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Toxicology Letters 169 (2007) 129–136
Toxicology Letters
www.elsevier.com/locate/toxlet

Detrimental effects of anabolic steroids on human endothelial cells

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Received 7 November 2006; received in revised form 19 December 2006; accepted 20 December 2006
Available online 3 January 2007

Abstract

The aim of this study is to investigate the effects *in vitro* induced by androgenic anabolic steroids (AAS) (testosterone, nandrolone, androstenedione, norandrostenedione, and norandrostenediol) used illicitly in sport competitions, on the proliferation ability, apoptosis and the intracellular calcium concentration ($[Ca^{2+}]_i$) in human umbilical vein endothelial cells (HUVECs), selected as a prototype of a biological target system whose structure and function can be affected by steroids.

For this purpose, we evaluated the proliferation inhibition by cytotoxic assay expressed as the concentration of drug inducing a 50% decrease in growth (IC_{50}). The IC_{50} was reached for testosterone at 100 μM, androstenedione at 375 μM, nandrolone at 9 μM, norandrostenedione at 500 μM. The IC_{50} value for norandrostenediol was not reached until a concentration of 6000 μM. The apoptotic effect was evaluated by flow cytometry at IC_{50} for each drug. We observed that testosterone induced 31% of apoptotic cells, norandrostenedione 25%, androstenedione 15% and nandrolone 18%. We have analyzed the effects of these drugs on $[Ca^{2+}]_i$ both in the immediate and long-term continuous presence of each compound. Our data show a statistically significant increase of $[Ca^{2+}]_i$ in the acute condition and in long-term treated cultures, suggesting that androgen steroids modulate intracellular levels of calcium independent of incubation time or compound identity. As a whole, this study demonstrates that AAS might alter endothelial homeostasis, predisposing to the early endothelial cell activation that is responsible for vascular complications observed frequently in AAS users.

Keywords: Androgenic steroids; Endothelial cells; Apoptosis; Calcium

1. Introduction

The abuse of androgenic anabolic steroids (AAS) is prevalent in sport competitions. Generally, AAS are used by athletes to improve their muscle mass and enhance exercise performance. Several studies have described the

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doi:10.1016/j.toxlet.2006.12.008

adverse effects on reproductive (Dohle et al., 2003; Eklof et al., 2003), hepatic (Velazquez and Alter, 2004) and endocrine function (Shaidi, 2001; Snyder, 2001), as well as on musculoskeletal features (Al-Ismail et al., 2002), in AAS users. Although there is evidence that AAS abuse may be associated with impaired vascular reactivity, few data are available describing the toxic effects of AAS on human endothelial cells.

Endothelial cells have an important function in preventing intravascular coagulation under normal conditions, but of promoting blood clotting and inflammation at sites of vessel damage (Mann, 1997). To achieve these opposite functions, endothelial cells secrete several proteins that regulate blood clotting, blood flow and local immune responses. In the cardiovascular system, the endothelial cells play key regulatory roles by producing several potent vasoactive agents and modulating coagulation states. A dysfunction has been implicated in many cardiovascular diseases, and the modulation of endothelial functions could be a promising therapeutic approach. These diverse functions make endothelial cells indispensable in the body's normal homeostasis and in several pathological conditions (Sullivan et al., 1998).

Most important endothelial cell functions depend, to various extents, on changes in the intracellular concentration of calcium $[Ca^{2+}]_i$. An increased $[Ca^{2+}]_i$ is a sensitive indicator of the early phase of endothelial cell activation, leading to the production and release of some important vasoactive molecules, such as prostaglandins, platelet activating factor (PAF), nitric oxide and endothelin (Watanabe et al., 1996).

The aim of this study was to evaluate the *in vitro* toxicity of some steroids usually used in sport competitions on freshly prepared human endothelial cells. Particularly, it is focused on the detrimental effects of testosterone, nandrolone, androstenedione, and norandrostenedione on pivotal endothelial cell functions such as proliferation, apoptosis and changes in intracellular calcium concentration.

