SYNERGISTIC EFFECTS AGAINST POST-ISCHEMIC CARDIAC DYSFUNCTION BY SUB-CHRONIC NANDROLONE PRETREATMENT AND POSTCONDITIONING: ROLE OF $\beta_2$-ADRENORECEPTORS

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$\beta_2$-adrenoreceptor overexpression is beneficial against ischemia/reperfusion (I/R) injury. Whether $\beta$-adrenoreceptors are involved in postconditioning (PostC) is unknown. We investigated whether nandrolone-decanoate (ND)-pretreatment can modulate (1) $\beta$-adrenoreceptor expression and (2) post-ischemic cardiac function in response to I/R and PostC. Finally, we tested whether cardioprotection can be prevented by the inhibition of $\beta_2$-adrenoreceptors. Isolated rat hearts from ND-pretreated (15 mg/kg/day i.m., for 14 days) and untreated-animals underwent 30-min ischemia and 120-min reperfusion. In subgroups, at the end of ischemia a PostC protocol (five cycles of 10-s reperfusion and 10-s ischemia) was applied and/or a $\beta_2$-adrenoreceptor blocker, ICI-118.551 (10 µM), was infused. Left ventricular pressure (LVP) was measured with an electromanometer, and infarct-size was evaluated using nitro-blue-tetrazolium staining. ND-pretreatment increased $\beta_2$-adrenoreceptor expression, but did not alter cardiac-weight, LVP and maximum rate of increase of LVP ($dP/dt_{max}$). After I/R, infarct-size was smaller in ND-pretreatment than in untreated-animals. Contracture was less marked in ND-pretreated animals. PostC reduced contracture in both ND-pretreated and untreated hearts. Moreover, PostC improved post-ischemic recovery of developed LVP and $dP/dt_{max}$ much more in hearts of ND-pretreated than untreated-animals. ICI-118.551 abolished ND-protection and PostC-protection both in ND-pretreated and untreated hearts. Data show that two-weeks ND-pretreatment induces 1) an overexpression of $\beta_2$-ARs without cardiac hypertrophy and 2) improves the post-ischemic diastolic and systolic cardiac function. Intriguingly, ND-pretreatment potentiates the improvement of systolic function induced by postconditioning via $\beta_2$-adrenoreceptor activation.

Key words: beta-receptors, cardioprotection, ischemia/reperfusion, nandrolone, postconditioning.
INTRODUCTION

Ischemia/reperfusion (I/R) of the heart causes cell injury and death which results in infarction, myocardial stunning and cardiac contractile dysfunction. It has been reported that Postconditioning (PostC), i.e. repetitive cycles of reperfusion and coronary occlusion following an ischemic insult, cause massive salvage of the myocardium exposed to I/R. The extension of protection obtained with PostC and the transduction pathways involved in PostC are similar to those of ischemic preconditioning (IP) (1-7).

Stimulation of α-adrenoreceptors (ARs) and/or β-ARs has different effects (either null, detrimental or beneficial effects) on I/R injury, depending on time and duration of the stimulus. Studies that considered AR-stimulation in the pre-ischemic phase indicate that both α-ARs and β-ARs (particularly β₂-AR) may be involved in preconditioning-induced cardioprotection (8-11).

It is not known yet if catecholamines and/or ARs are involved in PostC. It has been suggested that β₁-AR stimulation may be detrimental in the reperfusion phase, thus increasing infarct size (12,13). There are indications, however, that β₂-AR activation during reperfusion has beneficial effect which can counteract the detrimental effect of β₁-AR stimulation (14). Further lines of evidence for a cardioprotective role of β₂-ARs arise from studies on transgenic mice over-expressing β₂-ARs or knockout for β₂-ARs (8, 15).

We recently developed a model of rats which over-express cardiac β₂-ARs in the absence of cardiac hypertrophy after two weeks of nandrolone decanoate (ND) treatment (16).

On the basis of the above studies, we hypothesized that hearts from ND-pretreated rats may show a better response to I/R and to PostC stimulus. In particular we argued that this better response may be due to β₂-AR stimulation. To verify these hypotheses we performed experiments on isolated rat hearts, in which infarct size and post-ischemic cardiac function are studied in response to I/R and PostC either in the absence or presence of β₂-AR blockade.

