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Ischemia/Reperfusion Injury Is Increased and Cardioprotection by a Postconditioning Protocol Is Lost as Cardiac Hypertrophy Develops in Nandrolone Treated Rats

Penna C[1,2], Tullio F[1], Perrelli M-G[1], Moro F[1,2], Abbadessa G[1], Piccione F[1],

Carriero V[1], Racca S[1] and Pagliaro P[1,2*]

[1]Department of Clinical and Biological Sciences, "S. Luigi Gonzaga" Hospital, University of

Turin, Regione Gonzole 10, 10043 Orbassano, Italy

[2]Italian Institute for Cardiovascular Research (INRC), Via Irnerio 48, 40126 Bologna, Italy

Running head: Nandrolone, Cardiac Hypertrophy and Ischemia/Reperfusion

***Address for the correspondence:**
Dr Pasquale Pagliaro,
Dipartimento di Scienze Cliniche e Biologiche
Università di Torino
Regione Gonzole 10,
10043 ORBASSANO (TO), Italy
Tel: 39-11 6705430/5450
Fax: 39-11 9038639
e-mail: pasquale.pagliaro@unito.it

Abstract

We hypothesized that Nandrolone (ND)-abuse induces cardiac hypertrophy, increases myocardial susceptibility to ischemia/reperfusion (I/R) injury, and reduces responsiveness to postconditioning (PostC) cardioprotection. Wistar-rats were ND-treated for 2-weeks (short_ND) or 10-weeks (long_ND). *Vehicle*-treated rats served as controls. Hearts were retrogradely perfused and left ventricular pressure (LVP) was measured before and after 30-min global ischemia. In subgroups of hearts, to induce cardioprotection a PostC protocol (five cycles of 10-s reperfusion and 10-s ischemia) was performed. β-adrenoreceptors, kinases (Akt and GSk-3β) and phosphatases (PP2A sub A and PP2A sub B) were examined by Western blot before and after ischemia. After 120-min reperfusion infarct-size was measured. Short_ND slightly increased cardiac/body weight ratio, but did not affect cardiac baseline nor post-ischemic contractile function or infarct-size when compared to *vehicle* hearts. However, PostC limited cardiac dysfunction much more in short_ND hearts than other groups. Although cardiac/body weight ratio markedly increased after long_ND, baseline LVP was not affected. Yet, post-ischemic contracture and infarct-size were exacerbated and PostC was unable to reduce infarct-size and ventricular dysfunction. While short_ND increased phosphatases, non-phosphorylated and phosphorylated Akt, long_ND reduced phosphatase-expression and Akt-phosphorylation. Both short_ND and long_ND had no effect on the GSK-3β-phosphorylation but increased the expression of β_2-adrenoreceptors. In reperfusion, PostC increased Akt-phosphorylation regardless of protective effects, but reduced phosphatase-expression in protected hearts only. In conclusion: short_ND improves post-ischemic myocardial performance in postconditioned hearts. However, long_ND increases myocardial susceptibility to I/R injury and abolishes cardioprotection by PostC. This increased susceptibility might be related to steroid-induced hypertrophy and/or to altered enzyme expression/phosphorylation.

Key words: Cardioprotection; Ischemia/Reperfusion; Nandrolone; Postconditioning.

Introduction

Ischemia/reperfusion (I/R) of the heart causes cell injury and death, which result in cardiac contractile dysfunction (myocardial contracture and stunning) and infarction. Clearly postconditioning (PostC, *i.e.,* repetitive cycles of reperfusion and coronary occlusion following an ischemic insult) causes massive salvage of the myocardium exposed to I/R. The extension of protection by PostC and the transduction pathways involved in PostC are similar to those of ischemic preconditioning (IP) [3,10,21,34,62,65]. In particular the protective cascades may involve post-translational modifications mainly mediated by the activity of several kinases [3,10,12,21,22,34] and phosphatases [7,2134,62] in reperfusion. Several signaling pathways of cardioprotection converge to increase the threshold for mitochondrial permeability transition pore opening [3,4,11,23,24,34,43]. However, the details of the signal transduction of postconditioning remain unclear [21-23,55].

There is a growing body of evidence indicating an increasing prevalence of the abuse of anabolic-androgenic steroid (AAS) in both non-athletes and athletes. Likewise, the number of people abusing AAS and being admitted to hospital for infarction is increasing. These patients are displaying angiographically normal arteries [1,35]. Nandrolone (ND), an AAS strongly associated with detrimental cardiovascular effects including sudden cardiac death, is commonly abused by humans [1,35]. When administered chronically to rats, ND has been associated with accelerated development of hypertension in developing spontaneously hypertensive rats (SHR) [61] and with left ventricular hypertrophy [60,61] in sedentary rats. More recently left ventricular hypertrophy and increased myocardial susceptibility to I/R injury have also been shown in isolated hearts prepared from rats treated chronically with ND [13]. However, we recently have shown that sub-chronic treatment with ND for 14 days does not induce an evident left ventricular hypertrophy

and, paradoxically, improves PostC cardioprotection [36]. Since cardiac hypertrophy is accompanied with alteration of function and expression of several *proliferative modulators*, such as kinases and phosphatates [53], many of which are involved in cardioprotection [10,21,22,34], we wondered whether treatment with ND, which induces cardiac hypertrophy, would alter the expression/activity of these enzymes and then would suppress the protective response to a PostC protocol.

Therefore, we hypothesized that chronic ND-abuse would increase myocardial susceptibility to ischemia/reperfusion (I/R) injury, and would reduce responsiveness to cardioprotective maneuvers only when myocardial hypertrophy is evident. Moreover, to our knowledge, thus far no investigation has been performed to study the effect of chronic ND on both infarct size and post-ischemic mechanical recovery after I/R with and without PostC in the same hearts. Importantly, no studies have compared the effects of sub-chronic and chronic ND in terms of development of hypertrophy and alteration of proliferative modulators, and in terms of alterations of responsiveness to I/R or cardioprotective maneuvers.

Thus, this study was performed in isolated rat hearts, in which infarct size and post-ischemic cardiac function were studied in response to I/R and PostC. Hearts were harvested from rats, which had undergone two ND treatments with different duration (14 days or 10 weeks). At the end of ND-treatment (two or ten weeks) the activation/inactivation (phosphorylated/total ratio) of kinases (Akt and GSk-3β) and expression of phosphatases (PP2A sub A and PP2A sub B) were analyzed and compared with vehicle-treated hearts. Phosphorylation of kinases and levels of phosphatases were also analyzed during reperfusion.

Materials and Methods

The methods are similar to those previously described [36,37]. These studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. National Institutes of Health) and with Italian law (DL-116, Jan. 27, 1992) and approved by the ethical committee of the University of Turin.