2. Materials and methods

2.1. Chemicals and reagents

Nandrolone (19-nor-4-androsten-17β-ol-3-one) and testosterone (17β-hydroxy-4-androsten-3-one) were obtained from Sigma–Aldrich (Milan, Italy); androstenedione (4-androsten-3,17-dione), 19-norandrostenediol and 19-norandrostenedione (19-nor-4-androsten-3,17-dione) were obtained from Steraloids (Steraloids Inc., Newport, RI). Working standard solutions were prepared daily from stock solutions, which were stored in screw-cap vials in the dark at −20 °C for a maximum of 1 month.

2.2. Cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord veins filled with 0.27 U/ml collagenase as described (Jaffe et al., 1973). After incubation for 30 min at 37 °C, umbilical cord veins were perfused with DMEM-containing antibiotics for the collection of cells. Then, after centrifugation for 8 min at 900 × *g*, the cell pellet was resuspended in DMEM supplemented with 20% (v/v) newborn calf serum, 10% (v/v) heat-inactivated fetal calf serum (FCS), 20 mM Hepes and 6 U/ml of heparin. Serum was charcoal/dextran-stripped to remove endogenous steroids. HUVECs were grown on 1% (w/v) gelatin-coated flasks in the same medium supplemented with 50 μg/ml of endothelial cell growth factor (crude extract from bovine brain). Characterization of HUVEC was identified by the CD31 (DeLisser et al., 1993) and the von Willebrand factor (Sadler, 1991). Cells were used between the third and fifth passage.

2.3. Growth inhibition assay

All cytotoxicity experiments were performed in triplicate and cell viability was determined for each drug by the XTT/PMS viable cell dye assay (Sigma Chemicals, Milano, IT) (Scudiero et al., 1988). We determined the appropriate seeding density (1.5×10^3 cells/well) on the basis of the linear relationship between absorbance and the number of cells in the growth curve of each cell line, as well as the optimal time of drug exposure (from 24 to 72 h) for evaluation of IC_{50}. Briefly, cells were plated into flat-bottomed 96-well plates (Nunc, Denmark) and cultured in DMEM supplemented with 10% FCS, 20% newborn calf serum, 20 mM Hepes, 6 U/ml of heparin, 2 mM glutamine, 50 μg/ml of endothelial cell growth factor in the presence of drugs before the addition of 50 μl of XTT/PMS solution (final concentrations 50 and 0.38 μg/ml, respectively) and incubated for 4 h at 37 °C. Plates were read on a microplate ELISA reader at 450 nm. The IC_{50} value, defined as the concentration of drug inducing a 50% decrease in growth (as determined by measurement of $A_{450\,nm}$) of treated cells compared to controls, was reported as a percentage with respect to untreated cells. Data (mean ± S.D.) are representative of at least three independent experiments for each drug. The drugs were tested at the indicated range: testosterone, 10–200 μM; androstenedione, 50–400 μM; nandrolone, 5–100 μM; norandrostenedione, 125–750 μM; and norandrostenediol, 50–6000 μM.

2.4. Apoptosis assay

The ability of the drugs to induce apoptosis in primary endothelial cells was evaluated by flow cytometry. HUVECs were treated with drugs at the concentration corresponding to the IC_{50} value. The adherent cells were trypsinized, pooled with

the culture supernatant containing the apoptotic cells detached from the dish, and centrifuged. Cells (1×10^6) were washed in PBS and fixed for 30 min by the addition of 1 ml of 70% (v/v) ethanol. Then the cells were pelleted by centrifugation ($720 \times g$; 5 min), resuspended in 1 ml of DNA staining solution (PBS-containing 200 μg/ml of RNAse A, 20 μg/ml of propidium iodide plus 0.1% (v/v) Triton X-100) and stained by incubation at room temperature for 60 min. All cells were then measured on a FACScan flow cytometer (Becton Dickinson, UK) with an argon laser at 488 nm for excitation and analyzed using Cell Quest software (Becton Dickinson). All the flow cytometric measurements were done at the same instrument settings and at least 10,000 cells were measured for each sample. The detection of apoptotic cells was estimated by a quantifiable peak in the sub-G1 phase corresponding to the red fluorescence light emitted by sub-diploid nuclei of cells, and the results were expressed as the percentage of death by apoptosis induced by a particular treatment.

