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Myocardial Pharmacological Preconditioning by Norepinephrine in Rabbit. A Role for Beta-3 Adrenergic Agonists?

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Abstract: High doses of norepinephrine abolish the infarct-limiting effect of Ischemic Preconditioning (IP) in rabbits. We wished to define the role of the beta-3 receptors in this observation. Rabbits were randomized into 5 groups of 8 animals each. In Control Group (CTRL), anaesthetized rabbits were subjected to 30 min left coronary marginal branch occlusion and 180 min reperfusion. In NEHI group, rabbits received a 10 μg kg^{-1} norepinephrine injection 10 min before coronary occlusion and reperfusion. In NELOW group, rabbits received 0.25 μg/kg/min of norepinephrine for 5 min and then had 10 min of washout before coronary occlusion and reperfusion. In NELOWBRL group, rabbits had BRL37344 (8.3 μg kg^{-1}) injection 1 min prior to a norepinephrine perfusion comparable to NELOW group, followed by ischemia and reperfusion. In BRL group, rabbits had BRL37344 (8.3 μg kg^{-1}) injection 10 min before coronary occlusion and reperfusion. At termination of the experiment, Left Ventricular Volume (LVV), myocardial Volume At Risk (VAR) and Infarct Volume (IV) were determined with methylene blue and tetrazolium staining and measured using planimetry. LVV was not significantly different among groups. Myocardial VAR/LVV was not significantly different between groups (CTRL, 13.52±5.17%; NEHI, 12.11±4.23%; NELOW, 12.45±11.35%; NELOWBRL, 14.71±4.74; BRL, 8.39±4.60; p = ns). IV/VAR was significantly reduced in the NELOW group as compared with CTRL, NEHI, NELOWBRL and BRL (respectively, 16.52±5.50% vs 56.42±14.40%, 55.71±8.38%, 50.95±11.62%, 50.18±10.20, $p<0.001$). There was no significant difference in IV/VAR between CTRL, NEHI, NELOWBRL and BRL. The absence of a preconditioning protective effect during a massive injection of norepinephrine could be in relation with a beta-3 stimulation that interferes with the preconditioning pathways.

Key words: Preconditioning, norepinephrine, beta-3, myocardium, LVV, CTRL

INTRODUCTION

Ischemic Preconditioning (IP) has been described as a rapid, adaptive response of myocardium to a brief ischemic insult, slowing down the rate of cell death during a subsequent period of prolonged ischemia (Murry *et al*., 1986). This concept has since been extended to include the protection against other deleterious effects of the ischemia-reperfusion sequence and to also include different pharmacological triggers that induce preconditioning such as k-ATP channels openers, bradykinine, opioïds and catecholamines (Murry *et al*., 1994). Low doses of norepinephrine have been proposed as a putative mediator of preconditioning through α or β receptors (Bankwala *et al*., 1994).

Kirsch *et al*. (2000) have described that the cardioprotective effect of IP can be altered or even abolished after acute brain death in rabbit compared to a same sequence of IP in anesthetized animals. This observation can be in relation with the massive release of norepinephrine occurring during brain death (Farhat *et al*., 2001). Since low doses of exogenous norepinephrine can mediate preconditioning, whereas high doses interfere in the IP pathways, leading to negative effects upon cardiac protection, we hypothesized that norepinephrine can act at different concentrations through different mediators for contradictory results. It have been reported that high doses of norepinephrine stimulate the β-3 cellular receptors in addition to β-1 and β-2 (Gauthier *et al*., 2000a, b). Thus, we wished to explore the β-3 myocardial receptors that to establish their implication in the absence of preconditioning after injection of high doses of norepinephrine. We studied the action of norepinephrine upon the reduction of infarct size in an experimental model

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of regional ischemia in rabbit heart. We have voluntarily restricted our study to the infarct-limiting effect of norepinephrine since other end-points such as reduction of post-ischemic contractile dysfunction, dysrhythmias or endothelial dysfunction may not be manifestations of preconditioning as originally defined.