All animals were housed in our animal facilities at the age of 3 months, weighed and randomly assigned to one of the following three groups.

The 1st group: 3 months old male rats (*Chronic, long_ND*, n= 14) were treated twice a week for 10 weeks with an *i.m.* injection of 0.5 ml/kg of peanut oil solution containing 5 mg/kg of ND [60].

The 2nd group: 3 months old male rats (*Sub-Chronic, short_ND*, n= 14) were housed in our animal facility until the animals were 5 months old, then was treated daily for 2 weeks with an *i.m.* injection of 0.5 ml/kg of peanut oil solution containing 15 mg/kg of ND [30,36,37].

The 3th group: 3 months old male rats (*vehicle*, n= 12) were treated twice a week for 10 weeks with an i.m. injection of 0.5 ml/kg of peanut oil and served as *control group*. A group of short_vehicle controls was not considered because we had observed that 14 days of 0.5 ml/(kg/day) peanut-oil was ineffective in changing the studied parameters (body weight, organ weights, including the heart weight, heart performance and infarct size following I/R) when compared to untreated animals (unpublished observations). In particular, in our previous study [37] the infarct size and post-ischemic mechanical function of the short vehicle group were similar to those observed several times in untreated animals in our laboratory [38,40,44-46].

Animal Sacrifice and Isolated Heart Perfusion

Methods for isolated rat hearts were similar to those previously described [38,40,42,44-46]. In brief, at the end of treatment (animal age= 5.5 months) each animal was weighed and

treated with heparin (800 U/100 g b.w., i.m.). Then, 10-min afterwards, animals were sacrificed; the heart was rapidly excised, placed in ice-cold buffer solution and weighed. Several organs (liver, kidney, prostate, spleen, pituitary and adrenal gland) were also harvested and weighed.

Isolated hearts were retrogradely perfused with oxygenated Krebs–Henseleit buffer (127 mM NaCl, 17.7 mM $NaHCO_3$, 5.1 mM KCl, 1.5 mM $CaCl_2$, 1.26 mM $MgCl_2$, 11 mM D-glucose and gassed with 95% O_2 and 5% CO_2) at constant flow (9±1 ml/min/g), paced at 280 bpm and kept in a temperature-controlled chamber (37°C) [38,40,42,44-46]. Coronary perfusion pressure and left ventricular pressure were monitored to assess the preparation conditions. 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 (LVEDP) of 5 mmHg. LVP was analyzed offline with Lab View software (*National Instruments*), which allowed the determination of LVEDP, as well as the developed LVP (dLVP) and the maximum rate of increase (dP/dt_{max}) and decrease (dP/dt_{min}) of systolic LVP. While LVEDP and dP/dt_{min} are used as indices of diastolic function, dLVP and dP/dt_{max} are considered indices of contractile state. In particular LVEDP is used to monitor contracture, which can be defined as an increase in intrachamber pressure of 4 mmHg above pre-ischemic (baseline) LVEDP values [33]. The cardiac/body weight ratio was used as an index of cardiac hypertrophy [13,60].

Experimental protocols

After a stabilization period (40 min), hearts of the three groups (*long_ND, short_ND, and vehicle*) were subjected to a specific protocol, which included a period of 30-min of global no-flow ischemia and a subsequent period of 120-min of reperfusion (I/R). In subgroups,

immediately after the 30-min of ischemia, the hearts underwent a protocol of PostC. This consisted of five cycles of 10-s reperfusion and 10-s ischemia at the beginning of reperfusion [38,40,44-46].

Therefore the following six subgroups were studied (three subgroups of hearts underwent I/R only and three received PostC immediately after 30-min ischemia):

1) *vehicle*+I/R (n= 6); 2) *vehicle*+PostC (n= 6), which served as controls;

3) *short*_ND+I/R (n= 7); 4) *short*_ND+PostC (n= 7);

5) *long*_ND+I/R (n= 7); 6) *long*_ND+PostC (n= 7).

Western blotting analysis

To investigate the level of phosphatases (PP2A sub A and PP2A sub B) and the ratio of phosphorylated/total form of kinases (Akt and GSK-3β) after ischemia, in the six treatment groups above reported, samples of left ventricles were collected and freeze-clamped for Western blotting at the 60^{th} min of reperfusion. To study the levels of these enzymes in the baseline conditions additional *vehicle, short_ND and long_ND* hearts (n=4 for each condition) were examined. These hearts were mounted on the Langendorff perfusion apparatus, perfused for the stabilization time and then the left ventricle was collected. Samples were homogenized on ice in RIPA Lysis buffer (Santa Cruz Biotechnology), using a polytron tissue grinder. The homogenate was centrifuged at 4°C for 30-min at 13,000 g. Protein level was determined by Bradford's method [8]. About 50-100 μg of protein extracts were separated by SDS-PAGE on 10% acrylamide gels (for Akt, p-Akt, GSK-3β; p- GSK-3β; PP2A sub A; PP2A sub B) and transferred to PVDF membranes (GE-Healthcare). These were then incubated overnight at 4°C with the following primary antibodies: anti-PP2A sub A, anti-PP2A sub B, anti-Akt, anti-phospho-(Ser473)-Akt, anti-GSK-3β, anti-phospho-(Ser-9)-GSK-3β (Cell Signalling). To confirm equal protein loading, membranes were incubated with an anti-α-actin antibody (Sigma).

Immunoblotted proteins were visualized by Immuno-Star HRP Substrate Kit (BioRad) and quantified by Kodak Image Station 440CF. Image analyses were performed with Kodak 1D 3.5 software [41,43].

Positive control: since ND treatment is accompanied by alteration of the expression of adrenoreceptors (AR) [36,37 and references therein], rat left ventricle lysates were also immunoprecipitated and immunoblotted with anti-β_1- and anti-β_2-AR polyclonal antibodies (Santa Cruz Biotech) according to the method previously reported [36,37].

Assessment of myocardial injury

At the end of the experiment, *i.e.*, directly after 120-min reperfusion, each heart was rapidly removed from the perfusion apparatus and the left ventricle (LV) was dissected into 2–3 mm circumferential slices. Following 15-min of incubation at 37°C in 0.1% solution of nitro-blue-tetrazolium in phosphate buffer [38,40,42,44-46], unstained necrotic tissue was carefully separated from stained viable tissue by an independent observer who was not aware of the nature of the intervention. The weights of the necrotic and non-necrotic tissues were then determined and the necrotic mass was expressed as a percentage of total left ventricular mass, which is considered the risk area.

Chemicals

We used a commercially available ND solution of 50 mg/ml (Deca-Durabolin, Organon, Italy). All other chemicals were purchased from Sigma (USA) if not otherwise specified.