2.5. Measurement of the cytosolic calcium concentration

To determine the effects of AAS on basal calcium levels, both in the acute condition and long-term exposition, HUVECs suspended in HCM buffer (10 mM Hepes (pH 7.4), 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 137 mM NaCl, 4 mM KCl, 11 mM D-glucose,) at 1×10^6 cells/ml were loaded with 5 μM fura 2-AM (Sigma, Milan, IT) and incubated for 30 min at 37 °C in a waterbath. After loading, extracellular dye was removed by washing and cells were resuspended in HCM buffer. Intracellular levels of free calcium were determined by the method of Grynkiewicz et al. (1985). Fluorescence was measured with an Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo, Japan) using excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm. The cuvette containing 0.5×10^6 cells/ml in the presence of each drug was thermostatically controlled at 37 °C, and the cell suspension was stirred continuously. In every sample, calibration was performed with detergent (Triton X-100) to calculate the maximum intensity of fluorescence, and with the calcium chelator EGTA to obtain the minimum level of fluorescence.

2.6. Statistical analysis

All data are expressed as mean ± S.D. from at least three independent experiments. Data were analyzed using the Mann–Whitney *U*-test and ANOVA with GraphPad PRISM 4 software. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Growth inhibition assay

Growth inhibition assay as expression of preliminary cytotoxicity was analyzed for HUVECs incubation for 24, 48 and 72 h with different concentrations of drug: nandrolone, 5–100 μM; norandrostenedione, 125–750 μM; norandrostenediol, 50–6000 μM; testosterone 10–200 μM; and androstenedione, 50–400 μM. The value of IC_{50} (μM) for each drug was determined at 72 h (Table 1). As reported in Fig. 1, nandrolone expressed an $IC_{50} = 9(\pm 0.1)$ μM; norandrostenedione, $IC_{50} = 500(\pm 30)$ μM; testosterone, $IC_{50} = 100(\pm 0.2)$ μM; androstenedione, $IC_{50} = 375(\pm 20)$ μM. The IC_{50} value for norandrostenediol was not reached until a concentration of 6000 μM.

Table 1
IC_{50} (μM) values of drugs on HUVECs

	HUVECs
Nandrolone	9 ± 0.1
Testosterone	100 ± 0.2
Androstenedione	375 ± 20
Norandrostenedione	500 ± 30
Norandrostenediol	>6000

Data are the mean ± S.D. of three independent experiments performed in triplicate.

3.2. Apoptosis assay

To determine if the inhibition of the proliferation was associated with a process of late death, we cultured HUVECs for 72 h in the presence of each drug at the IC_{50} concentration (see above). Using flow cytometry, we found the highest percentage of apoptotic cells was induced by testosterone (31%), followed by norandrostenedione (25%), androstenedione (15%), and finally nandrolone (18%) (Fig. 2). Exposure of HUVECs to norandrostenediol did not promote apoptosis.

3.3. Intracellular levels of calcium

The intracellular basal level of calcium in untreated HUVECs was 100.2(±9.1) nM. When HUVECs suspensions were exposed to nandrolone (9 μM), testosterone (100 μM), and norandrostenedione (500 μM) we observed a significant increase in $[Ca^{2+}]_i$ that reached 140.4(±13.0), 149.8(±9.1), and 144.8(±8.7) nM, respectively. The effect of AAS on $[Ca^{2+}]_i$ was detected 10–20 s after direct exposure to each compound in spectrofluorimetric cuvette of HUVECs. In Fig. 3, data of five independent experiments are reported. In Fig. 4, the kinetic of calcium rice induced by nandrolone 9 μM is reported such as a representative experiment. Approximately 10 s later nandrolone was added to yield final concentration in cuvette of 9 μM,