MATERIALS AND METHODS

Animals: Adult New Zealand white rabbits weighing from 2.0 to 2.5 kg were used. All animals received human care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

General surgical preparation: Rabbits were premedicated with 0.25 mL kg^{-1} intramuscular 2% xylasine (Rompun®). Venous access was obtained through a 24-Gauge IV catheter placed in a marginal ear vein. Anesthesia was induced and maintained using intravenous nesdonal (20 mg kg^{-1} and 20 mg/kg/h, respectively). A continuous perfusion of hydroxyethyl amidon (6%, 15 mL h^{-1}) was started and maintained all through the experiment. After general heparinization (500 UI kg^{-1}), animals were ventilated through a tracheotomy with an endotracheal tube at a rate of 40 strokes per min and 40% oxygen fraction. The expired partial O_2 and CO_2 were measured after equilibration. Body temperature was maintained at 38°C throughout the experiments by a heating lamp and measured by an intrarectal temperature catheter. A 20-Gauge catheter was placed in the right carotid artery for continuous monitoring of systemic Mean Arterial Pressure (MAP), Heart Rate (HR) and blood sample withdrawal.

Experimental protocol: The experimental protocol is summarized in Fig. 1. Rabbits were randomized into five experimental groups of eight animals each. In control group (CTRL), anesthetized rabbits were subjected to 30 min of coronary occlusion and 180 min of reperfusion without any pretreatment. In NEHI group, anesthetized rabbits were subject to an intra-venous bolus of 10 μg kg^{-1} of norepinephrine 10 min before the 30 min of coronary occlusion followed by the 180 min of reperfusion. In NELOW group, anesthetized rabbits were subject to a continuous intravenous perfusion of 0.25 μg/Kg/min of norepinephrine for 5 min before 10 min of washout (Bankwala *et al*., 1994). This sequence was followed by the 30 min of coronary occlusion then the 180 min of reperfusion periods. In NELOWBRL, anesthetized rabbits had 8.3 μg kg^{-1} IV injection of

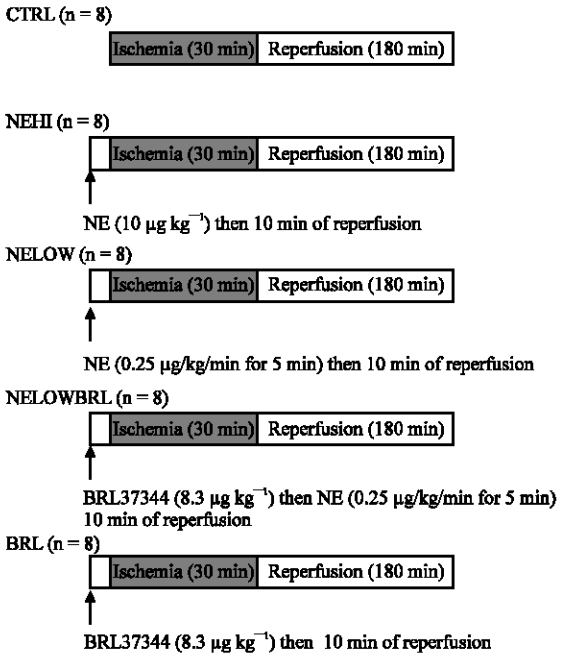

Fig. 1: Schematic of experimental protocol. The norepinephrine IV and the BRL37344 injections were followed by 10 min of washout before coronary occlusion and reperfusion. CTRL, control group; NEHI, norepinephrine high dose group; NELOW, norepinephrine low dose group; NELOWBRL, norepinephrine low dose and BRL37344 group; BRL, BRL37344 alone group

BRL37344, a beta-3 receptors agonist (Shen *et al*., 1994) (Laboratoire Sigma Aldrich, St Quentin Fallavier, France) 1 min prior to the start of the norepinephrine perfusion as described in NELOW group, followed by the same sequences. In BRL group, anesthetized rabbits had an IV injection of 8.3 μg kg^{-1} of BRL37344, 10 min prior to the 30 min of coronary occlusion and the 180 min of reperfusion periods. We didn't use any ischemic preconditioning in any group.

Blood sampling: Arterial blood samples for catecholamine level determination were taken at baseline, 1 min after norepinephrine injection in NEHI, at the end of the 5 min norepinephrine perfusion in NELOW and NELOWBRL and 1 min after the BRL37344 injection in BRL (End NE) and at the end of the reperfusion period. Blood samples were centrifuged in a cooling (4°C) centrifuge at 3000 rpm for 15 min. Plasma was then removed and stored at -80°C until analysis. Plasma catecholamines norepinephrine and epinephrine were analyzed by high-performance liquid chromatography with electrochemical (coulometric) detection (Revol *et al*., 1994).

