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Short running title: Isoprostanes and myocardial preconditioning

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Objective. Myocardial ischemia-reperfusion in humans is associated with increased formation of the isoprostanes 15-F_{2t}-IsoP and 5-F_{2t}-IsoP. Whether this formation is clinically relevant remains controversial. This study was performed in order to evaluate the ability of isoprostanes 15-F_{2t}-IsoP and 5-F_{2t}-IsoP to reduce myocardial ischaemic injury in isolated rat heart.

Methods. Rats were divided into six groups. Their hearts were excised, retrogradely perfused and pretreated with vehicle (ethanol 5.10^{-7} and 2.10^{-9} M, n = 6), subjected to ischaemic preconditioning (n = 8) or pretreated with the isoprostanes 15-F_{2t}-IsoP (3.10^{-10} and 3.10^{-7} M, n=8) or 5-F_{2t}-IsoP (10^{-9} M, n = 8). After a 5 min treatment-5 min wash-out period, the hearts were submitted to 30 min global ischaemia, followed by a 120 min reperfusion period.

Results. Infarct-to-ventricle zone ratio was significantly reduced in ischaemic preconditioned (20.6 +/- 2.6%) compared to vehicle groups (44.5 +/- 4.3% and 51.3 +/- 2.5% in groups ethanol 5.10^{-7} and 2.10^{-9} M respectively). Pretreatment with both isoprostanes had no cardioprotective effect, the infarct-to-ventricle ratio were respectively 43.1 +/- 2.2%, 49.4 +/- 5.9% and 44.5 +/- 5.0% in groups treated with 15-F_{2t}-IsoP (3.10^{-10} M), 15-F_{2t}-IsoP (3.10^{-7} M) and 5-F_{2t}-IsoP (10^{-9} M).

Conclusion. These data provide evidence that the isoprostanes 15-F_{2t}-IsoP and 5-F_{2t}-IsoP are not implicated in early myocardial preconditioning at concentrations similar to those found in the human coronary sinus following coronary angioplasty.

KEY WORDS

Isoprostane

Lipid peroxidation

Myocardial ischaemia

Preconditioning

Discipline: experimental; **Object of study:** heart; **Level:** isolated organ; **Field of study:** pharmacology.

ABBREVIATIONS

5-F_{2t}-IsoP	5-F_{2t}-Isoprostane
15-F_{2t}-IsoP	15-F_{2t}-Isoprostane
CF	coronary flow
HR	heart rate
I	infarct size
K-H buffer	Krebs-Henseleit buffer
LVDP	left ventricular developed pressure
LVEDP	left ventricular end-diastolic pressure
TP-receptor,	thromboxane/PGH_2-receptor
V	ventricular area

INTRODUCTION

Isoprostanes are a family of compounds produced from arachidonic acid by a free-radical catalysed mechanism. Unlike prostaglandins, the non-enzymatic metabolism of arachidonic acid leads to a large number of isomeric isoprostanes that are esterified in cell membranes, cleaved presumably by phospholipase A_2, and released in free forms in the blood stream. Among these compounds, most attention has been focused on the 15- and the 5-series, which are the most abundant *in vivo* [1], are quantifiable in humans, and are currently used as a biomarker of lipid peroxidation [2]. These isoprostanes are released in response to an ischaemia-reperfusion sequence [3, 4]. Some 15-series F_2-isoprostanes are biologically active and mediate vasoconstriction associated with an endothelium-dependant relaxation, mediated by nitric oxide release [5], whereas preliminary reports show that the 5-series do not possess any biological activity, at least in vessels [6]. A currently unresolved key question is whether the isoprostanes that are liberated following myocardial ischaemia-reperfusion play a pathogenic role in myocardial injury. Recent reports have shown that coronary angioplasty in humans induces coronary sinus increase in both 15-F_{2t}-IsoP and 5-F_{2t}-IsoP formation [7, 8]. Whether this formation is clinically relevant remains controversial [9].