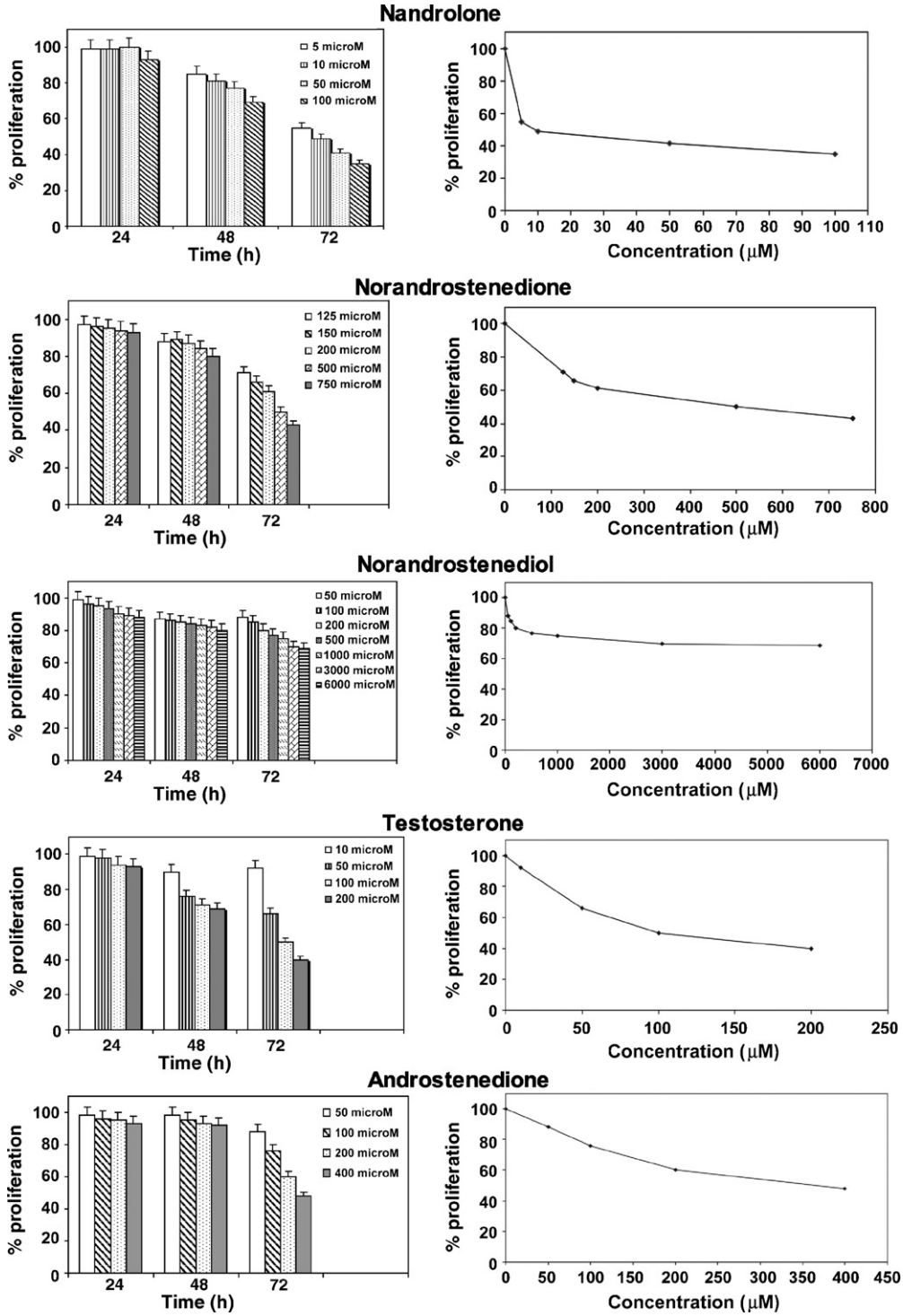

Fig. 1. Proliferation rate of HUVECs. (Left) Endothelial cells were seeded in 96-well plates and then incubated with nandrolone (5–100 μM), norandrostenedione (125–750 μM), norandrostenediol (50–6000 μM), testosterone (10–200 μM) and androstenedione (50–400 μM) for 24, 48, and 72 h. Cell viability was assessed by XTT/PMS as described in Section 2. (Right) Determination of IC_{50} value for each drug after incubation for 72 h. All the drugs reached the concentration required for 50% inhibition of cell growth (IC_{50}), except norandrostenediol, which shows a decrease of about 30% in cell proliferation at a concentration of 6000 μM. The results are expressed as a percentage with respect to untreated cells (control). Data (mean ± S.D.) are representative of at least three independent experiments for each set of conditions.

