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Abstract

Nandrolone decanoate (ND), an anabolic-androgenic steroid prohibited in collegiate and professional sports, is associated with detrimental cardiovascular effects through redox-dependent mechanisms. We previously observed that high-dose short-term ND administration (15 mg/kg for 2 weeks) did not induce left heart ventricular hypertrophy and, paradoxically, improved postischemic response, whereas chronic ND treatment (5 mg/kg twice a week for 10 weeks) significantly reduced the cardioprotective effect of postconditioning, with an increase in infarct size and a decrease in cardiac performance.

We wanted to determine whether short-term ND administration could affect the oxidative redox status in animals exposed to acute restraint stress. Our hypothesis was that, depending on treatment schedule, ND may have a double-edged sword effect.

Measurement of malondialdehyde and 4-hydroxynonenal, two oxidative stress markers, in rat plasma and left heart ventricular tissue, revealed that the levels of both markers were increased in animals exposed to restraint stress, whereas no increase in marker levels was noted in animals pretreated with ND, indicating a possible protective action of ND against stress-induced oxidative damage. Furthermore, isolation and identification of proteins extracted from the left heart ventricular tissue samples of rats pretreated or not with ND and exposed to acute stress showed a prevalent expression of enzymes involved in amino acid synthesis and energy metabolism. Among other proteins, peroxiredoxin 6 and alpha B-crystallin, both involved in the oxidative stress response, were predominantly expressed in the left heart ventricular tissues of the ND-pretreated rats.

In conclusion, ND seems to reduce oxidative stress by inducing the expression of antioxidant proteins in the hearts of restraint-stressed animals, thus contributing to amelioration of postischemic heart performance.

Keywords: anabolic-androgenic steroids; heart; proteomics; oxidative stress; restraint stress

Introduction

Anabolic-androgenic steroids (AASs), synthetic derivatives of testosterone, exert both androgenic and anabolic actions [1] through their binding to the androgen receptor, acting at both the genomic and non genomic levels. AAS abuse, because of their anabolic effects, is detrimental to health [2-5]; nonetheless, AASs are widely used by athletes to increase muscle mass and power or enhance physical endurance [6]. Human and animal studies have reported discordant results regarding the cardiac and metabolic complications of AAS abuse [5,7-11], with some suggesting that testosterone

improves myocardial lipid metabolism [12] and may act as a cardioprotectant and coronary vasodilator comparable to conventional anti-ischemic drugs [13,14].

In cardiovascular diseases, both protective and damaging mechanisms derive from redox conditions. For example, exercise protects the heart by increasing myocardial resistance to ischemia-reperfusion (I/R) injury [15,16]. Yet, as a consequence of I/R injury, myocardial redox status is altered because of increased reactive oxygen species (ROS) production . This effect can be counteracted by exercise, which improves antioxidant defenses and reduces infarct size [15-17].

Nandrolone decanoate (ND), one of the most extensively studied AASs, may interfere with redox conditions [18] and its abuse can limit the cardioprotective effects of exercise. Indeed, chronic ND administration for 6 to 10 weeks at supraphysiological doses causes heart hypertrophy [19-21], increases cardiac susceptibility to I/R injury [17,22,23], activates the local renin-angiotensin system [24], and induces ventricular repolarization disturbances [25].

In two previous studies we reported that subchronic treatment with high-dose ND (15 mg/kg for 14 days) does not induce evident left ventricular hypertrophy but, paradoxically, improves cardiac postischemic response [26], whereas chronic ND treatment (10 weeks) significantly reduces the cardioprotective effect of postconditioning, with an increase in infarct size and decrease in cardiac performance [27]. We hypothesized that the double-edged sword effect of ND may be related to treatment schedule and redox conditions. To test this hypothesis, we investigated the effects of subchronic ND treatment on left heart ventricular tissue and on systemic and cardiac redox status in rats exposed or not to restraint stress. Experimental evidence supports the notion that stressful conditions produce oxidative damage and the rationale for using restraint to investigate alterations in redox activities in different tissues. Restraint conditions have been associated with impairment of antioxidant enzyme activity (e.g., superoxide dismutase) and depletion of reduced glutathione [28,29]. Moreover, we previously reported that restraint conditions induce downregulation of myocardial contractility response to isoproterenol stimulation which is reversed by ND pretreatment [30].