MATERIALS AND METHODS

The methods were similar to those previously described (16). In brief, the animals received care in compliance with Italian law (DL-116, Jan. 27, 1992). A first set of 3 months old male rats (ND treated, n= 26) was treated for 14 days with an i.m. injection of 0.5 ml/kg of peanut oil solution containing 15mg/(kg/day) of ND (16-23). This protocol of ND treatment was chosen because it does not induce cardiac hypertrophy (16,17). A second set of rats (untreated rats, n= 34) received for 14 days 0,5 ml/(kg/day) peanut-oil and served as control group.

This study was approved by the local Ethics Committee of University of Torino, Italy.

Animal Sacrifice and Isolated Heart Preparation

On day 15th of the experiments each animal was weighed and then treated with heparin (800 U/100 g b.w., i.m.). Then, 10 min after, animals were sacrificed, the heart was rapidly excised,
placed in ice-cold buffer solution and weighed. Several organs (brain, liver, kidney, adrenal gland, prostate) were also harvested and weighed.

Isolated rat hearts were immediately attached to the perfusion apparatus and retrogradely perfused with oxygenated Krebs–Henseleit buffer (2, 4-6, 16). The hearts were instrumented as previously described and pump-perfused at constant-flow (2, 4-6, 16). During the stabilization period the flow was titrated to reach a coronary perfusion pressure (CPP) of about 85 mmHg. Left ventricular pressure (LVP) was recorded by a polyvinyl chloride balloon placed in the left ventricle via the mitral valve and connected to an electromanometer (Monitoring Kit mk 5-02 DTBNVF, Abbott, Milan, Italy). The balloon was saline-filled to achieve an end-diastolic LVP of 5 mmHg. The hearts were electrically paced at 280 bpm and kept in a temperature-controlled chamber (37°C). Coronary flow, CPP and LVP were monitored to assess the preparation conditions. LVP was analyzed offline with Lab View software (National Instruments), which allowed the determination of end-diastolic LVP, index of contracture, as well as the developed LVP and the maximum rate of increase of systolic LVP (dP/dt\text{\max}), indices of contractile state.

**Experimental protocols (Fig. 1 A and B)**

After a stabilization period (20 min), hearts were subjected to a specific protocol, which included in all groups 30 min of global no-flow ischemia. A period of 120 min of reperfusion followed the 30 min ischemia in all groups (see below).

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**Fig. 1.** Schematic representation of experimental protocols. *Panel A* = Groups without β\text{-}2-adrenoreceptor (AR) blocker; *Panel B* = Groups with β\text{-}2-AR blocker. ICI-118.551 (ICI) = β\text{-}2-AR blocker; ND = Nandrolone decanoate; PostC = postconditioning.
As can be seen in Fig. 1A, after stabilization, hearts of untreated animals Control_I/R group (Group 1, n= 11) and those of ND-pretreated animals (ND+I/R group, Group 2, n= 7) were exposed to 30 min ischemia and then to 120 min reperfusion only.

In Group 3 (PostC group; n=11) after the 30 min ischemia, the hearts of untreated animals underwent a protocol of PostC. This consisted of five cycles of 10 s reperfusion and 10 s global ischemia at the beginning of reperfusion (2-6). In Group 4 (ND+PostC group; n=7) after the 30 min ischemia, the hearts of ND-pretreated animals underwent an identical PostC protocol.

To study the role of β2-ARs we used the specific β2-AR inhibitor, ICI-118.551 (ICI, 10µM) (24), in the following groups (Fig. 1B):

- in Group 5 (I/R+ICI; n= 6) and Group 6 (ND+I/R+ICI n= 6), hearts of untreated animals and ND-pretreated animals, respectively, were exposed to 30 min ischemia and then to the infusion for five minutes of ICI at the beginning of the 120 min reperfusion.
- In Group 7 (PostC+ICI, n= 6) and Group 8 (ND+PostC+ICI; n= 6) hearts of untreated and ND-pretreated animals, respectively, were infused with the β2-AR inhibitor, ICI, during the initial five minutes of reperfusion, while performing the PostC maneuvers.

At the end of the experiment infarct areas were assessed as previously described (2,4-6) using a solution of nitro-blue tetrazolium in phosphate buffer.

Rat left ventricle lysates were immunoprecipitated and immunoblotted with anti-β1- and anti-β2-AR polyclonal antibodies according to the method previously described (18).