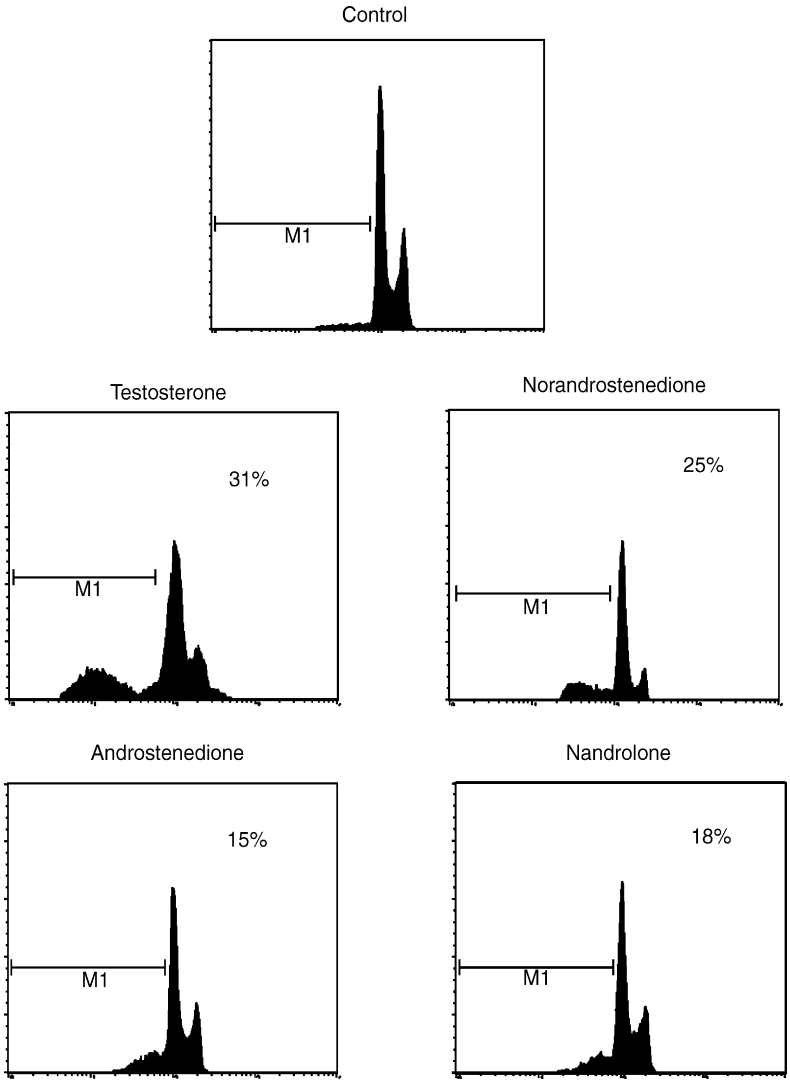

Fig. 2. Flow cytometric analysis of apoptotic cell death. Endothelial cells were cultured in the absence (control) or in the presence of the indicated drugs at a concentration corresponding to the IC_{50} value for 72 h, then the cells were processed as described in Section 2. Right upper panel, percentage of apoptotic cells. Histograms are from one representative experiment of the three performed.

we observed a growing $[Ca^{2+}]_i$ increase until to reach a steady state value of 140.4 nM. No modification of calcium values respect to immediate nandrolone (9 μM) addition was observed when HUVECs were incubated for 1–2 h. Similar results were obtained when HUVECs suspensions were exposed to testosterone (100 μM) and norandrostenedione (500 μM) (data not shown). To investigate whether the drug-mediated $[Ca^{2+}]_i$ rice was

Table 2
Effects of drugs on intracellular $[Ca^{2+}]_i$ (nM) movement in HUVECs

	Time		
	24 h	48 h	72 h
HUVEC	99.4 ± 12.0*	102.8 ± 9.5*	102.0 ± 7.2*
+Nandrolone (9 μM)	137.2 ± 15.0*	134.8 ± 17.9*	141.8 ± 15.6*
+Norandrostenedione (500 μM)	144.2 ± 11.6*	148.2 ± 10.5*	142.8 ± 7.8*
+Testosterone (100 μM)	146.2 ± 12.8*	148.4 ± 13.9*	149.6 ± 9.0*

Data are the mean ± S.D. of five independent experiments at the indicated time of exposure. *$P < 0.05$.