Although oxygen free radicals contribute to myocardial reperfusion injury, they also trigger myocardial preconditioning [10-12]. Which mediators are implicated in this mechanism remains under debate. In addition, it has been suggested that phospholipase A_2 derived lipid second messengers are involved in myocardial ischaemic preconditioning [13]. Considering these data, the F_2-isoprostane, free radical-derived metabolites of arachidonic acid that are released following ischaemia-reperfusion, could be candidate molecules for myocardial preconditioning.

Therefore, this study was performed in order to evaluate the ability of the isoprostanes 15-F_{2t}-IsoP and 5-F_{2t}-IsoP to reduce myocardial ischaemic injury in isolated rat heart.

MATERIALS AND METHODS

Isolated preparations

In accordance with the French law and the local ethical committee guidelines for animal research, male Wistar rats (280-300 g IFFA CREDO, Lyon, France) were housed in climate controlled conditions and provided with standard rat chow. Animals were anaesthetized with an intraperitoneal injection of 60 mg kg^{-1} sodium pentobarbital (Sanofi, Libourne, France). Heparin (500 U kg^{-1}, Sanofi Winthrop, Gentilly, France) was injected intravenously. Then, hearts were rapidly excised and immediately immersed in 4°C Krebs-Henseleit (K-H) buffer solution (NaCl 118, KCl 4.7, $CaCl_2$ 1.8, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.2 and glucose 11 mM). The aortic stumps were then cannulated and hearts were perfused retrogradely using the Langendorff technique at a constant pressure (75 mm Hg) with oxygenated K-H buffer.

A water filled balloon (Hugo Sachs, N° 4), coupled to a pressure transducer, was inserted in the left ventricular cavity *via* the left atrium for pressure recording. Left ventricular end-diastolic pressure (LVEDP) was adjusted to 5 mmHg. Myocardial temperature was measured by a thermoprobe inserted into the left ventricle and was maintained constant close to 37°C. Coronary flow (CF) was measured throughout the experiment, by collecting the effluent. Heart rate (HR) and left ventricular developed pressure (LVDP=difference between left ventricular systolic pressure and LVEDP) were continuously recorded (8 channels, MacLab ADInstruments). All drugs were administered through a Y connector in the aortic cannula.

Experimental treatment groups

The rats were assigned randomly to one of 6 groups: groups I and II: vehicle-pretreated groups (i.e. pretreatment with the vehicle ethanol at concentrations equivalent to those in the isoprostane groups), group III: ischaemic-preconditioned control group, and groups IV to VI: pretreatment with isoprostanes. In all groups, hearts were submitted to a 30 min stabilization period, followed by 30 min of global ischaemia before a 120 min reperfusion period. *Group I and II* (vehicle, n=6) hearts were perfused with ethanol (at concentrations equivalent to those in the group IV and VI, 5.10^{-7} and 2.10^{-9} M respectively) for 5 min followed by a 5 min wash-out period before ischaemia; *Group III* (ischaemic preconditioning, n=8) hearts were submitted to 5 min global ischaemia followed by 5 min reperfusion with K-H solution before the sustained ischaemia; *Group IV and V* (15-F_{2t}-IsoP, n=8) hearts were perfused with 15-F_{2t}-IsoP (3.10^{-10} and 3.10^{-7} M) for 5 min followed by a 5 min wash-out period before ischaemia; *Group VI* (5-F_{2t}-IsoP, n=8) hearts were perfused with 5-F_{2t}-IsoP (10^{-9} M) for 5 min followed by a 5 min wash-out period before ischaemia.

The experimental protocol is summarised in Figure 1.

Measurement of infarct size

At the end of the ischaemia-reperfusion protocol, atria were removed and the heart was frozen at -80°C for 10 min. It was then cut into 2 mm transverse sections from apex to base (6-7 slices/heart).