Ischemia-reperfusion injury: After induction of anesthesia, the heart was exposed by means of a thoracotomy in the fourth left intercostal space and the pericardium was opened. A prominent marginal branch of the circumflex coronary artery was identified. A 5-0 polypropylene suture was passed around the vessel and the ends of the suture were threaded through a small vinyl tube to make a snare. The coronary branch was occluded by pulling the snare, which was then fixed by clamping the tube. Myocardial ischemia was confirmed by regional cyanosis of the myocardial surface. Thereafter, the coronary branch was occluded for 30 min and then reperfused for 180 min. Hemodynamic measures were recorded after BRL37344 and norepinephrine injection and all through the experimentation.

Determination of myocardial infarct size: After the reperfusion period, the coronary branch was re-occluded with the snare and the rabbit was perfused in intravenous with 5 mL of a solution containing 1% methylene blue. The anatomic area at risk was demarcated by the absence of methylene blue dye. This area at risk represents the region perfused by the concerned coronary branch and that subsequently necroses after coronary occlusion. The heart was then rapidly explanted and rinsed in saline solution. The left ventricle was dissected free from aorta, pulmonary artery, atria and right ventricle and cut transversely into approximately 2 mm thick slices. Each slice was stained by incubation for 20 min at 37°C in 50 mL of phosphate-buffered 1% Triphenyl Tetrazolium Chloride (TTC, Laboratoire Sigma Aldrich, St Quentin Fallavier, France). TTC staining has been shown to demarcate viable tissue by reacting with myocardial dehydrogenase enzymes to form a brick red stain (Fishbein *et al*., 1981). TTC negative regions, representing irreversibly injured myocardium, appeared as pale yellow. The heart slices were conserved for 24 h in a 10% formaldehyde solution and then frozen for one week to enhance the contrast between the necrotic and the non necrotic area (Farhat *et al*., 2001). Thereafter, they were mounted in a glass press, which compressed the slices into uniform 2 mm thickness, for examination. Areas of infarct and risk zone were determined by computer-assisted planimetry. Infarct and risk zone volumes were then calculated by multiplying each area by the slice thickness and summing the products.

Statistical analysis: All results were expressed as mean±standard deviation. Intragroup haemodynamic and hormonal changes were assessed by one-way analysis of variance for repeated measures followed by Dunnet's test. Histological results were analyzed by one way ANOVA followed by Tukey's post-hoc correction for multiple comparisons. A p value of less than 0.05 was considered significant. All statistical procedures were performed by use of Prism 3.0 software (GraphPad Software, Inc. San Diego, USA).

RESULTS

Hemodynamic data: Evolution of Mean Arterial Blood Pressure (MAP) and Heart Rate (HR) are shown in Table 1. Rabbits, in CTRL and NELOW groups, exhibited a moderate reduction in MAP during the study period. In contrast, the norepinephrine bolus in the NEHI group induced an immediate and significant increase in MAP, followed after 2 to 3 min by a rapid decrease and a return to the level of baseline values until the end of the reperfusion period. In NELOWBRL and BRL groups, a sustained and significant drop of the MAP was observed until the end of the experiment. No statistical differences were noted in HR in the different group through the examination period.

Table 1: Evolution of heart rate during experimentation

Group	Baseline	End BRL	End NE	Ischemia (30 min)	Reperfusion (60 min)	Reperfusion (120 min)	Reperfusion (180 min)
CTRL							
HR	227±17	-	-	203±22	197±33	198±32	193±39
MAP	82±23	-	-	69±28	67±31	72±37	63±29*
NEHI							
HR	254±40	-	225±50	241±44	228±45	226±44	220±44
MAP	65±12	-	138±9*	53±13	60±17	60±21	45±14*
NELOW							
HR	223±26	-	209±67	212±23	235±41	236±49	210±49
MAP	66±22	-	74±27	50±8	48±14	50±14	44±15*
NELOWBRL							
HR	240±43	232±57	220±44	249±42	224±23	235±29	240±30
MAP	73±12	60±14	70±14	40±14*	40±15*	40±15*	44±14*
BRL							
HR	245±12	253±27	-	227±31	225±34	247±29	230±47
MAP	69±15	47±9*	-	50±7*	52±11	43±21*	45±24*

All data are mean±standard deviation, CTRL, Control group; NEHI, Norepinephrine high dose; NELOW, Norepinephrine Low Dose; NELOWBRL, Norepinephrine Low dose and BRL37344; BRL, BRL37344 alone; End BRL, 1 min after the BRL37344 injection in NELOWBRL and BRL groups; End NE, end of norepinephrine perfusion in NELOW and NELOWBRL, 1 min after norepinephrine bolus in NEHI; HR, heart rate, MAP: Mean Arterial Pressure (in mmHg), * $p<0.05$ vs baseline by ANOVA for repeated measures