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) were 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 over organs and body weight (Table 1)

At three months old, when animals were assigned to the different groups, they all had similar body weights, while at the end of treatment, just before the sacrifice (*i.e.* at 5.5 months of age), the body weights of *long*_ND treated animals resulted significantly ($p< 0.01$, for both) less than those of *vehicle*-treated animals and *short*_ND treated animals (*vehicle vs short*_ND p= NS). The inhibition of weight gain by ND has been associated to ND-induced modulation of proopiomelanocortin (POMC) expression and alteration of the central melanocortin system [30]. At the end of treatment, cardiac weight increased from 1.42±0.04 in *vehicle* group, to 1.61±0.05 in *short_ND* and to 1.65±0.03 *g* in *long_ND group*. Moreover, cardiac to body weight ratio increased from 0.31±0.001 in *vehicle*s to 0.36±0.009 (+15%, $p< 0.05$) in *short_ND*, and to 0.41±0.09 *g/100g bw* (+31%, $p<0.001$) in *long_ND* animals (*short_ND vs long_ND p<0.05)*. Therefore, there was evidence of a slight cardiac hypertrophy in *short*_ND treated animals and a marked hypertrophy in animals treated with ND for long period, compared with *vehicle*s animals.

The kidney and prostate of *short*_ND and *long*_ND treated animals were found to be heavier than those of untreated animals. While the prostate weight variations are not consistent in the various protocols, the kidney weight increase is typical in rats ND-treated with similar schedules and doses [19,30,36,61].

Effect of ND pre-treatment over baseline hemodynamic parameters

In baseline conditions hearts of the three groups had similar cardiac performance, *i.e.* no significant differences were observed in hemodynamic parameters among groups (Table 1).

Effect of I/R and PostC on cardiac function and injury in ND-pre-treated and vehicle-pre-treated animals

Ischemia and the subsequent reperfusion caused *diastolic dysfunction* characterized by an increase in LVEDP and a reduction in dP/dt_{min}, and a *systolic dysfunction* evidenced by a decrease in dP/dt_{max} and in developed LVP. These parameters were differently affected by both I/R and PostC in the different experimental groups.

Post-ischemic Diastolic function (Fig. 1)

Diastolic dysfunction was analyzed by variations of LVEDP (Fig. 1, panels A and B) and dP/dt_{min} (Fig. 1, panels C and D) during reperfusion, in I/R (panels A and C) and PostC (panels B and D). LVEDP analysis revealed that the time-course of contracture was different between long_ND+I/R from one side, and short_ND+I/R and vehicle+I/R hearts on the other side (Fig. 1A). In particular, LVEDP was higher in hearts of long_ND+I/R compared to both vehicle+I/R and short_ND+I/R subgroups; in fact at the end of reperfusion the values were 78±24 *vs* 48±11and 48±15 mmHg, respectively ($p<0.05$, long_ND+I/R *vs* both vehicle+I/R and short_ND+I/R). Differences were not significant among I/R groups in terms of lusitropic effect as indicated by dP/dt_{min} (Fig. 1C).

Postconditioning confirmed its cardioprotective effect in the *vehicle*+PostC hearts, in fact in this subgroup at the end of reperfusion the LVEDP was significantly ($p<0.05$) reduced to 29±7 mmHg. PostC maneuvers also reduced LVEDP to 35±9 mmHg in the *short*_ND *treated* hearts ($p<0.05$ *vs short*_ND+I/R; p=NS *vs Vehicle*+PostC). However, in the *long*_ND+PostC the LVEDP was 80±12 mmHg at the end of reperfusion (Fig. 1B), *i.e.* not different from that observed in *long*_ND+I/R (Fig. 1A).

The dP/dt_{min} variations present a similar trend: only the *vehicle*+PostC and *short*_ND+PostC show a significant improvement of dP/dt_{min} recovery by PostC with respect to the correspondent I/R protocols ($p<0.05$ *vs* I/R; Fig. 1, panels C and D).

Post-ischemic Systolic function (Fig. 2)

Post-ischemic systolic dysfunction was analyzed by dLVP (Fig. 2, panels A and B) and dP/dt_{max} reduction in reperfusion (Fig. 2, panels C and D). No differences of these two parameters were seen among groups during post-ischemic periods in hearts subjected to I/R only (Fig. 2 panels A and C).

However, post-ischemic systolic function was slightly improved by *postconditioning* in *vehicle* subgroup, and markedly improved in *short*_ND+PostC hearts. In particular, *short*_ND+PostC showed higher post-ischemic dLVP and dP/dt_{max} with respect to hearts of both *Vehicle*- and *long*_ND treated groups (Fig 2, panels B and D). In fact, in the *short*_ND+PostC hearts, at the end of reperfusion, the recovery of dLVP and dP/dt_{max} were above 70% of baseline levels. The worsening of cardiac performance in the *long*_ND+PostC was similar to that of *long*_ND+I/R hearts, *i.e*. PostC was not protective (Fig. 2, panels C and D).

Infarct size (Fig.3)

Total infarct size, expressed as a percentage of left ventricular mass was 61±5% of risk area in hearts of *vehicle* pre-treated animals. *Short*_ND pre-treatment did not significantly affect infarct size of hearts subjected to I/R, infarct size being 53±10% of the risk area. However, *long*_ND pretreatment increased infarct size of hearts subjected to I/R, infarct size being 81±5% of the risk area ($p< 0.05$ *vs vehicle* groups).

The *postconditioning* maneuvers induced a significant reduction of infarct size both in hearts of *vehicle* pre-treated animals and in *short*_ND treated, infarct size being 35±7 and 30±3%

($p<0.05$ with respect to correspondent I/R; p=NS *vs* each other). However, in the *long*_ND+PostC, PostC was unable to reduce infarct size; in fact it was 72±5% of the risk area.

Western blotting analysis

The immunoprecipitated and immunoblotted assay, as seen in Fig. 4, confirmed the overexpression of β_2-ARs during both short and long treatment with ND, as previously shown for short treatment only [36,37]. No changes in β_1-AR expression were observed either in short or long treatment (data not shown) as previously reported for short treatment only [36,37].

In Fig. 5 the ratios of phosphorylated/total kinases (Akt and GSK-3β) in baseline conditions are reported. After sub-chronic treatment (*Short*_ND) no appreciable changes in Akt and GSK-3β ratios were observed. However, after chronic treatment (*Long*_ND) only Akt ratio was significantly reduced. Of note, in *Short*_ND hearts the maintained Akt ratio is due to an increase of both the total and phosphorylated forms. However, in *Long*_ND hearts the marked Akt ratio reduction is due to both an increase in total Akt and the almost complete absence of phosphorylation. Apparently there is also a slight tendency of total GSK-3β increase in *Long*_ND hearts.