Once thawed, the slices were incubated at 37°C with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) for 10 min and fixed in 10% formaldehyde solution to distinguish the clearly stained viable tissue from unstained necrotic tissue. Infarct size (I) was determined using a

computerized planimetric technique (Scion image for Windows) and expressed as the percentage of the ventricular area (V).

Drugs

15-F_{2t}-IsoP (8-iso-prostaglandin $F_{2\alpha}$) was purchased from Cayman (Ann Arbor, USA). 5-F_{2t}-IsoP was synthesised according to our procedure [14]. In this study, all isoprostanes were dissolved in ethanol at 10^{-2} M. Stock solutions were then diluted in K-H solution before being added to the organ baths.

The long-term stability of 5-F_{2t}-isoP in ethanol or methanol as well as the short term stability in Krebs'solution was previously checked [6].

Statistical analysis

Data are presented as mean ± s.e.mean. Infarct size values were compared using one-way ANOVA. Haemodynamic data were analysed using a two-way ANOVA, with *post-hoc* multiple comparison Tuckey test. *P* values < 0.05 were considered significant.

RESULTS

Myocardial function

Pre-ischaemic haemodynamic data are given in Table 1. 15-F_{2t}-IsoP perfusion (3.10^{-7} and 3.10^{-9} M) significantly decreased the coronary flow without affecting heart rate, LVDP and dP/dt.

Myocardial infarction

Figure 2 shows myocardial infarct size, expressed as the percentage of the ventricular (V) area in the different groups. The infarct size was smaller in the ischaemic preconditioned group, compared with that in hearts from other groups, whereas isoprostane pretreatment had no effect.

Functional recovery

As shown in Figure 3, a 30-min global ischaemia was accompanied by a marked reduction in dP/dt in all hearts. Most hearts from vehicle groups (I and II) did not recover during the reperfusion period. In contrast, functional ventricular recovery was greatly improved in hearts from the ischaemic preconditioning group (group III)(*P<0.05 vs the other groups (one way ANOVA)). In hearts treated with isoprostanes, the functional recovery did not differ from the vehicle groups.

DISCUSSION

This study demonstrates that the isoprostanes 15-F_{2t}-IsoP and 5-F_{2t}-IsoP, that are liberated following myocardial reperfusion, are not implicated in early myocardial preconditioning.

F_2-isoprostanes are metabolites of arachidonic acid through a free radical dependent mechanism. These isoprostanes are released in a large number of vascular diseases including unstable angina [15], coronary angioplasty [3, 7, 8] and reperfusion following myocardial infarction [3, 4]. A key question that remains unresolved is whether isoprostanes play a pathogenic role in such pathological conditions. Considering that other vasoconstrictors such as cathecolamines, angiotensin II and endothelin-1 trigger myocardial preconditioning [16-19], we tested the effect of a pretreatment with two F_2-isoprostane isomers on the myocardial infarct size following global ischaemia. The choice of the concentrations used was based on a recent study where both 15-F_{2t}-IsoP and 5-F_{2t}-IsoP levels were markedly increased in the coronary sinus following coronary angioplasty in humans [7]. At baseline, the concentrations of 15-F_{2t}-IsoP and 5-F_{2t}-IsoP in the left main coronary artery were similar to those in the coronary sinus. After coronary angioplasty, the concentrations in the coronary sinus were 0.35 and 0.83 nmol/l for 15-F_{2t}-IsoP and 5-F_{2t}-IsoP respectively [7]. Based on these data, we chose to test the properties of these compounds at similar concentrations (0.5 and 1 nmol/l respectively). Iuliano's report is the only study that investigated local concentrations following an ischaemia-reperfusion sequence in humans. It is possible that local concentrations in the coronary microvessels located in the myocardial ischaemia area are even more elevated. However, no study confirms this hypothesis. Furthermore, preconditionning is not limited to the infarct area. In line with these data, the choice of other 15-F_{2t}-IsoP and 5-F_{2t}-IsoP concentrations would have been very speculative and questionable.