In this experimental model of subchronic ND treatment in modulating redox status, we measured two oxidative stress markers in plasma and left heart ventricular tissue samples: malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), the aldehydes resulting from polyunsaturated fatty acid lipid peroxidation. Using a proteomic approach, we identified the protein profile signatures in the left heart ventricular tissues of rats pretreated or not with ND and exposed to acute restraint stress .

Methods

Animals and treatments

Treatment schedule: 70 two-month-old male Sprague–Dawley rats weighing 300 g (Harlan Laboratories, San Pietro al Natisone, Udine, Italy) were housed in groups of two in polycarbonate cages with ad libitum access to food and tap water and maintained in a controlled environment (12:12 light–dark cycle, room temperature 20-24° C, and humidity 50-60%). They were allowed 1 week of acclimatization before the experiment began and were handled daily during this period. To avoid circadian variability, all tests were performed between 10:00 and 12:00, when plasma hormones levels are relatively low. Institutional ethical committee approval was obtained for this study; all experimental procedures were performed in accordance with EC Directive 86/609/EEC and Italian law regulating experiments on animals.

One group of rats (n=32) received once a day for 14 days an intramuscular (i.m.) injection of 0.5 mL of peanut oil (vehicle) containing 15 mg/kg of ND (DECA-Durabolin®, Organon), which is equivalent to the abuse dose of AAS in humans [31]. Sixteen of these rats (ND group) remained in their cages until sacrifice, and the 16 other animals were exposed to 1 h of restraint stress 24 h after the last injection and immediately before sacrifice (ND+S group) [32]. A second group (n=32) received 0.5 mL peanut oil i.m. injection once a day for 14 days. This group was divided into two subgroups: 16 were treated with the vehicle only (V group) and were sacrificed, and the 16 other animals were exposed to the same acute restraint stress protocol described above (V+S group). The control group (n=6) received i.m. injections of normal saline solution daily for 14 days to check for possible peanut oil effects. The experimental design is presented in Table 1. At the end of 14 days of treatment, all animals were sacrificed by decapitation and trunk blood was collected. The hearts were rapidly removed, rinsed with saline buffer solution, and dissected to collect the left heart ventricle. All samples were snap frozen and stored at -80° C until use.

Oxidative stress marker measurement in plasma and left heart ventricles

Plasma oxidative stress damage was determined by measuring the fluorescent adducts formed between lipid peroxidation-derived aldehydes and plasma proteins. Plasma samples were extracted in ethanol/ether (3:1 v/v) and then centrifuged at 1000 *g* for 10 min. Pellets were resuspended in ethanol/ether (3:1 v/v); this procedure was repeated three times. Protein adducts were dried under nitrogen and resuspended in a 5% SDS solution. Hydroxynoneal (4-HNE) protein adducts (355 nm excitation/460 nm emission) and malondialdehyde (MDA) protein adducts (390 nm excitation/460 nm emission) were measured using a spectrofluorometer (SFM 25 Kontron Instruments AG, Zurich, Switzerland). Data are expressed as arbitrary unit fluorescence (AUF)/mg plasma proteins [33].

In the left heart ventricle lysates, free MDA levels were determined by measuring thiobarbituric acid-reactive substances [34]. Briefly, left heart ventricle tissues were disintegrated with a Mikro-Dismembrator II (B. Braun, Melsungen, Germany) and dissolved in Tris-HCl buffer (0.1 M, pH 7.4) using a Potter-Elvehjem homogenizer (Sigma-Aldrich, St. Louis, MO, USA) The MDA concentrations determined by the thiobarbituric acid reaction are expressed as nmol/g tissue.

Tissue damage evaluation

Tissue damage was evaluated by measuring two cytotoxic markers, lactate dehydrogenase (LDH) (Abbott Laboratories, Abbott Park, IL, USA, 2P56-21) and creatine kinase MB isozyme (CK-MB) (Abbott, 6K25-30), in blood samples according to the manufacturer's instructions with the aid of a clinical chemistry analyzer (Abbott, Architect C8000 Plus).