Table 2: Plasmatic norepinephrine and epinephrine levels through the experimental protocol

Group	Baseline	End NE	End reperfusion
CTRL			
Norepinephrine	365±253	-	1106±1522
Epinephrine	45±20	-	827±1503
NEHI			
Norepinephrine	112±72	24420±22390*	1026±859
Epinephrine	30±16	55±60	780±1745
NELOW			
Norepinephrine	178±127	965±651	300±225
Epinephrine	36±16	226±553	304±534
NELOWBRL			
Norepinephrine	36±14	620±366	935±1057*
Epinephrine	24±22	25±26	1863±4186
BRL			
Norepinephrine	52±20	163±313	1309±1114*
Epinephrine	38±19	117±253	953±1273

All data are mean±standard deviation and expressed in picogram per milliliter (pg mL^{-1}), CTRL, Control group; NEHI, Norepinephrine High-dose group; NELOW, Norepinephrine Low-dose group; NELOWBRL, Norepinephrine Low-dose and BRL37344 group; BRL, BRL37344 alone group; End NE, end of norepinephrine perfusion in NELOW and NELOWBRL groups, 1 min after norepinephrine bolus in NEHI group and 1 min after BRL37344 injection in BRL group.* $p<0.05$ vs baseline by ANOVA for repeated measures

Plasma catecholamines: Plasma catecholamines levels remained stable throughout the study period in rabbits of the CTRL and NELOW groups (Table 2). In contrast, the norepinephrine plasmatic increased significantly 1 min after bolus in NEHI group and returned to baseline at the end of the experimentation. In BRL and NELOWBRL groups, the norepinephrine levels remained comparable to baseline after the end of drug infusion, but were significantly higher at the end of the reperfusion period ($p<0.05$). There were no significant changes in plasmatic epinephrine levels during the study period in these 5 groups.

Infarct size: The effects of coronary occlusion on infarct size are shown in Fig. 2. Infarct volume, expressed as a percentage of volume at risk, was significantly reduced in the NELOW group as compared to CTRL, NEHI, NELOWBRL and BRL groups (respectively, 16.52±5.50% vs 56.42±14.40%, 55.71±8.38%, 50.95±11.62% and 50.18±10.20%, $p<0.001$). There was no significant difference in infarct volume, expressed as a percentage of volume at risk, between the CTRL, the NEHI, the NELOWBRL and the BRL groups (p = ns). Similarly, infarct volume, expressed as a percentage of left ventricular volume, was significantly reduced in the NELOW group as compared with the CTRL, NEHI, NELOWBRL and BRL groups (respectively, 2.27±2.04% vs 7.68±3.15%, 7.38±3.16%, 7.24±2.17% and 4.29±2.44, $p<0.01$). There was no significant difference in infarct volume, expressed as a percentage of left ventricular volume, between the CTRL, the NEHI, the NELOWBRL and the BRL groups (p = ns). The left ventricular volume

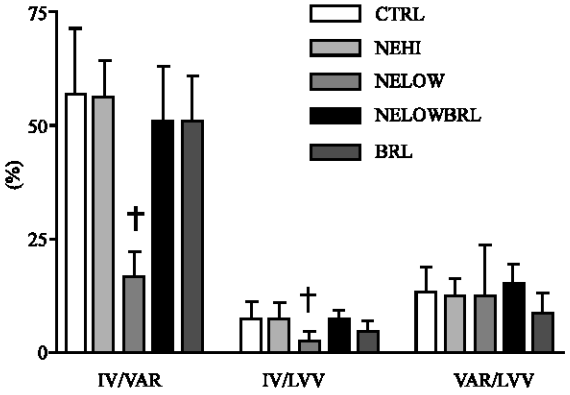

Fig. 2: Bar graph comparing the effects of ischemia on myocardial infarct volume. All hearts underwent 30 min of regional ischemia followed by 180 min of reperfusion. Infarct volume was significantly reduced in NELOW group, but not in NEHI, NELOWBRL and BRL groups, in comparison to CTRL. Volume at risk of infarction was not significantly different among the four groups. Data are expressed as mean±standard deviation.† $p<0.001$ in NELOW vs CTRL, NEHI, NELOWBRL and BRL groups. IV, infarct volume; VAR, volume at risk; LVV, left ventricular volume

was not significantly different among the five experimental groups (CTRL, 3.73±0.44 cm^3; NEHI, 3.27±0.48 cm^3; NELOW, 3.22±0.47 cm^3; NELOWBRL, 3.28±0.39 cm^3; BRL, 3.24±0.13 cm^3, p = ns). Furthermore, the myocardial volume at risk, expressed as a percentage of left ventricular volume, was not significantly different between groups (CTRL, 13.52±5.17%; NEHI, 12.11±4.23%; NELOW, 12.45±11.35%; NELOWBRL, 14.71±4.74; BRL, 8.39±4.60, p = ns).