15-F_{2t}-IsoP is a vasoconstrictor of pig and cow coronary arteries, with an EC_{50} value equivalent to 0.7 and 1.3 µM respectively [20]. In guinea pig isolated heart, the EC_{50} value 15-F_{2t}-IsoP on the coronary flow was 0.1 µM [21]. Although no data are available for human coronary arteries, the EC_{50} value has been shown to be 0.5 µM in human internal mammary arteries [22]. Therefore, we also chose to test the potential preconditioning effect of 15-F_{2t}-IsoP at a concentration (0.3 µM) close to which induces vasoconstriction *in vitro*. No further 5-F_{2t}-IsoP concentration was tested because unlike 15-F_{2t}-IsoP, 5-F_{2t}-IsoP does not possess any vasomotor activity in rat or human vessels [6]. The isoprostanes used in the present study were dissolved in ethanol. In order to ensure that the vehicle, ethanol, was not implicated in any potential preconditioning effect, we tested the effect of ethanol at two concentrations, so as to rule out this potential interference.

15-F_{2t}-IsoP is a vasoconstrictor in most species and vascular beds [5]. However, data on the vasoconstrictor effect of 15-F_{2t}-IsoP on coronary arteries are conflicting. 15-F_{2t}-IsoP induces a dose-dependent coronary artery vasoconstriction in guinea pig [21], cow [20]. In contrast, no effect was observed in sheep [20] and rat in basal conditions [23]. Data on the vascular effect of 15-F_{2t}-IsoP in pig are conflicting as a dose-dependent coronary artery vasoconstriction was found by Kromer [20] but not by [24]. However, in the latter study, a dose-dependent vasoconstriction was found in pigs following experimental hypercholesterolemia. Similarly, 15-F_{2t}-IsoP caused a dose-dependent drop in rat coronary flow following an ischemia-reperfusion procedure as well as a xanthine/xanthine oxidase perfusion [23]. In summary, it appears that 15-F_{2t}-IsoP coronary vasoconstriction is highly dependent both on the species and the basal conditions. In some species, the vasoconstriction is increased in pathological conditions including ischemia-reperfusion. With respect to these data, our study found that 15-F_{2t}-IsoP induced a weak but

significant drop in coronary flow, revealing a moderate coronary microvessels vasoconstriction. Our study was not aimed at testing a putative dose-dependent effect. The lower dose probably induced the weak maximal contraction observed, explaining the lack of difference between the two doses. In accordance with this hypothesis, 15-F_{2t}-IsoP was shown to be more potent on microvessels than conductance vessels [5].

In contrast, 5-F_{2t}-IsoP had no effect, further supporting the previous data showing that the 5-series isoprostanes, which are the most abundant in human plasma and urine, have no vascular effects [6].

Our data were obtained from an animal model of myocardial ischaemia-reperfusion, and should be extrapolated to humans with caution. However, they provide evidence that the isoprostanes 15-F_{2t}-IsoP and 5-F_{2t}-IsoP, at concentrations similar to those found in the human coronary sinus following coronary angioplasty, are not implicated in the early myocardial preconditioning.

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Table 1. Pre-ischaemic haemodynamic values in the different experimental groups

	Groups	*n*	*Stabilisation*	*Following 5 min treatment*	*Following 5 min wash-out*
CF (ml min^{-1})	**I**	6	13.7 ± 0.2	13.4 ± 0.2	12.8 ± 0.4
	II	6	14.7 ± 0.8	14.7 ± 0.7	14.6 ± 0.8
	III	8	14.1 ± 0.6	-	13.7 ± 0.7
	IV	8	16.0 ± 0.6	14.2 ± 0.7*	14.5 ± 0.7
	V	8	14.6 ± 0.3	13.4 ± 0.4*	13.7 ± 0.4
	VI	8	15.2 ± 0.6	15.4 ± 0.6	15.4 ± 0.5
HR (bpm)	**I**	6	271 ± 14	262 ± 18	268 ± 18
	II	6	273 ± 7	273 ± 16	262 ± 14
	III	8	262 ± 15	-	247 ± 10
	IV	8	280 ± 10	274 ± 11	290 ± 9
	V	8	282 ± 10	290 ± 7	277 ± 9
	VI	8	276 ± 10	274 ± 7	277 ± 11
LVDP (mmHg)	**I**	6	111 ± 5	107 ± 12	105 ± 8
	II	6	108 ± 10	101 ± 9	98 ± 8
	III	8	102 ± 4	-	95 ± 4
	IV	8	110 ± 6	101 ± 4	99 ± 6
	V	8	106 ± 5	106 ± 6	99 ± 4
	VI	8	112 ± 7	111 ± 9	115 ± 7