In Fig. 6 the ratios of phosphorylated/total kinases (Akt and GSK-3β) in reperfusion in both I/R and PostC subgroups are reported. As can be seen, in I/R subgroup Akt ratio shows a trend similar to that observed in baseline conditions in response to treatments, *i.e.*, with a significant reduction of this ratio in Long_ND animals only. However, in PostC the phosphorylated/total ratios are higher than in the I/R hearts. On the other hand GSK-3β ratios were higher in I/R and PostC than those observed in baseline conditions, but PostC induced no appreciable changes in these ratios in the three treatment groups.

In Fig. 7 the expression of phosphatases (PP2A sub A and PP2A sub B) in baseline conditions is reported. The expression of both subunits increased after *Short*_ND, and decreased

after *Long*_ND, the subunits being PP2A sub A below and sub B at *Vehicle* levels in the *Long*_ND group.

In Fig. 8 the expression of phosphatases (PP2A sub A and PP2A sub B) in reperfusion is reported. As can be seen the expression of both subunits shows similar trends in reperfusion to those observed in baseline conditions in the three groups. Comparing I/R with PostC we observe a significant reduction for PP2A sub A in protected hearts (Vehicle and Short_ND) and, in particular, for both subunits (sub A and sub B) in PostC subgroups of the Short_ND animals.

Discussion

Here we report that sub-chronic treatment with the potentially toxic Nandrolone renders the postconditioned heart more resistant to ischemic injury, improving post-ischemic mechanical recovery. However, this protection is lost a few weeks afterwards, and I/R injury is increased, possibly as hypertrophic cardiomyopathy develops. Clearly the use of chronic models better reflects the clinical situation of compulsive abuse observed in humans AAS addicts, in which detrimental cardiovascular effects, including sudden cardiac death is commonly observed [36]. The increased protection in sub-chronic ND-treated heart and the increased susceptibility of the chronically ND-treated heart to I/R injury may be related to developing hypertrophy, which in an initial phase is characterized by preservation/potentiation of protective mechanisms; then, these mechanisms are exhausted and I/R injury increases. This shift from potentiation to worsening is reminiscent of the observed increase/reduction of the resistance to I/R injury when diabetes is induced by streptozotocin or alloxan administration [18]. Actually, dose- and time-dependency in determining detrimental *vs* beneficial effects has been observed for other compounds, including PAF [41,42] and TNFα [21,26].

We did not find any differences between the basal pre-ischemic function of the hearts from *vehicle*- and ND-treated animals with either treatment regimes (sub-chronic and chronic). This lack of effect of ND may be related to the model we used and/or to the dose of the steroid we choose from the literature [30,57]. In the studies where baseline mechanical function of the hearts was compromised, the doses of different AAS were somewhat higher [27,50]. In particular, while LeGros *et al.* [27] used isolated rat hearts in which an intra-ventricular balloon was filled to achieve an identical diastolic volume in the different experimental groups, we instead, filled the balloon to achieve the same baseline LVEDP, as is usually done in I/R studies [20,38,40,42,44-46]. Therefore, we cannot rule out whether chronic treatment with anabolic steroids may reduce left ventricular compliance, as shown by LeGros *et al.* [27].

We did, however, find that the hearts from animals that were on a chronic treatment program developed higher susceptibility to I/R injury and they lost the possibility to reduce infarct size with a well-characterized protective protocol [38,40,44-46]. The *long*_ND treatment significantly reduced the cardioprotective effect by PostC, with an increase of infarct size and reduction of cardiac performance. Paradoxically, the short_ND pre-treatment enhanced the cardioprotective effect of PostC, improving both diastolic and systolic functions during reperfusion. This is not associated with a further reduction of infarct size, suggesting a better improvement of stunning with respect to PostC in the control group. This aspect is very intriguing and has been attributed to the over-expression of β_2 adrenergic receptors, which are involved in the cardioprotection induced by PostC [37]. In fact, the release of catecholamines from sympathetic terminal nerves and β-AR stimulation play an important role in cardioprotection against necrosis and/or stunning [31,47,59,64]. We previously reported that the β_2-AR antagonist, ICI-118.551, abolishes PostC-protection both in ND- and vehicle-pretreated hearts [37]. Also

Penson *et al.* [48] suggested that PostC-protection can be prevented by the same β_2-AR antagonist in isolated rat hearts.

β_2-AR over-expression may be taken as an index of ND treatment [36,37]. Yet this over-expression may represent a first modification that will lead to cardiac hypertrophy, as is the case for transgenic mice that overexpress β_2-ARs [29]. These receptors are even more upregulated in the long_ND hearts, where I/R injury are exacerbated and cardioprotection by PostC lost. In fact, in our model hypertrophy develops progressively during treatment with ND, arriving at *borderline levels* after two weeks and very evident after ten weeks of treatment. Actually, with the two week regime treatment, the increase of heart/body weight ratio was not significant in our previous study [37] and it is slightly significant in the present study. Nevertheless, only when hypertrophy is evident cardioprotection is lost. The gain or loss of protection may be related to discrete alterations in intracellular enzymes and pathways (see below).

Our data confirm results from a recent study showing that chronic ND treatment induced cardiac hypertrophy and increased the susceptibility of hearts to I/R injury [13] and are in agreement with a more recent study showing that a 8 week treatment with ND impairs exercise-induced cardioprotection [9]. Data of the present study are also in line with our previous observation that PostC protection is impaired in hypertrophic hearts of SHR [46]. However, it is not clear whether hypertrophic hearts can be protected by PostC. In hearts from 2-year old mice (a model that displays definitive morphometric and molecular hallmarks of cardiovascular aging, including hypertrophy), no benefit was seen in any of the multiple PostC algorithms that were evaluated [52]. However, Fantinelli & Mosca [16] reported that PostC was as effective as preconditioning in improving the post-ischemic dysfunction of hearts isolated from SHR. Yet in two different models of overload myocardial hypertrophy, Zhu et *al.* [66] in post-ischemic

remodeled myocardium and Li *et al.* [28] in transverse aortic constriction in mice reported preserved PostC cardioprotection.

