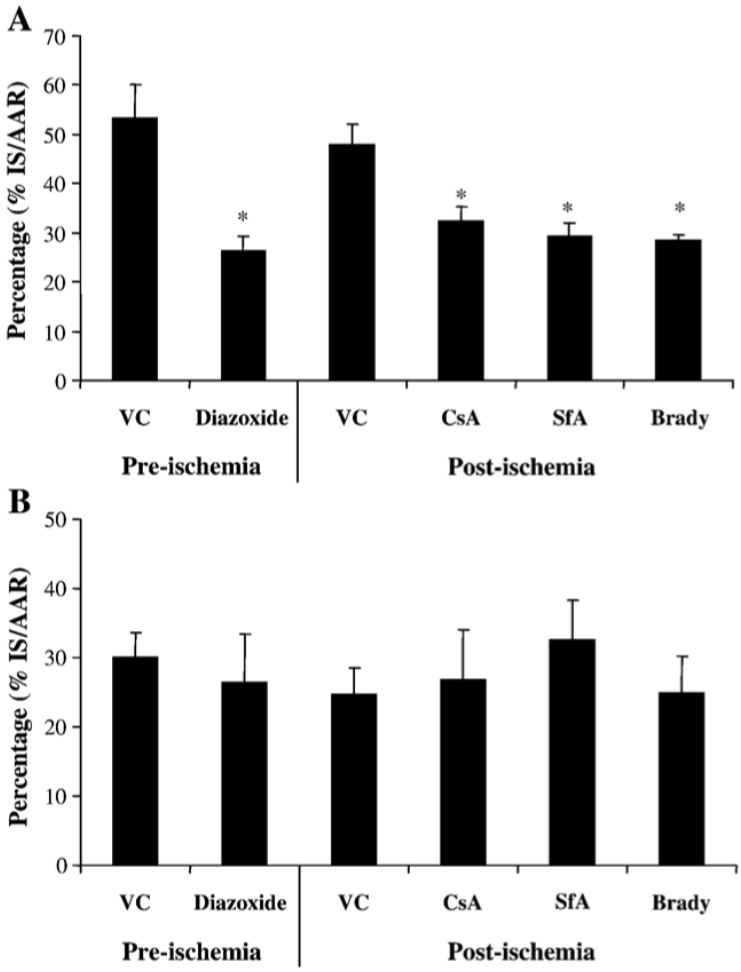

Fig. 4.

CYP-D-/- mice are resistant to pharmacological preconditioning and postconditioning. Infarct size (IS) expressed as a % of the area at risk (AAR) in wild-type (A) or Cyp-D-/- (B) mice receiving vehicle (VC) or diazoxide pretreatment or vehicle (VC), cyclosporin-A (CsA), sanglifehrin-A (SfA), bradykinin (Brady) at time of reperfusion (n=6-8/group). *P<0.01 vs. control.

Table 1

Hemodynamic variables in wild type and CYP-D-/- mice

Group	MABP (mm Hg)/HR (bpm)			
	I, 0 min	I, 15 min	R, 30 min	R, 120 min
Wild type				
Control	127±5/409±10	106±6/395±8	81±4/368±8	53±2/408±13
IPC	128±3/442±7*	106±4/405±8	83±4/377±9	58±4/413±20
IPost-1	129±2/407±10	109±2/386±6	77±4/356±13	54±4/436±15
IPost-2	130±2/411±10	108±3/400±6	76±2/377±7	54±5/420±26
CYP-D-/-				
Control	130±6/425±7	112±7/411±7	81±5/389±19	43±5/379±19
IPC	120±12/423±22	106±10/405±17	84±6/386±15	48±6/385±11
IPost-1	111±9/415±18	90±7/377±7	76±5/370±15	49±4/367±22
IPost-2	113±9/402±13	96±8/394±14	73±3/385±10	49±8/389±24
Wild type				
Control	133±4/416±6	102±5/401±5	75±4/381±7	41±5/392±19
Dzx	133±4/398±9	106±9/382±8	77±6/363±6	47±4/371±19
Control	129±4/411±7	107± ±5/400±6	78±3/393±8	49±4/392±17
CsA	134±3/414±8	111±5/389±7	83±3/400±10	58±3/406±22
SfA	124±5/412±6	101±5/394±12	82±3/367±6	48±4/443±18
Bradykinin	132±3/428±7	111±6/407±9	84±2/393±9	53±4/392±17
CYP-D-/-				
Control	103±6/427±15	88±6/418±12	74±3/411±10	45±4/386±14
Dzx	96±7/428±14	79±3/432±21	70±3/420±14	34±7/378±17
Control	124±8/430±12	112±8/422±18	87±3/413±11	47±7/386±9
CsA	103±7/403±13	94±8/381±15	73±4/390±10	34±6/372±15
SfA	114±9/428±14	101±9/420±18	78±7/424±11	47±5/400±13
Bradykinin	117±6/420±10	103±5/404±11	78±4/413±7	41±5/393±18

Mean arterial blood pressure (MABP) and heart rate (HR) were taken at 0 and 15 min into occlusion (I, 0 min and I, 15 min), and at 30 and 120 min into reperfusion (R, 30 min and R, 120 min). n=6-9.

*P<0.05 vs. wild type control at the same time point.