Data are mean ± s.e. mean. **P* < 0.05 *vs* Stabilisation.

CF – coronary flow; HR – heart rate; LVDP – left ventricular developed pressure.

I – Vehicle: ethanol 5.10^{-7} M pretreated hearts; **II** – Vehicle: ethanol 2.10^{-9} M pretreated hearts; **III** – ischaemic preconditioning pretreated hearts; **IV** - 15-F_{2t}-IsoP 3.10^{-10} M pretreated hearts; **V** - 15-F_{2t}-IsoP 3.10^{-7} M pretreated hearts; **VI** - 5-F_{2t}-IsoP 10^{-9} M pretreated hearts.

FIGURE LEGENDS

Figure 1. Experimental protocol.

Group I and II - hearts were perfused with ethanol (at concentrations equivalent to those in the group IV and VI, 5.10^{-7} and 2.10^{-9} M respectively) for 5 min followed by a 5 min wash-out period before ischaemia; *Group III* – hearts were submitted to a 5 min global ischaemia-5 min reperfusion period (Ischaemic Preconditioning) before the sustained ischaemia; *Group IV and V* - hearts were perfused with 15-F_{2t}-IsoP (3.10^{-10} and 3.10^{-7} M) for 5 min followed by a 5 min wash-out period before ischaemia; *Group VI* - hearts were perfused with 5-F_{2t}-IsoP (10^{-9} M) for 5 min followed by a 5 min wash-out period before ischaemia.

Stab - stabilisation; O - occlusion; R - reperfusion; T - treatment.

Figure 2. Infarct size is expressed as a percentage of ventricular area (I/V) assessed following global ischaemia (30 min) in the six groups. Drug perfusions are initiated 10 min before ischaemia, maintained during 5 min and followed by 5 min washing period. Open circles represent individual hearts, whereas solid circles represent mean ± s.e.mean. **P* < 0.001 vs group I and II (one way ANOVA).

Figure 3. Functional recovery assessed by the percentage of maximum dP/dt from the pre-ischaemic value, during ischaemia (30 min)-reperfusion (120 min) sequence. **P*<0.05 vs other groups (one way ANOVA).