DISCUSSION

The results of the present study suggest that exogenous norepinephrine can mimic the cardioprotective effects of an Ischemic Preconditioning (IP) in rabbit when used in low doses, but seems ineffective in case of association with a selective beta-3 adrenergic agonist. Furthermore, a severe overload of the myocardium by high doses of norepinephrine does not procure protection against ischemic injury and elective beta-3 stimulation does not enhance myocardial ischemic lesions or procure cardioprotection by the mean of a peripheral vasodilatation.

Although the mechanisms underlying the cellular protection procured by myocardial preconditioning are not completely clear, there are some redundant

evidences that they are due to receptor-mediated effect (Kloner *et al*., 1998; Yanaguchi *et al*., 1997). Different pharmacological triggers can induce preconditioning such as k-ATP channels openers, bradykinine, opioïds and catecholamines(Murry *et al*., 1994; Bankwala *et al*., 1994). Low doses of norepinephrine have been proposed as a putative mediator of preconditioning through α or β receptors (Bankwala *et al*., 1994). Furthermore, previous selective α1-adrenergic blockade completely eliminates the effects of preconditioning (Toombs *et al*., 1993). Norepinephrine acts by activating the intracellular protein kinase C which has a major implication in the preconditioning pathways (Tsuchida *et al*., 1994). We have recently reported that the loss of the cytoprotective effects of IP after brain death in rabbit heart is probably related to the massive release of norepinephrine that occurs due to brain death induction (Farhat *et al*., 2001). De Zeeuw *et al*. (2001) have reported that the cerebral ischemia-induced release of norepinephrine does not induce cardioprotection by the mean of a pharmacological preconditioning. Thus, norepinephrine seems to act at different concentrations with different results upon myocardial protection. At this point, several points are to underscore:

- Low doses of norepinephrine can provide an IP-like cardioprotection.
- A massive exogenous infusion of norepinephrine does not provide cardioprotection against ischemia.
- A massive endogenous release of norepinephrine accompanies brain death induction.
- Brain death promotes severe myocardial injuries and subsequently heart failure.
- Beta-3 adrenergic receptors are over expressed in failing heart.
- A massive dose of norepinephrine stimulates beta-3 adrenergic receptors in addition to beta-1 and 2.

In this study, major hemodynamic modifications were observed in the NEHI group. Comparably to what has been previously reported in brain death models, we could distinguish two phases (Novitzky *et al*., 1984). The first phase followed the norepinephrine injection, lasting about 5 min, was characterized by a significant increase of the Mean Arterial blood Pressure (MAP) but with no consequence upon the heart rate. The second phase showed a sustained drop of the MAP through the rest of the experimentation. These observations coincide with the evolution of the plasmatic norepinephrine levels. The massive overload of norepinephrine results in increased cardiac work and vascular resistance, in association to an inflow of calcium into myocardial cells (Rona *et al*., 1985; Bittner *et al*., 1995). This subsequently activates cellular enzymes, especially nitric oxide synthetase and enhances mitochondrial damage (Rona *et al*., 1985; Bittner *et al*., 1995; Pratschke *et al*., 1999; Kitakaze *et al*., 1994). Burch *et al*. (1967) showed necrotic zones in mice myocardium after cerebral hemorrhage, relating them to the massive release of norepinephrine. These lesions could be prevented by a previous injection of reserpine. In the CTRL and NELOW groups, the MAP fell slowly all through the experimentation. In NELOWBRL and BRL groups, a significant drop of MAP was observed after the beginning of the ischemic period and until the end of the experimentation. This could be in relation with the vasodilatation consequence to the β_3 stimulation by BRL37344 (Shen *et al*., 1994). Nevertheless, we did not notice compensatory tachycardia in this group. Second, there was no difference in MAP comparatively between NELOW and NELOWBRL groups, with meanwhile a difference upon the infarct reduction effect of norepinephrine favorable to NELOW. For this reason, we can hypothesize that the BRL37344 counterbalanced the cardioprotective effect of norepinephrine in NELOWBRL comparatively to NELOW. No significant changes in the plasmatic catecholamines were observed in CTRL, NELOW, NELOWBRL and BRL before the regional ischemia, but we noticed a significant increase of norepinephrine levels in NELOWBRL and BRL groups at the end of the reperfusion period in comparison to baseline. The implication of this observation in the absence of reduction of the infarct size in these two groups remains questionable.