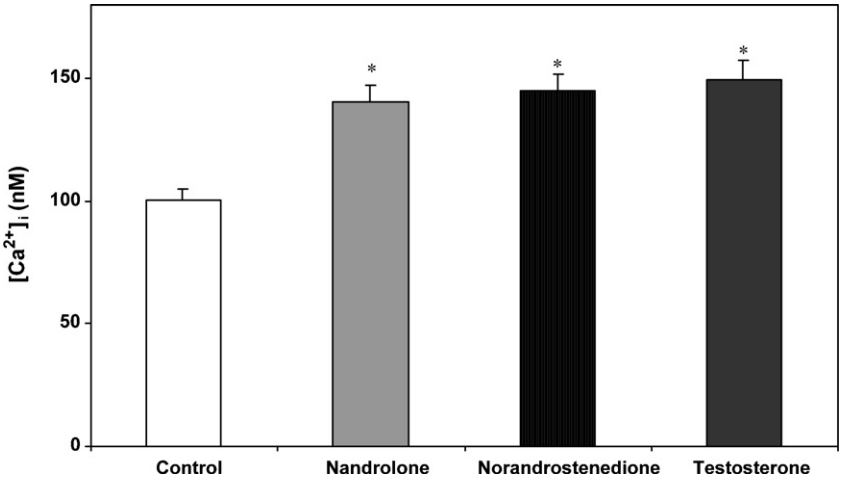

Fig. 3. Acute effects of AAS on intracellular concentration of calcium. Nandrolone (9 μM), testosterone (100 μM), and norandrostenedione (500 μM) added quickly to HUVECs increased the intracellular level of calcium with respect to untreated cells ($^{*}P < 0.05$). Data (mean ± S.D.) are representative of five independent experiments.

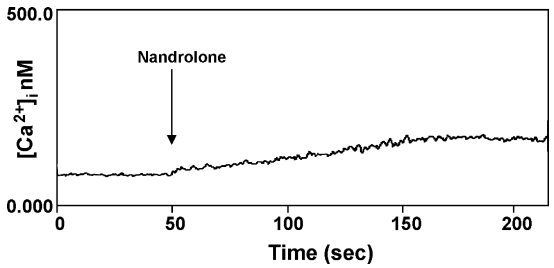

Fig. 4. Effect of nandrolone on calcium signal in endothelial cells. The drug, at a concentration corresponding to IC_{50} (9 μM), caused a growing increase of calcium level until to reach a steady state. Intracellular calcium level was measured as described in Section 2. The trace shown is from a representative experiment.

dependent on a prolonged incubation time higher than 2 h, experiments were carried out incubating HUVECs with each tested drug at concentration corresponding to its IC_{50} for 24, 48 and 72 h (Table 2). The results show a significantly increased level of calcium ($P < 0.05$) for each drug with respect to the baseline level in the control sample, and this observed increase of $[Ca^{2+}]_i$ appears to be independent of incubation time as well as on the steroid tested (Table 2). Furthermore, the reported AAS-mediated $[Ca^{2+}]_i$ modifications are observed only at steroid's concentrations able to determine 50% growth inhibition.

4. Discussion

Although it remains to be ascertained if the relationship between adverse health effects and AAS abuse is a chance observation, some evidence indicates a higher incidence of vascular reactivity alterations and premature cardiovascular complications in individuals using or having used high-dosage androgen without preceding androgen deficiency (McCredie et al., 1998). Many case reports underline cardiomyopathy, lower high-density lipoprotein cholesterol (Sader et al., 2001) levels and pro-atherogenic effect in anabolic steroid users, particularly in body-builders using androgens (Strauss and Yesalis, 1991).