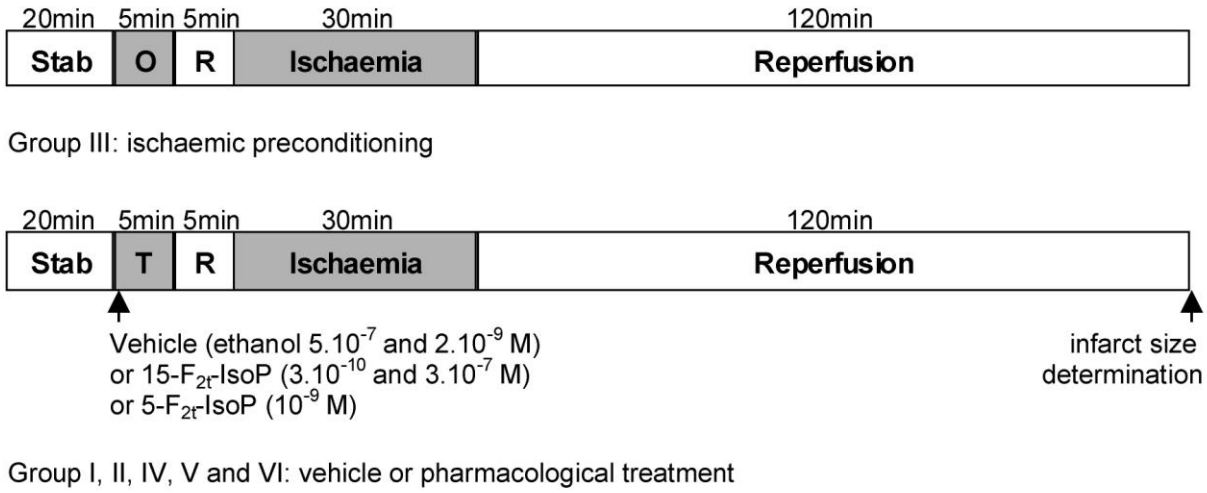
20min
5min
5min
30min
120min
Stab
O
R
Ischaemia
Reperfusion
Group III: ischaemic preconditioning
20min
5min
5min
30min
120min
Stab
T
R
Ischaemia
Reperfusion
Vehicle (ethanol 5.10-7 and 2.10-9 M)
or 15-F2t-IsoP (3.10-10 and 3.10-7 M)
or 5-F2t-IsoP (10-9 M)
infarct size
determination
Group I, II, IV, V and VI: vehicle or pharmacological treatment

Figure 1.

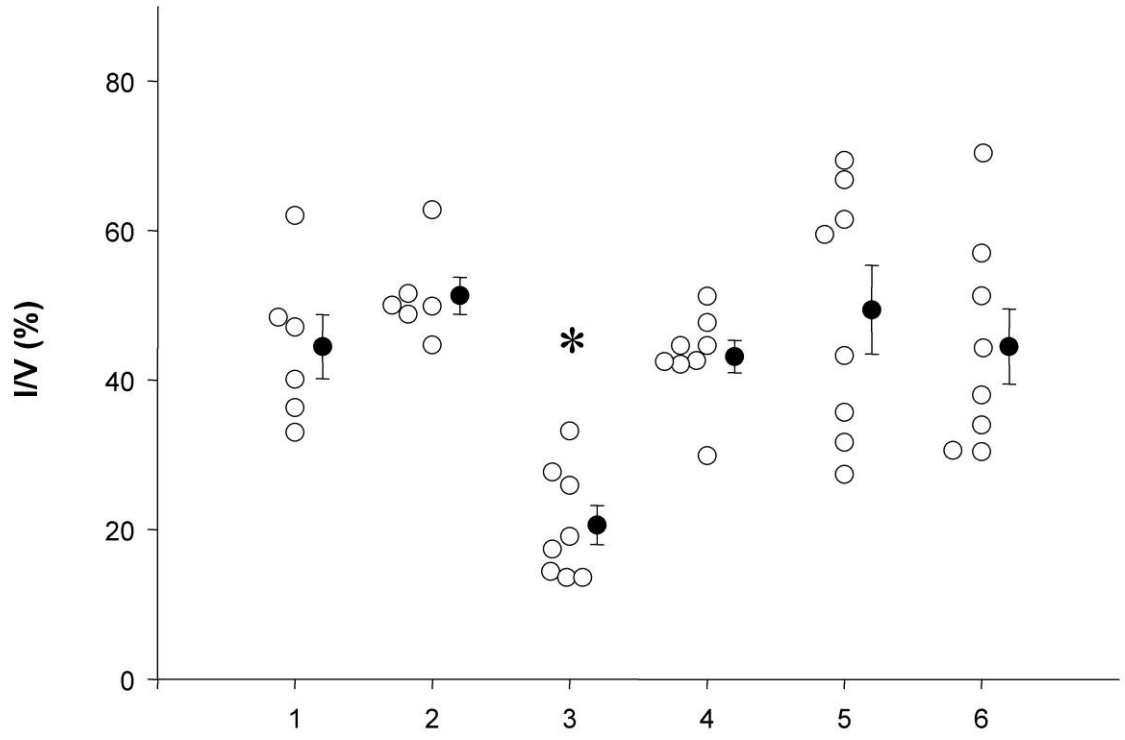

Figure 2.