A limit to the present study is that we used a single PostC protocol, but did not check whether or not this stimulus was submaximal in hypertrophic hearts. Our study only suggests that a specific PostC protocol that is ideal for normo-trophic hearts is not working in hyper-trophic hearts of animals after chronic treatment with ND. Therefore, we cannot rule out that protective PostC protocol also exists for ND-treated hypertrophic hearts. Nevertheless, we should consider that it is not easy to ascertain whether or not increasing or reducing the numbers and/or the duration of postconditioning I/R cycles would be protective [25,56]. In fact, reducing the "additive ischemia" (cumulative coronary re-occlusions during PostC) from 2 to 1% of index ischemia in aging mice fully reestablished the protection [2,5,56]. Yet, in porcine hearts an increase in "additive ischemia" was necessary to show effectiveness [39,55,56]. Finally, as said, in aging hypertrophic mouse heart no benefit was seen in any of the multiple PostC algorithms that were evaluated [52]. Whether infarct size reduction with PostC is, indeed, preserved in hypertrophic myocardium remains to be assessed in future studies. On the basis of the present study, we can argue that preserved PostC-induced protection is typical of moderate/initial hypertrophy.

It has been suggested that in rodent models contracture development, rather than systolic function, may be a more appropriate end-point to study the protective effects of cardioprotective maneuvers [20,45]. The present study confirms this point of view; in fact contracture development matches very well with infarct size in all the experimental conditions. In particular, a higher level of contracture is observed in hypertrophic *long*_ND treated hearts, which develops greater infarct size and looses PostC protection.

Heart hypertrophy and failure are associated with diminished β-adrenergic responsiveness, altered protein phosphatase activity, and altered protein phosphorylation [14,63]. We previously reported altered β-adrenergic responsiveness in *short*_ND treated rats [36]. Here we observe reduced phosphorylated/total ratio of Akt only after chronic treatment with ND. However, phosphatases levels (both sub A and sub B of PP2A) increased after sub-chronic ND and then decreased after chronic ND treatments. Whether these enzyme alterations play a causal role in both hypertrophy and altered responses to I/R cannot be inferred. Nevertheless, data show that the impact on baseline levels of kinases and phosphatases is different, depending on the duration of ND treatment. We can argue that after *short*_ND-treatment up-regulation of PP2A may represent a feedback regulator of the increased activation of the PI3K/Akt pathway, as revealed by increased Akt level and phosphorylation (Fig. 5). Yet reduced phospho-Akt and increased GSK-3β total levels in *long*_ND-treatment might act as negative modulators of proliferation and protection. [12,58]. After ischemia/reperfusion Akt phosphorylation is particularly low in more vulnerable hearts (*i.e.*, Long_ND group). However, albeit starting from different levels, the increase in Akt phosphorylation is observed after PostC in all groups, regardless of protective effects. This supports the hypothesis that Akt phosphorylation in PostC needs to reach a threshold to be protective or alternatively that it may be an epiphenomenon [54,55]. Moreover, GSK-3β ratios are not correlated to protective effects; in fact the role of GSK-3β phosphorylation in PostC has been questioned [32]. Finally, PostC reduces the levels of PP2A when is protective. In particular, in short_ND, PostC reduces both PP2A subunits. Whether these reductions are somehow correlated to protection and to the maintained level of Akt phosphorylation needs to be confirmed. Actually, this different framework in the expression/activity of enzymes in the two ND-treatment regimes with and without I/R and PostC deserves further studies. Nevertheless, our results are in line with a recent study [7] showing that other phosphatases (PTEN, MKP-3, and

PP2C) are significantly decreased in wild-type mice by PostC protection, but are increased by PostC in ob/ob mice, which cannot be protected. Although we did not study the catalytic subunit of PP2A and did not directly measure PP2A activity, one could speculate that a reduced phosphatase activity could be responsible for the enhanced phosphorylation of protecting kinases and therefore contributes to beneficial effect of PostC. Intriguingly, alterations in phosphatase expression or activity limit the efficacy of both pre- and postconditioning during aging and cardiac hypertrophy [17,52].

In conclusion, sub-chronic use of high concentrations of ND, improves post-ischemic cardiac function in postconditioned hearts. However, chronic treatment with ND induces marked myocardial hypertrophy, increases cardiac susceptibility to I/R injury and abolishes the possibility to induce postconditioning protection. Moreover, both sub-chronic and chronic ND treatments increase β_2-AR expression. Yet only chronic ND treatment markedly decreases the activity/expression of important enzymes (phosphorylated Akt and PP2A levels) involved in both cardiomyocyte growth and protection. It seems that maintenance of adequate PP2A expression and appropriate ratio of phosphorylated/total form of Akt in basal conditions are necessary to preserve the possibility for future PostC cardioprotection. Starting from these adequate levels a concomitant increase in Akt phosphorylation and a reduced level of PP2A occur with PostC protection in this model. Yet the increase in Akt phosphorylation is not always associated to cardioprotection, especially if it starts from inadequately low pre-ischemic levels and/or occurs without concomitant PP2A downregulation. Nevertheless, whether the observed enzyme alterations play a causal role in the shift from protection to exacerbation of I/R injury remains to be clarified.

Acknowledgments

The authors were supported by Compagnia di S. Paolo, National Institutes of Cardiovascular Research (INRC, FM, PP); Regione Piemonte (CP, PP, SR), PRIN (CP, PP), ex-60% (CP, PP, SR). We would thank Prof. D. Gattullo and Dr D. Mancardi for their invaluable support and help.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig 1. Diastolic function. Left ventricular end diastolic pressure (LVEDP, mmHg, panel A) and percent variation of dp/dt_{min} with respect to baseline level (panel B), during the 30 min ischemia and 120 min reperfusion. Time 0 correspond to the beginning of reperfusion. I/R, Ischemia/Reperfusion; PostC, Postconditioning. † $p< 0.05$ *vs* Vehicle; * $p<0.05$, *vs* I/R.

Fig 2. Systolic function. Percent variation of developed left ventricular pressure (dLVP, panel A) and percent variation of dp/dt_{max} with respect to baseline (panel B) during the 30 min ischemia and 120 min reperfusion. Time 0 correspond to the beginning of reperfusion. I/R, Ischemia/Reperfusion; PostC, Postconditioning. † $p< 0.05$ *vs* Vehicle; * $p<0.05$, *vs* I/R; ** $p< 0.01$ *vs* I/R.

Fig. 3 Infarct size (IS). The amount of necrotic tissue is expressed as percent of the left ventricle (LV), which is considered the risk area. I/R, Ischemia/Reperfusion; PostC, Postconditioning.
* $p<0.05$, *vs* I/R; † $p< 0.05$ *vs* Vehicle.

Fig 4. Expression of β_2-Adrenoceptor (β_2-AR). Immunoblots are from representative experiments. β_2-AR levels assessed in the left ventricle are expressed in arbitrary units (a.u.). † $p< 0.05$ *vs* Vehicle; ‡ $p< 0.01$ *vs* Vehicle.