Protein sample preparation

Frozen left heart ventricular tissue samples were disintegrated with a Mikro-Dismembrator II and lysed in RIPA buffer (20 mM HEPES, 150 mM NaCl, 5m M EDTA, 1 mM DTT, 1% Triton X-100, 10% glycerol, 1 µg/mL leupeptin, 1 µg/mL aprotinin, 1 µg/mL phenylmethyl–sulfonyl fluoride, 1 mM sodium orthovanadate) at 4° C. The lysates were then spun-down to remove insoluble cellular debris. Protein concentration was measured by the Lowry method [35].

Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis (2-DE) was performed according to the manufacturer's instructions (GE Healthcare, Milan, Italy) with slight modifications. Samples containing 0.125 mg proteins were in-gel rehydrated for 12 h onto 7-cm immobilized non-linear pH gradient strips (pH 3-10), electrofocused at 200 V, 500 V, and 1000 V each for 1 h (step-N-hold), 1000-5000 V (gradient) for 30 min, and 5000 V (step-N-hold) for 3 h using an IPGphor system (GE Healthcare). The strips were then reduced for 30 min in SDS equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS) containing 1% (w/v) DTT, followed by 30 min alkylation in the same equilibration buffer with 2.5% (w/v) iodoacetamide instead of DTT. Electrophoresis was run on an Ettan DALT II system (GE Healthcare). The gels were stained with colloidal Coomassie Blue G250 (Bio-Rad, Hercules, CA, USA), scanned using image scanner and the gel images were analyzed with ImageMaster 2D Platinum 6.0 software (GE Healthcare).

Mass spectrometry and protein identification

Protein spots were excised and in-gel trypsin (Sigma-Aldrich) digested according to the manufacturer's instructions. Aliquots of 1 µL of each sample were mixed with an equal volume of matrix (α-cyano-4-hydroxycinnamic acid), saturated in 50% acetonitrile (Sigma Aldrich), and the mixture was dropped onto the metal target plate of a Microflex LRF MALDI-TOF mass spectrometer (Bruker Daltonics, Germany). Mass spectrometry analysis was carried out in reflector mode. The adrenocorticotropic hormone (2464.1989 m/z) and the angiotensin II fragments (1045.5423 m/z) were used for internal mass calibration to maximize mass accuracy. The peptide mass fingerprint spectra were searched against the MASCOT (Matrix Science) and National Center for Biotechnology Information (NCBI, www.ncbi.nlm.gov) protein databases.

Western blot analysis (WB)

Total cellular proteins (25 µg) were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membrane (Hybond™ ECL™, GE Healthcare) for immunoblotting. The membranes were probed with the following antibodies: rabbit polyclonal anti-glutathione-S-transferase (GST) (1:2000, Alpha Diagnostic International, San Antonio, TX, USA, GSTM11-S), rabbit monoclonal anti-CRYAB (1:5000, GeneTex, Irvine CA, USA, GTX62094), rabbit monoclonal anti-PRDX6 (1:1000, GeneTex GTX62281), mouse monoclonal anti-alpha tubulin (TUB) (1:10000, Santa Cruz Biotechnology, Dallas, TX, USA, SC-23948) and goat polyclonal anti-vinculin (VCL) (1:1000, Santa Cruz Biotechnology sc-7649). The blots were then incubated with their appropriate secondary antibodies. Protein signals were detected using enhanced chemiluminescence ECL^{TM} WB detection reagents (GE Healthcare). WB images were analyzed using ImageJ software (http://imagej.nih.gov/ij/). CRYAB and PRDX6 were quantified by normalizing them against TUB selected as the reference housekeeping protein; GST was normalized using VCL.

Statistical analysis

All data are presented as the mean and the median as a central measure and interquartile range (IQR) as a measure of spread. The Shapiro-Wilk test was applied to check the normality assumption. Since the MDA concentrations in the left heart ventricular tissue samples had skewed distribution, natural log transformation was applied to achieve normality assumption for standard parametric analysis. Two-way analysis of variance (ANOVA) and Tukey's post hoc test were carried out in the event of a significant F ratio. Statistical significance was set at $p < 0.05$. Analyses were performed using R version 3.02 (www.r-project.org).