**Chemicals**

We used a commercially available ND solution of 50 mg/ml (Deca-Durabolin, Organon, Italy). All other chemicals were purchased from Sigma (USA).

**Statistical analysis**

All data are means±S.E.M. One-way ANOVA and One-way ANOVA for multiple measures (post test: Newman-Keuls Multiple Comparison Test and t Test with Bonferroni correction) have been used for the analysis of infarct size and LVP data, respectively. A p value < 0.05 was considered significant.

**RESULTS**

**Effect of ND pre-treatment on β1- and β2-AR expression.**

As can be seen in Fig. 2, immunoprecipitated and immunoblotted assay confirmed the overexpression of β2-ARs, as previously showed by immunoblotting of the whole heart lysate (16). No changes in β1-AR expression were observed.

**Effect of ND pre-treatment over organs and body weight.**

At the end of treatments, cardiac weight (1.95±0.13 vs 1.90±0.07 g ) and cardiac to body weight ratio (0.571±0.04 vs 0.540±0.04 g/100g b.w.) were similar in the ND-treated and untreated animals (p= not significant for both). Therefore, there was no evidence of cardiac hypertrophy in ND treated animals compared with untreated animals. The body weight of ND-treated animals was
Fig. 2A. β₁-Adrenoreceptor (AR) expression. B. β₂-AR expression. β₁- and β₂- AR expression in the left ventricle of rat heart from untreated and nandrolone decanoate (ND)-treated animals. The immunoblots shown in the figure are from one representative experiment out of three, for each group. AR levels are expressed as arbitrary units obtained from densitometric scanning analysis of the immunoreactive bands.

Data are mean±S.E.M. *p<0.05 vs untreated.
slightly but significantly smaller (340±8 in ND treated vs 365±6 g in untreated animals, p< 0.05). However, the kidneys of ND treated animals were bigger than those of untreated animals (0.40±0.03 vs 0.35±0.01 g/100 bw, p< 0.01). These variations of body and kidney weights are typical in rats treated with a similar ND-treatment schedule and dose (16, 17). The weight of all other organs was similar in the two groups.

Infarct size

The results of infarct size expressed as percent of risk area are shown in Fig. 3. We found that infarct area induced by I/R was reduced by ND-pretreatment with respect to Control hearts of untreated animals. Infarct size was also reduced by PostC both in Control and ND pretreated groups (Fig. 3A).

As can be seen in Fig. 3B, the infusion of the β2-AR antagonist, ICI, in the Control hearts (I/R+ICI, Groups 5) did not significantly modify the infarct size. However, in hearts of ND-treated animals (ND-I/R+ICI, Groups 6) the β2-AR antagonist, ICI, avoided the reduction of infarct size induced by ND-treatment only (ND+I/R, Group 2).

The cardio-protection by PostC was abolished by ICI in hearts of untreated animals (PostC+ICI, Group 7) as well as in hearts of ND-treated animals (ND+PostC+ ICI, Group 8).

Post-ischemic Cardiac function

In baseline conditions all hearts had a similar LVP and maximum rate of increase of LVP (dP/dt_max).

Ischemic and post-ischemic diastolic function analyzed by end-diastolic LVP revealed that time-course of contracture was different between hearts treated with ND and untreated hearts (Fig. 4). Significant (p< 0.05) differences in end-diastolic LVP between ND-treated and untreated hearts are already appreciable during ischemia. During reperfusion contracture was less in ND-treated hearts, and PostC reduced post-ischemic contracture both in hearts of ND-treated and untreated animals. As can be seen in Fig. 5, the inhibition of β2-AR during reperfusion blunted the reduction in contracture induced by ND-treatment and by PostC.

Ischemic and post-ischemic systolic function was analyzed by developed LVP (Fig. 6A) and dP/dt_max (Fig. 6B). While no difference were appreciate during ischemic period, data revealed that both ND and PostC improved post-ischemic systolic function. In particular, ND-pretreatment _per se_ improved post-ischemic dP/dt_max with respect to hearts of untreated animals. Yet, PostC increased developed LVP and improved dP/dt_max more in hearts of ND-pretreated than untreated animals.