Planimetric examination showed a significant reduction of infarct size in NELOW group in comparison to CTRL, NEHI, NELOWBRL and BRL. There was no statistical difference between these four groups. In opposition to NELOW group, we did not obtain myocardial protection in NEHI, NELOWBRL and BRL, but we did not observe emphasized ischemic lesions in comparison to CTRL. Beta-3 selective stimulation in NELOWBRL has interfered with the cytoprotective effects of norepinephrine infusion as observed in NELOW. It have been previously reported that isoproterenol induces negative inotropic effects when associated to nadolol, a potent β_1 and β_2 adrenoceptor antagonist with low β_3 properties (Gauthier *et al*., 1996; Galitzky *et al*., 1993; Emorine *et al*., 1989). Beta-3 receptors are known to produce, among different peripheral effects, arterial vasodilatation, negative inotropic effects and participate to the pathogenesis of cardiac failure (Gauthier *et al*., 1996). In failing hearts, the positive inotropic effects of isoproterenol are severely reduced whereas membranous expression of β_3 adrenoceptors is increased

(Moniotte *et al*., 2001). Beta-3 action implicates the inhibitory G protein $G_{i/o}$ and involves the myocardial production of the endothelial isoform of Nitric acid Oxyde Synthetase (eNOS) (Shen *et al*., 1994; Gauthier *et al*., 1996). NO may regulate cardiac function through a cGMP-independent manner through the activation of key proteins such as cytochrome c oxydase, creatine kinase C or L-type calcium channels. Moniotte *et al* (2001) have reported that the response to inotropic amines such as isoproterenol was reduced by 75% in case of failing heart (ejection fraction 18.6±2.0%) whereas the β_3 expression as well as the $G\alpha_{i-2}$ protein that couples β_3 were 2 to 3-fold increased. Dekker *et al*. (1996) have reported that IP does not reduce infarct size in failing myocardium. Nevertheless, the implication of β_3 in the preconditioning mechanism has never been described yet. Contrary to β_1 and β_2 adrenoceptors, β_3 adrenoceptors lack phosphorilation sites for cAMP-dependant protein kinase or β-adrenoceptor kinase (Gauthier *et al*., 1998). Protein kinase C takes a major place in the preconditioning pathways (Tsuchida *et al*., 1994). According to this, the non-preconditioning effects observed in NEHI group could be a β_3-adrenoceptors stimulation counterbalancing the β_1 and β_2 stimulations. Comparatively, beta-1 downregulation is well established in the failing human heart (contrary to the β_2 subtype) and norepinephrine has a high affinity to the β_3 adrenoceptors (again contrary to the β_2 subtype) (Moniotte *et al*., 2001).

Peripheral vascular effects of a selective β_3 stimulation have been studied previously in conscious dogs. Shen *et al*. (1994) have reported that β_3 injections significantly reduce mean arterial blood pressure, increases peripheral conductance and heart rate. Beta-3 induces positive chronotropic effects in conscious but not in denervated animals. It was concluded that the tachycardia results from a baroreceptor-mediated reflex in response to the drop of the MAP due to the β_3 action. In our study, the administration of BRL37344 was followed by a significant drop of the MAP but HR remained stable all through the experiment. Nevertheless, our model does not procure exact analyze of the hemodynamic parameters because of lack of pre and post-load control. Theses parameters could be procured by a totally perfused isolated myocardium study (Langendorff model), or by the use of isoproterenol to completely eliminate the α stimulation effects.

We hypothesize that the β_3 receptors are stimulated after a massive overload of norepinephrine. β_3, by the mean of the G protein, could directly interfere with the Protein Kinase C (PKC) and thus interrupt the preconditioning pathways rendering the myocardium unresponsive to the preconditioning stimulus. A further study upon intracellular expression of PKC and β_3-mRNA could bring the light upon the exact mechanism underlying these observations.

CONCLUSION

The results of the present study suggest that norepinephrine can be cardioprotective by the mean of a pharmacological preconditioning when used in low doses. Furthermore, it has no effect upon cardioprotection when administrated in high doses. The non protective effect could be in relation with a beta-3 stimulation that interferes with the intracellular pathways of preconditioning.

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