Fig 5. Phosphorylated/total ratio of kinases (Akt and GSK-3β) in baseline conditions. Blots are from representative experiments and ratios are assessed from kinase form levels detected in the left ventricle. † $p< 0.05$ *vs* Vehicle; # $p< 0.01$ *vs* Short_ND.

Fig 6. Phosphorylated/total ratio of kinases (Akt and GSK-3β) in reperfusion. Blots are from representative experiments and ratios are assessed from kinase form levels detected in the left ventricle. I/R, Ischemia/Reperfusion; PostC, Postconditioning.

* p< 0.05 vs I/R; † p< 0.05 vs Vehicle; # p< 0.05 vs Short_ND.

Fig 7. Expression of phosphatases (PP2A sub A and PP2A sub B) in baseline conditions. Blots are from representative experiments. Phosphatases levels assessed in the left ventricle are expressed in arbitrary units (a.u.). † p< 0.05 *vs* Vehicle; # p< 0.01 *vs* Short_ND.

Fig 8. Expression of phosphatases (PP2A sub A and PP2A sub B) in reperfusion. Blots are from representative experiments. Phosphatases levels assessed in the left ventricle are expressed in arbitrary units (a.u.). I/R, Ischemia/Reperfusion; PostC, Postconditioning.

* p< 0.05 vs I/R; † p< 0.05 vs Vehicle; # p< 0.01 vs Short_ND.

Figure 1

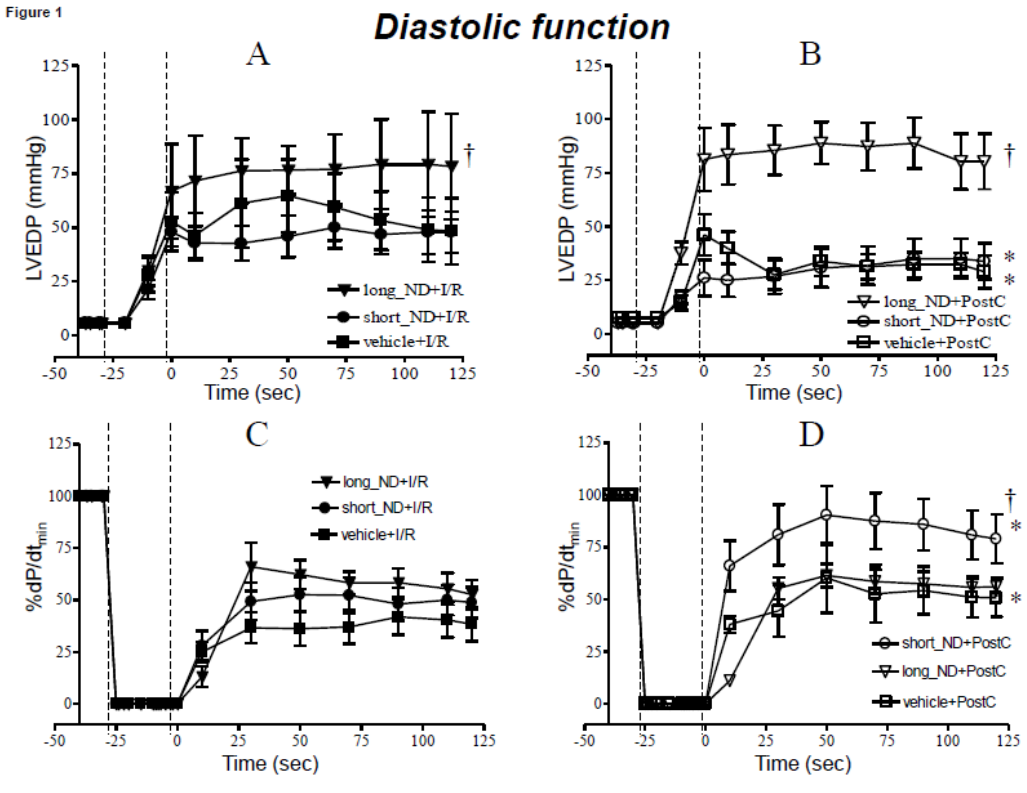
Diastolic function
A
B
C
D
LVEDP (mmHg)
%dP/dtmin
Time (sec)
long_ND+I/R
short_ND+I/R
vehicle+I/R
long_ND+PostC
short_ND+PostC
vehicle+PostC
†
*