Results

Nandrolone prevents HNE and MDA accumulation

We measured HNE and MDA adducts in plasma and MDA in its free form in left heart ventricular specimens. Since HNE is quickly metabolized to less cytotoxic compounds in many tissues, and thus scantly detectable by photometric methods, we measured in left heart ventricular tissue samples the protein expression levels of GST, an enzyme involved in its detoxification. Indeed, through GST, HNE is rapidly and covalently conjugated to GSH, giving rise to GSH-HNE, which is more water soluble and thus more easily excreted.

A significant increase in the plasma levels of both HNE and MDA adducts was observed in the rats exposed to restraint stress (Fig. 1 and Table 2; p=0.04 and p=0.02, respectively). The aldehyde-protein adduct levels were lower in the ND+S group than in the V+S group, suggesting that ND may prevent against restraint-induced stress effects. However, two-way ANOVA found no significant interaction (Table 2). MDA in its free form in the left heart ventricular tissues was increased in the vehicle-treated group exposed to restraint stress (V *vs* V+S, p=0.002), whereas the stress-induced MDA increase was significantly lower in the ND+S group (Fig. 2).

Western blotting indicated a significant increase in GST protein levels after ND treatment in the left heart ventricular tissue samples from animals exposed or not to restraint stress, as compared to the tissues from animals that had not received ND (V and V+S groups) (Fig. 3).

The MDA levels in the control group and the V group were similar (data not shown).

Nandrolone treatment impairs blood LDH and CK-MB activity levels

HNE and MDA are highly toxic side products of lipid peroxidation. To determine whether these catabolites might have damaged or altered heart muscle viability, we evaluated the activity of lactate dehydrogenase (LDH), a classic indicator of tissue cytotoxicity, and creatine kinase MB isozyme (CK-MB), a cardiac-specific cytotoxicity marker. LDH and CK-MB levels were increased in the control group exposed to restraint stress as compared to the unstressed group (V *vs*. V+S groups), though the difference between-groups was not statistically significant. Interestingly, LDH and CK-MB levels were significantly lower in both ND-treated groups than in the control group exposed to restraint stress (V+S) (Fig. 4). Overall, these data strengthen the hypothesis that the changes in oxidative stress after exposure to restraint stress did not damage heart muscle integrity.

Left heart ventricular tissue from V+S and ND+S animals displays different proteomic signatures

To gain further insights into the molecular components involved in triggering differential responses to ND treatment in the animals exposed to restraint stress, 2-DE was performed on lysates of left

heart ventricular tissue samples from both groups (V+S and ND+S). Representative images are shown in Fig.5. Comparison between the two groups showed a higher number of protein spots in the ND+S than in the V+S samples (282 spots in ND+S *vs* 201 spots in V+S), especially within the range of 20-45 kDa. Eighty-five visible spots were manually excised from the gels and subjected to MALDI-TOF-MS analysis to identify the corresponding proteins. The spots unambiguously identified via MALDI-TOF-MS are numbered in Fig. 5 and the proteins identified (together with their relative scores, sequence coverage, and functional categories) are reported in Table 3. Several spots were identified by MALDI-TOF-MS analysis as being either different isoforms of the same protein or the same protein that migrated differently in other cases, suggesting post-translational modifications. Functional category analysis highlighted that the proteins involved in energy metabolism were the major components detected.

We identified three non-overlapping spots detectable only in the protein profile of the left heart ventricular tissue samples from the ND+S group. Mass spectrometry analysis identified these spots as succinyl–CoA transferase, alpha B-crystallin (CRYAB), and peroxiredoxin 6 (PRDX6), the last two of which are involved in cellular response to oxidative stress [36,37]. Indeed, CRYAB is a chaperone protein that prevents apoptosis induced by OS and PRDX6 protects against oxidative injury through its peroxidase activity.

These data were corroborated by WB analysis carried-out onof left heart ventricular tissue samples from the four groups (V, V+S, ND, ND+S). The comparison showed that PRDX6 expression levels were higher in both ND-treated groups, whether exposed to restraint stress or not, whereas CRYAB expression was increased in the two groups exposed to restraint stress, with a greater increase in the ND-treated group (Fig. 6).