The improvement of contractile functions induced by ND _per se_ and by PostC are abolished by the inhibition of β2-AR. In fact, as can be seen in Fig 7A and B at the end of 2 h reperfusion there were no differences among ICI-treated groups which were similar to I/R (Group 1) in terms of post-ischemic systolic function.
Fig. 3. Infarct size. The amount of necrotic tissue is expressed as percent of the risk area. In Panel A and Panel B Groups are as in Fig. 1, Panel A and Panel B, respectively. In panel B it is also reported Control (I/R) Group 1 for comparative purpose. Data are mean±S.E.M. Post-test one-way ANOVA: ** = p< 0.001 and *= p< 0.05 vs Control (I/R); # = p< 0.01 vs ND+I/R; NS= non significant.
The principal new findings in the present study are that 14 days treatment only with nandrolone decanoate (1) induces an overexpression of β$_2$-ARs without cardiac hypertrophy, (2) improves the post-ischemic diastolic and systolic cardiac contracture development. 

Fig. 4. Contracture development during the 30 min ischemia and 120 min reperfusion. End-diastolic left ventricular pressure (LVP) is used to assess contracture development during ischemia/reperfusion. Time 0 (vertical dashed line) correspond to the beginning of reperfusion. Groups as in Fig 1 Panel A. Data are mean±S.E.M. Post-test one-way ANOVA for multiple measures: ** = p< 0.001 vs Control (I/R); *= p< 0.01 vs ND+I/R; # = p< 0.05 between Control (I/R) and ND+I/R.

DISCUSSION

The principal new findings in the present study are that 14 days treatment only with nandrolone decanoate (1) induces an overexpression of β$_2$-ARs without cardiac hypertrophy, (2) improves the post-ischemic diastolic and systolic cardiac contracture development.
function, and (3) potentiates the improvement of systolic cardiac function induced by postconditioning. Protective effects mediated by ND-pretreatment and by PostC were abolished by \( \beta_2 \)-AR antagonist, suggesting that post-ischemic endogenous release of catecholamines and subsequent activation of \( \beta_2 \)-ARs may be involved in the mechanisms by which cardioprotection is achieved.

Fig. 5. Contracture development during the 30 min ischemia and 120 min reperfusion. End-diastolic left ventricular pressure (LVP) is used to assess contracture development during ischemia/reperfusion. Time 0 (vertical dashed line) correspond to the beginning of reperfusion. Groups as in Fig. 1B. Here it is also reported Control (I/R) Group 1 for comparative purpose. Data are mean±S.E.M. No significant differences were observed among groups.
Fig. 6. Systolic function during the 30 min ischemia and 120 min reperfusion. A: Percent variation of first derivative of left ventricular pressure (LVP) during systole (dP/dt_{max}) with respect to baseline level. B: Percent variation of Developed LVP with respect to baseline level. Time 0 (vertical dashed line) correspond to the beginning of reperfusion. Groups as in Fig. 1A. Data are mean±S.E.M. Post-test one-way ANOVA for multiple measures: ** = \( p < 0.001 \) vs Control (I/R); * = \( p < 0.01 \) vs ND+I/R; # = \( p < 0.05 \) between Control(I/R) and ND+I/R.
Fig. 7. Systolic function during the 30 min ischemia and 120 min reperfusion. A: Percent variation of first derivative of left ventricular pressure (LVP) during systole (dP/dt_{max}) with respect to baseline level. B: Percent variation of Developed LVP with respect to baseline level. Time 0 (vertical dashed line) correspond to the beginning of reperfusion. Groups as in Fig 1B. Here it is also reported Control (I/R) Group 1 for comparative purpose. Data are mean±S.E.M. No significant differences were observed among groups.
The cardioprotective effects induced by short-term pretreatment with Nandrolone are evidenced by reduced contracture during ischemia, by reduced contracture and improved recovery of systolic function during reperfusion, and by reduced infarct size. Synergistic cardioprotective effects of nandrolone and PostC are underlined by the greater recovery of systolic function. On this regard, we would like to stress that post-ischemic impairment of function is due to both necrosis and stunning of viable tissue. When both effects are present it is hard to distinguish whether the recovery of function is due to an improvement of stunning and/or to a reduction of necrosis. In the present study the only conditions that clearly improves post-ischemic heart performance is the co-treatment with ND and PostC. On the other hand, $\beta_2$-AR blockade clearly avoids recovery of heart performance induced by ND and PostC. On the base of these effects on heart performance, but keeping in mind the limits above reported, we can argue that $\beta_2$-AR overexpression, which accompanies ND-treatment, is involved not only in reducing infarct size, but also in improving heart function recovery of viable tissue (i.e. anti-stunning effect).