Figure 2

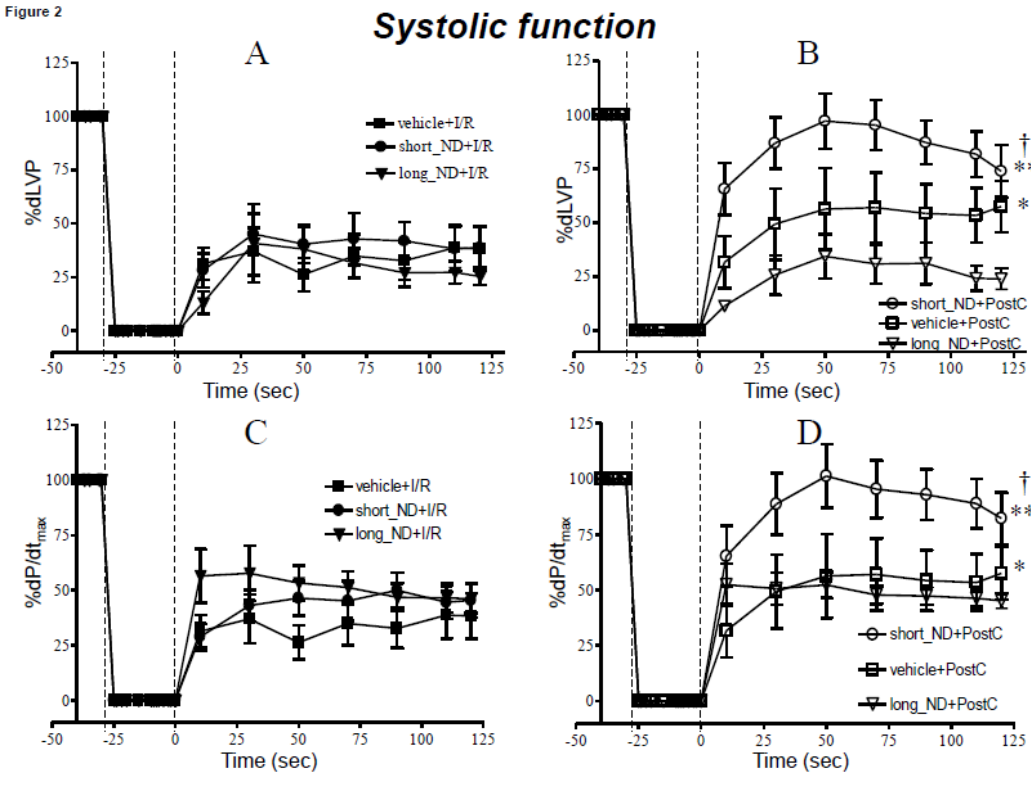
Systolic function
A
B
C
D
%dLVP
%dP/dtmax
Time (sec)
vehicle+I/R
short_ND+I/R
long_ND+I/R
short_ND+PostC
vehicle+PostC
long_ND+PostC
†
*
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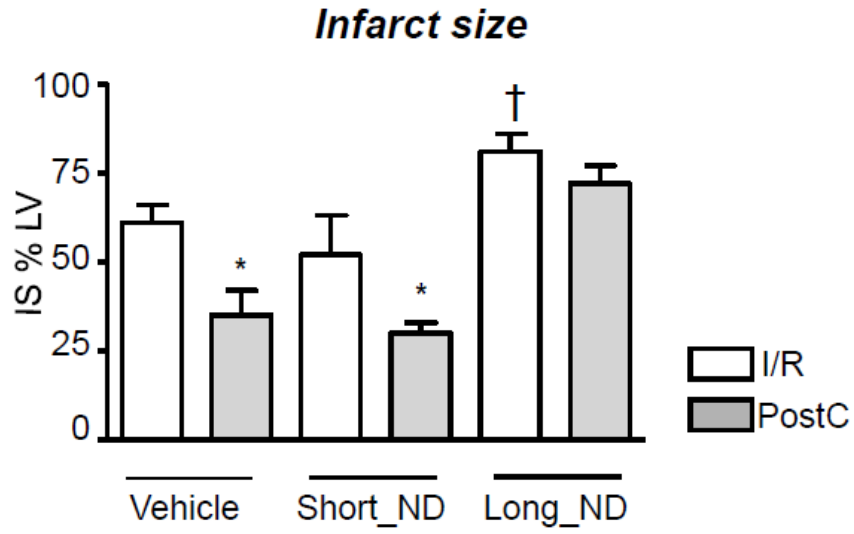
Figure 3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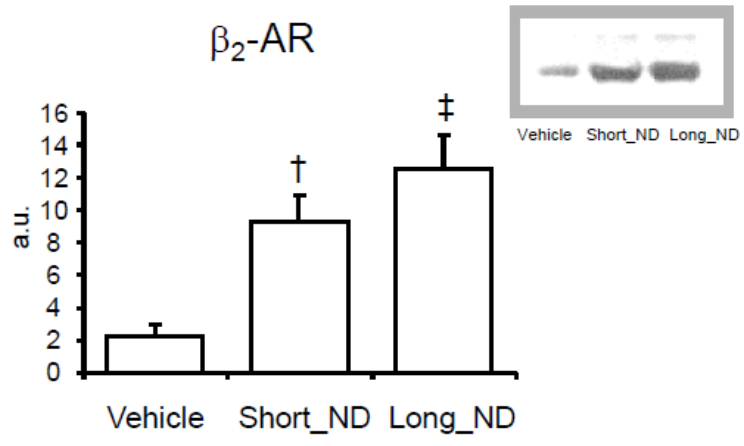
I/R
PostC
Vehicle
Short_ND
Long_ND

Figure 4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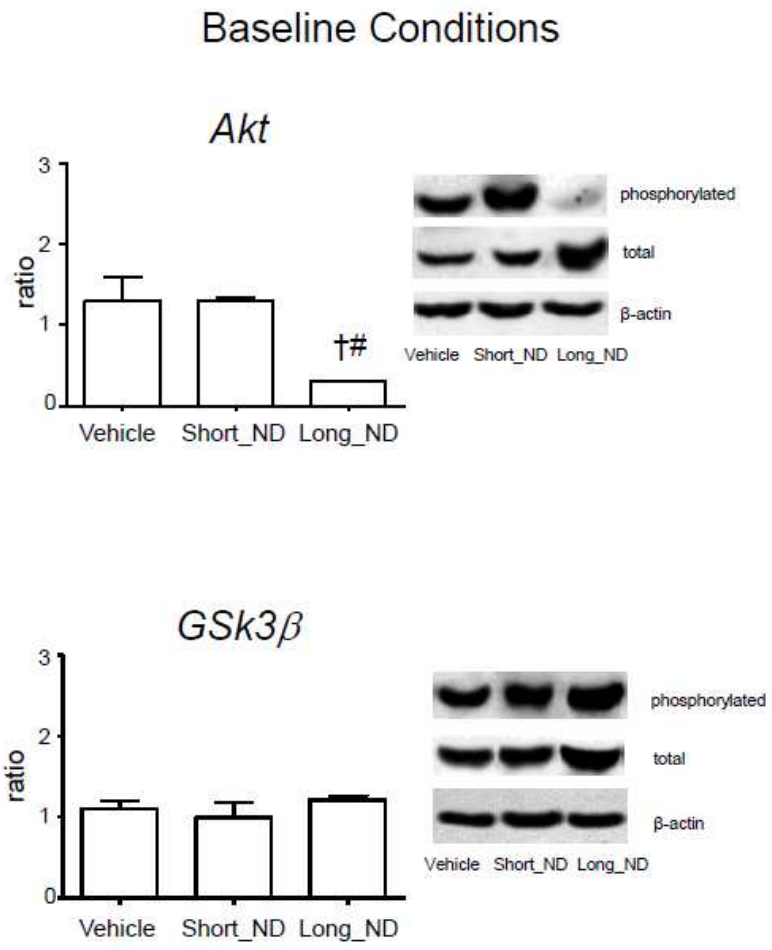
Short_ND
Long_ND