Discussion

Our findings indicate that restraint stress impacts on oxidative stress and that ND administration can alter the oxidative status of cardiac tissue in rats exposed to restraint stress. Previous experimental evidence has shown that short-term ND pretreatment can protect against pro-oxidant conditions, such as I/R injury [26]; therefore, we hypothesized that it may exert a protective action against oxidative stress. To settle this issue, and because stressful conditions have been found to cause oxidative damage in diverse tissues, we employed an *in vivo* model in which rats were exposed to restraint stress [28,29,38,39].

Our assumption seems to contrast with that of authors reporting a ND-mediated impairment of exercise-induced cardioprotection [17,24,22,40]. A possible explanation for the protective effect of

ND we observed in left heart ventricular tissue is that ND exerts a differential effect in relation to duration of treatment (14 days in our study *vs* 6 to 8 weeks in those mentioned above).

With the present study, we report that oxidative stress marker levels in plasma and left heart ventricular tissues are increased in rats exposed to restraint stress. Although ND *per se* does not directly modify oxidative stress markers, it appears to prevent an increase in free MDA in the left heart ventricular tissue of restrained animals and that it enables HNE detoxification by means of increased levels of GST. This observation is shared by previous results reporting that ND enhances GST activity [41]. A similar trend was observed for the accumulation of HNE and MDA adducts in the plasma samples. The lack of significant differences between the oxidative stress marker levels in the plasma samples may be due to several reasons; indeed, it cannot be ruled out that oxidative stress varies in different tissues of the body. Two data sets indicate restraint stress-induced lipid peroxidation: the increased HNE and MDA levels and the appreciable, though statistically not significant, blood accumulation of LDH and CK-MB detected in the V+S group.

Overall, the data indicate that, albeit restraint stress led to oxidative damage, it apparently does not affect tissue viability. Interestingly, the opposite behavior of the oxidative stress and cytotoxic markers detected in the ND+S group suggests that ND plays a protective role against the negative effects of oxidative stress.

The effects of ND on oxidative stress in the animals exposed to restraint stress may be related to its effect on the corticosterone (CORT) response to stressful conditions. In a previous study, we reported that in rats that received ND treatment identical to the schedule used in the present study CORT levels were reduced in both basal condition and after stress exposure [42]. Indeed, glucocorticoids may affect the redox status of different tissues (e.g., endothelial cells and brain) via different mechanisms, including an increase in superoxide cell production and impairment of tissue antioxidant capacity [43,44]. Therefore, one can assume that the increase in the two oxidative stress markers in the V+S group is a consequence of the high CORT levels induced by stress and because ND decreases the CORT levels in plasma after stress, it could conceivably exert a protective action against oxidative stress.

We identified two additional novel players implicated in oxidative stress response to subchronic ND pretreatment: CRYAB and PRDX6. These two proteins, which were expressed at higher levels in the ND+S as compared to the V+S samples, are both involved in oxidative response. PRDX6, a class of thiol-specific antioxidant enzymes, exerts peroxidase activity against a broad range of peroxides [36,45-47]. CRYAB is activated by phosphorylation during oxidative stress, thus protecting cells from apoptosis induced by oxidative damage [48-50]. Previous studies have shown that transgenic mice that ubiquitously overexpress CRYAB have increased tolerance to I/R injury

[51], whereas CRYAB/HSPB2-null mouse hearts display poorer functional recovery, a higher cell death rate [52], increased stiffness, and, hence, poor myocardial relaxation following I/R injury [53] as compared with wild-type controls. Of note, we previously observed an improvement in postischemic diastolic and systolic cardiac functions after subchronic ND treatment [26].

To elucidate to what extent the effect on PRDX6 and CRYAB expression was due to ND administration by itself or ND coupled to stress, we compared their expression in samples from the four groups (V, V+S, ND, ND+S) by WB analysis. ND treatment alone was enough to induce up-modulation of both PRDX6 and CRYAB.