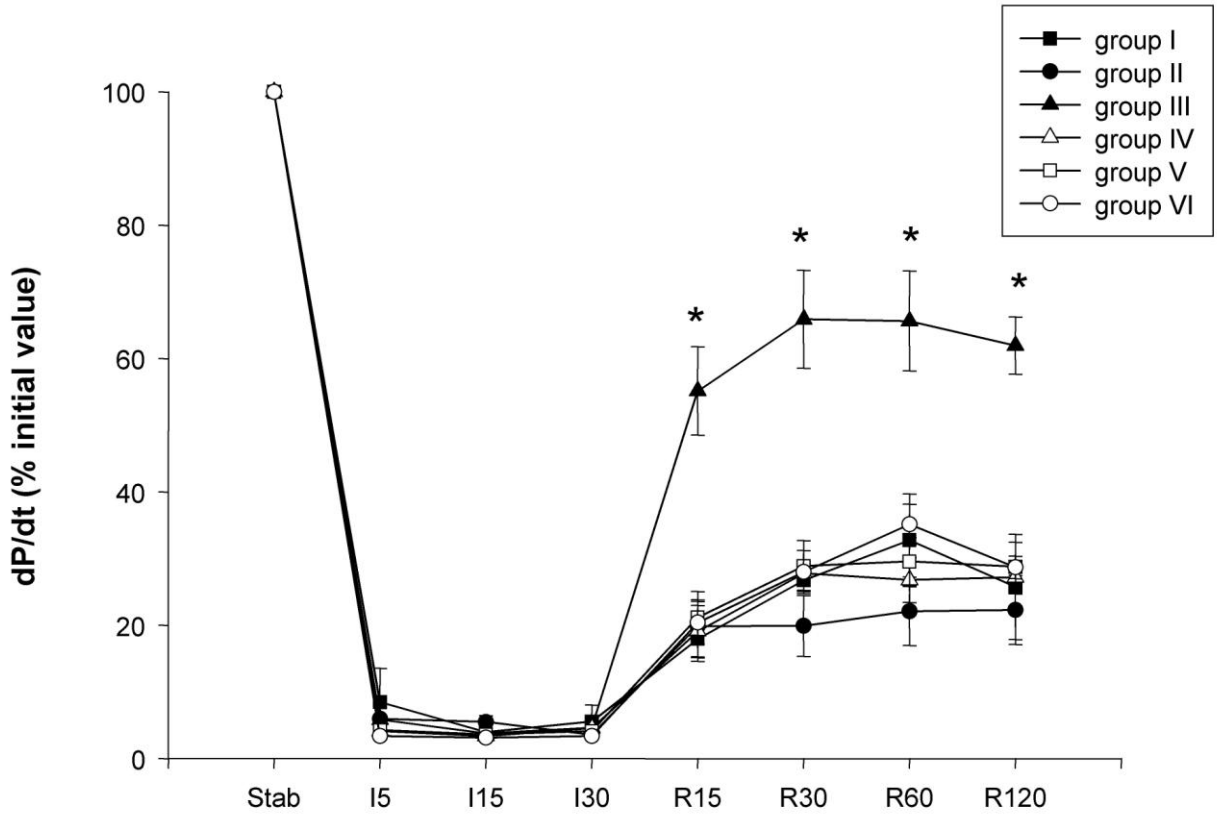
group I
group II
group III
group IV
group V
group VI
dP/dt (% initial value)
100
80
60
40
20
0
Stab
I5
I15
I30
R15
R30
R60
R120
*
*
*
*

Figure 3.