Figure 5


Baseline Conditions
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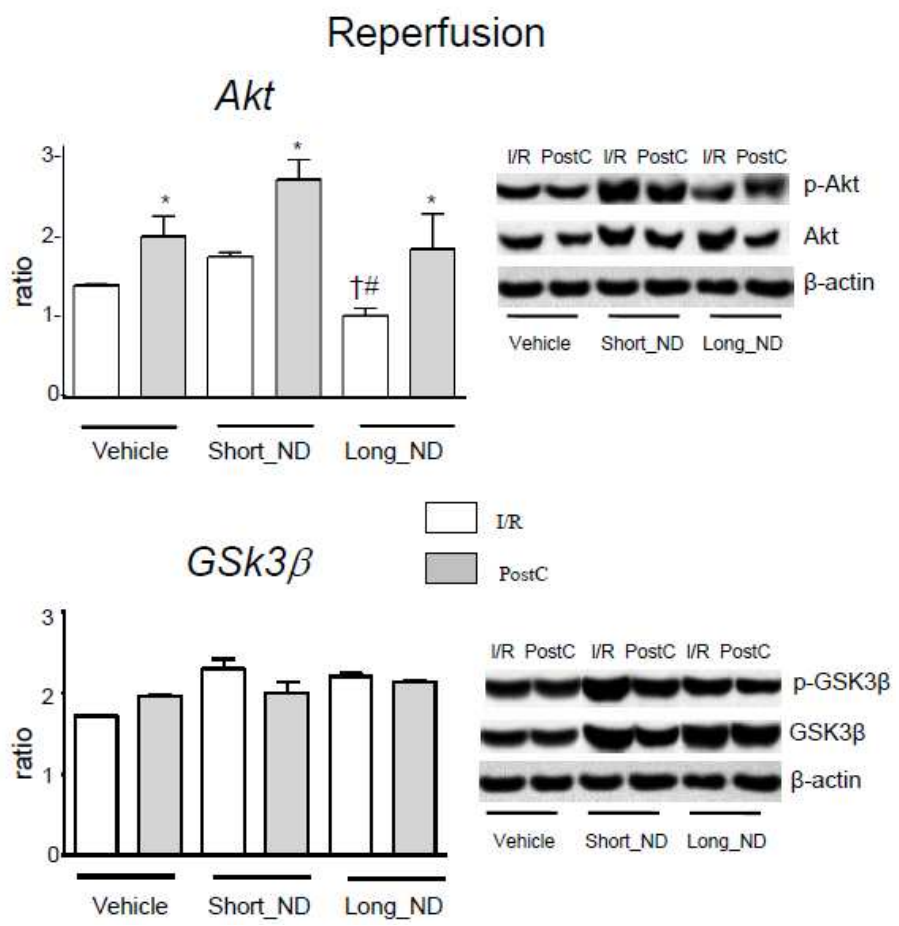
Vehicle Short_ND Long_ND

Figure 6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Vehicle
Short_ND
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I/R PostC I/R PostC I/R PostC
p-Akt
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β-actin
Vehicle
Short_ND
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GSk3β
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Short_ND
Long_ND
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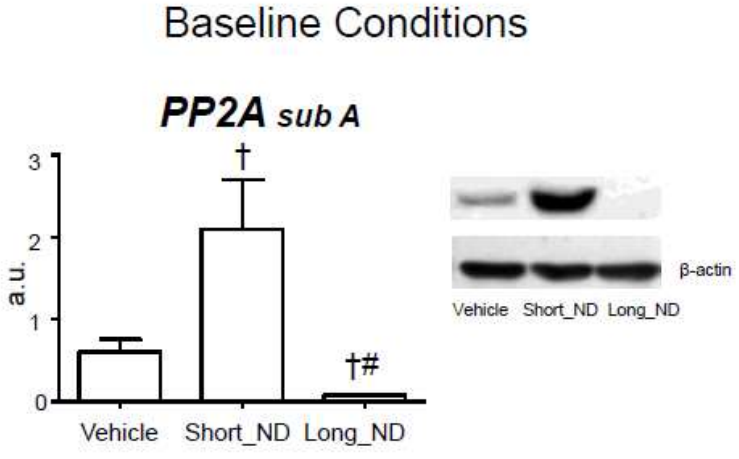
GSK3β
β-actin
Vehicle
Short_ND
Long_ND

Figure 7


Baseline Conditions
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PP2A sub B
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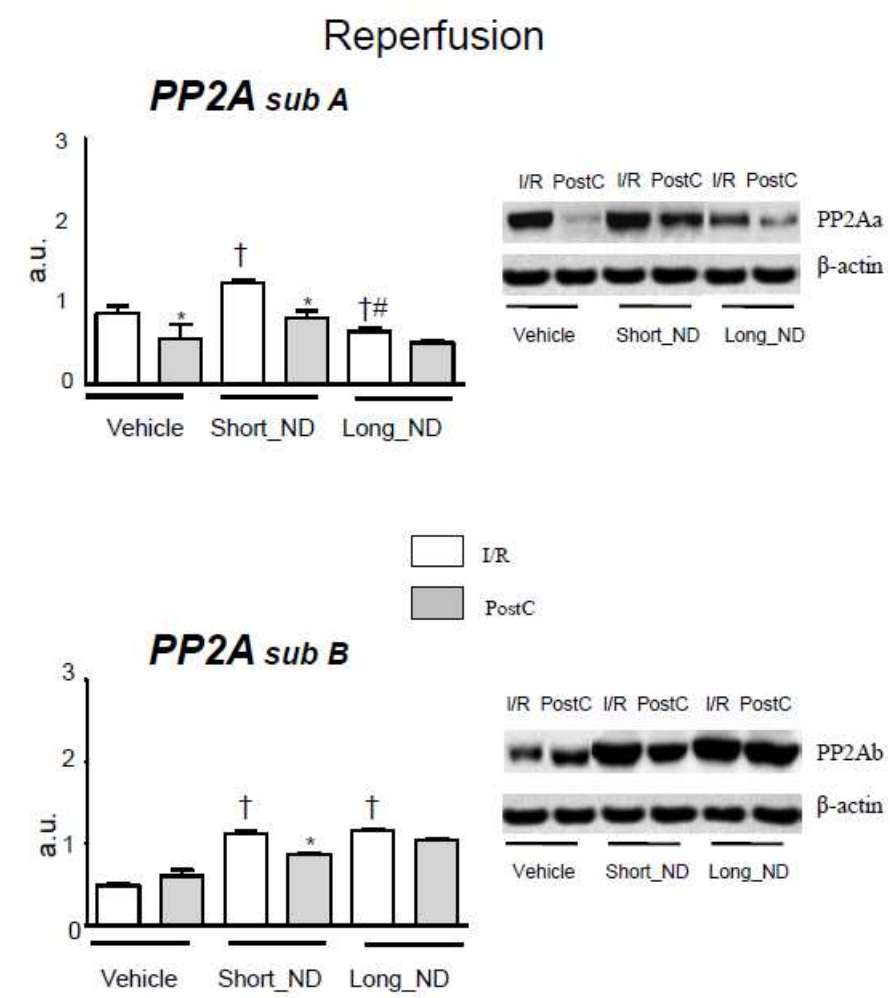
Vehicle Short_ND Long_ND

Figure 8


Reperfusion
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Vehicle
Short_ND
Long_ND
I/R PostC I/R PostC I/R PostC
PP2Aa
β-actin
Vehicle
Short_ND
Long_ND
I/R
PostC
PP2A sub B
3
2
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a.u.
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Vehicle
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Long_ND
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PP2Ab
β-actin
Vehicle
Short_ND
Long_ND