It has been reported that ischemia/reperfusion induces the release of catecholamines from sympathetic terminal nerves and that $\beta$-AR stimulation plays an important role in ischemia/reperfusion scenario (8, 11, 24, 25). In particular, it has been suggested that $\beta_1$- and $\beta_2$-ARs may have opposite effects: $\beta_1$-AR activation may trigger apoptosis via a protein kinase A (PKA) linked mechanism, whereas $\beta_2$-AR activation may induce Gi-protein kinase G (PKG)-ERK-mediated anti-apoptotic effects (8, 25). Yet, improved post-ischemic systolic function may be mediated by improved calcium handling via PKA-phospholamban-signaling (8, 11).

Here we show that short-term ND-pretreatment induces $\beta_2$-AR over-expression without cardiac hypertrophy, whereas $\beta_1$-ARs are not affected. Since $\beta_2$-ARs are coupled to both Gs- and Gi-protein, we suggest that their up-regulation may mediate PostC-cardioprotection via Gi-PKG and Gs-PKA pathways, which can sustain cardioprotection and contractile function, respectively (8). In fact, reduced contracture and cell death may be both signs of reduced calcium-overload via PKG-mediated L-type calcium channel inhibition. Whereas, improved systolic function may be mediated by improved calcium handling. As a matter of fact, when $\beta_2$-ARs are blocked with the selective $\beta_2$-AR antagonist, ICI-118,551, protection against infarct size contracture and stunning are lost. Our data are in line with the idea that the initial phase of reperfusion represent a crucial moment for triggering protection (3) and are in agreement with recent preliminary report by Penson et al. (24), who showed that PostC protection can be prevented by the same $\beta_2$-AR antagonist in Langendorff perfused rat hearts.

Although the improvement in systolic function may help a better recovery from post-ischemic conditions, we should not forget that the absence of the post-stress physiological down-regulation of adrenergic-response could represent an overlooked risk of cardiovascular complications for nandrolone addicts (16). Moreover, Phillis et al. (26) described, that nandrolone treatment just before
ischemia potentiates arrhythmogenic effects of cardiac ischemia and decreases the fraction of rats surviving ischemia significantly.

Ongoing experiments in our lab show that the Gi protein inhibitor, pertussis toxin, prevented the protective effect of ND plus PostC against infarct size. These data are consistent with the hypothesis that PKA-mediated switching of coupling of the β2-AR from Gs to Gi protein is responsible for the protection (8). While PKA may improve systolic function, Gi may exert protective effects against contracture and cell death (8).

**Methodological considerations**

Studies on murine hearts have shown that chronic treatment with anabolic steroids causes myocardial hypertrophy, inadequate vascularization of the hypertrophied myocardium, and/or tachycardia, and, then, increased susceptibility to I/R injury (19-23). The presence of hypertrophy, tachycardia and/or inadequate vascularization may exacerbate the effects of I/R. For instance, Chaves et al. (23) showed that 8 weeks nandrolone treatment induced cardiac hypertrophy and reduced cardioprotection upon ischemic events.

The absence of hypertrophy and the constant heart rate allowed us to study the pure effect of ND-pretreatment over cardiac response to I/R and PostC. Yet, we cannot exclude that the β2-AR over-expression may represent a first modification that will lead to cardiac hypertrophy, as it is the case for transgenic mice that overexpress β2-ARs (27). Nevertheless, we know that subchronic treatment (two weeks) with ND may be effective on inducing cardiac effect as it alters heart contractile response either to cocaine (17) or to sympathetic stimulation (16) without any signs of cardiac hypertrophy.

In conclusion, we show that sub-chronic nandrolone pretreatment induces an overexpression of β2-ARs without cardiac hypertrophy. In such a condition nandrolone pretreatment improves post-ischemic systolic function, especially in postconditioned hearts, supporting an important role for β2-ARs in cardioprotection. In fact, both PostC-cardioprotection and ND-induced improvement of post-ischemic function were abolished in the presence of β2-AR antagonist, thus suggesting that β2-AR activation is involved in the mechanism by which cardioprotection is achieved in reperfusion.

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