We previously reported that subchronic ND treatment improves systolic function induced by postconditioning cardioprotection via β2-adrenoreceptor activation [26] and probably, via the same pathway, prevents the decrease induced by restraint stress in heart contractile response to isoproterenol [30]. These findings appear to be in line with results from studies on other antioxidant substances [54,55]. Vitamin C is known to enhance the positive inotropic response to the β1-adrenoreceptor agonist dobutamine in the presence of high levels of reactive oxygen species [54] and, in the same condition, the free radical scavenger N-acetylcysteine was found to strengthen the inotropic effect of dobutamine in isolated heart tissue [55]. In our experimental conditions, it is unlikely that ND had a direct antioxidant action. ND-pretreatment affects the protein expression patterns, as revealed by 2-DE mapping.

In conclusion, our study provides further evidence for an enhanced oxidative response in animals exposed to restraint stress and reports the novel observation that subchronic ND-pretreatment limits myocardial redox response induced by restraint *via* the modification of anti-oxidant protein expression. These effects may explain the amelioration of postischemic heart performance observed after ND administration [26]. It can be argued that ND acts as a double-edged sword, with positive or negative effects on the heart depending on the treatment schedule.

Conflict of interest

The Authors declare that they have no conflict of interest.

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Fig. 1 Restraint stress increases plasma HNE and MDA adduct levels. The boxes indicate the lower and upper quartiles, and the central line is the median. The dashed line represents the mean. The whiskers above and below the boxes indicate a distance of 1.5 IQR. *p<0.05.
HNE denotes 4-hydroxynoneal; MDA malondialdehyde; IQR interquartile range.

Fig. 2 Free MDA levels in left heart ventricle are lowered by ND treatment upon restraint stress. Box plot of MDA levels in left heart ventricular tissue (log(nmol/g)) in ND-treated and control animals. The boxes indicate the lower and upper quartiles, and the central line is the median. The dashed line represents the mean. The whiskers above and below the boxes indicate a distance of 1.5 IQR. Two-way ANOVA and post hoc Tukey test showed significant differences between ND and ND+S (p=0.027), V and V+S (p=0.002), ND+S and V+S (p=0.001).
MDA denotes malondialdehyde; ND nandrolone decanoate; V vehicle-treated group; V+S vehicle-treated group exposed to restraint stress; ND nandrolone-treated group; ND+S nandrolone-treated group exposed to restraint stress; IQR interquartile range.

Fig. 3 Increased GST protein levels in left heart ventricular tissue samples from ND-treated animals. Representative Western blot image showing quantification of GST protein levels.
Fig. 4 Measurement of blood LDH and CK-MB activity. Box plot of LDH (panel A) and CK-MB isozyme (panel B) activity levels (U/L). The boxes indicate the lower and upper quartiles. The central and the dashed lines represent the median and the mean values, respectively. The whiskers above and below the boxes indicate a distance of 1.5 IQR. Two-way ANOVA and post hoc Tukey test showed significant differences between the V+S *vs* ND groups and the V+S *vs* ND+S groups.**Fig. 5** Two-dimensional gel electrophoresis (2-DE) displaying different patterns in the lysates obtained from left heart ventricular tissue samples from the two groups exposed to restraint stress (V+S and ND+S). Proteins from V+S (panel A) and ND+S (panel B) left heart ventricular tissues were separated by isoelectric focusing on 7-cm strips with a non linear 3 to 10 pH gradient and subjected to SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie Blue (G250). The numbers indicate the spots analyzed by mass spectrometry. Molecular size markers are indicated on the left.

Fig. 6 Immunoblots of PRDX6 and CRYAB proteins in left heart ventricular tissue. Histograms show the relative concentrations of the proteins normalized to the house-keeping protein tubulin (TUB).

PRDX6 denotes peroxiredoxin 6; CRYAB alpha B-crystallin; V vehicle-treated group; V+S vehicle-treated group exposed to restraint stress; ND nandrolone-treated group; ND+S nandrolone-treated group exposed to restraint stress. Two-way ANOVA and post hoc Tukey test showed significant differences between the groups. PRDX6 *$p<0.05$ *vs* V and V+S, n=3; CRYAB *$p<0.05$ *vs* V; #$p<0.05$ *vs* V+S, n=3.

The immunoblots are from one representative experiment.