Despite the marked predisposition to vasculature diseases of athletes, the AAS-related effects on endothelium have received little attention.

In this study, we demonstrate that exposure to AAS of HUVECs, the principal cell constituents of the vasculature, alters endothelial cell growth with a strong antiproliferative effect, induces apoptosis, and modifies intracellular levels of calcium. According to these findings, we suggest that the observed endothelial alterations may be considered as events predisposing to serious damage at the cell vasculature level.

Apoptosis is an important mechanism in cell development and differentiation, and its modulation results in cell dysfunction. The integrity and normal function of endothelial cells is indispensable for tissue repair and cell growth (Michiels, 2003). In particular, apoptosis of endothelial cells is considered an initial step in the development of atherosclerosis (Nakano et al., 2006). Recent studies indicate that the increase of $[Ca^{2+}]_i$ plays an important role in apoptosis (Guerini et al., 2005). However, data on the potential relationship between AAS-related apoptosis and altered calcium homeostasis in human endothelial cells have been scarce until now.

Endothelial cells have been widely used as a model to investigate the signal role of intracellular calcium in non-excitable cells. Many endogenous and exogenous substances (hormones, drugs, neurotrasmitters) act by modulating $[Ca^{2+}]_i$ in endothelial cells, and the increase in $[Ca^{2+}]_i$ is a sensitive and early indicator of endothelial cell activation (Jaffe et al., 1987). Endothelial activation is associated with a wide range of functional changes, including the release of procoagulant proteins (e.g. von Willebrand factor (vWf) and factor VIII), anticoagulant proteins (e.g. tissue plasminogen activator (t-PA), tissue factor pathway inhibitor (TFPI) and protein S (PS)), and vasoactive and inflammatory proteins (endothelin-1), calcitonin gene-related peptide (cGRP) and interleukin-8 (IL-8) (Zupancic et al., 2002). Thus, calcium plays an important role as an intracellular messenger in signal-transduction systems.

Here, we provide evidence that nandrolone, testosterone, and norandrostenedione are able to induce an increased $[Ca^{2+}]_i$ that seems to be independent of incubation time and compound identity. We suggest that this modified level of calcium might be induced by AAS binding to endothelial membrane-specific receptors that become activated. Subsequently, this binding might trigger the event cascade starting calcium release for storage compartments. It has been hypothesized that there is a messenger mediating depletion of intracellular Ca^{2+} stores to plasma-membrane Ca^{2+} channels (Takemura et al., 1989). Several investigators suggest a small phosphate-containing molecule (Randriamampita and Tsien, 1993; Parek et al., 1993) as a candidate for the putative messenger for the depletion-dependent Ca^{2+} entry. In skeletal muscle cells, both aldosterone and testosterone produce rapid increases of intracellular calcium involving release of calcium from inositol 1,4,5-trisphosphate (IP3)-sensitive stores (Estrada et al., 2003).

In this study, we did not analyse whether calcium increase was dependent on a modified membrane Ca^{2+}-influx, but it is conceivable that the initial depletion of Ca^{2+} from the intracellular stores could trigger the capacitative Ca^{2+}-influx through the plasma-membrane (Putney, 1990). Calcium signals elicited by steroid hormones may have an important role in the regulation of unknown process(es) in endothelial cells. However, the increase of calcium observed in our system appears to be insufficient to induce a massive modification of the level of intracellular calcium, but it may be considered as an early activation predisposing to subsequent endothelial damage.

The intracellular events accounting for the changes observed in our cell system require further studies. Experiments are in progress designed to examine the mechanisms that regulate the endothelial Ca^{2+} signalling events induced by AAS.

The deleterious effects of AAS reported in this study are induced by high levels of steroids. A limitation of our study is the lack of a direct and quantitative measurement of AAS in body-builders' serum due to their notorious reluctance to participate in medical research. However, taken together, this *in vitro* study could be a real representative condition consequent to a prolonged systemic AAS exposure similar to that may occur in athletes taking anabolic-androgenic steroids.

Acknowledgement

This work was supported by grants from Ministero della Salute to VD, AP and GSJ.

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