Endothelin-1 as a potential marker of melatonin’s therapeutic effects in smoking-induced vasculopathy

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Aims: Smoking is a significant independent risk factor for cardiovascular disease. Among the chemicals present in the cigarette smoke, nicotine is responsible for much of the damage; it induces marked vessel morphological dysfunction and vasoconstriction. Unfortunately, pharmacological or behavioural treatment is not useful against cigarette smoking. The purpose of this study is to test, in experimental conditions, the therapeutic ability of exogenous melatonin administered after smoking-induced vasculopathy and to evaluate the targets of its effects.

Main methods: Nicotine was orally administered for 28 days. Thereafter, the rats were orally treated with melatonin for another 28 days. Vessel damage, an important vasoconstrictor peptide (endothelin-1) and the oxidative stress markers were analysed.

Key findings: Nicotine treatment induced marked endothelial damage and an obvious vasoconstriction in the aorta as evaluated by an increased endothelin-1 (ET-1) expression. These alterations were correlated with a reduction of endothelial nitric oxide synthase (eNOS) and superoxide dismutase (SOD) and with increases of heat shock protein (Hsp70) and inducible nitric oxide synthase (iNOS) activities. Melatonin not only improved the impairment of endothelial-dependent relaxation, but also induced the increase of eNOS and SOD and the reduction of iNOS and Hsp70.

Significance: The findings indicate that nicotine is associated with an elevated synthesis of the vasoconstrictor peptide (ET-1); it also induces a reduction of nitric oxide-mediated vasodilatation (eNOS) and promotes oxidative stress in the vessel wall. We propose that melatonin should be considered as a therapeutic intervention for smokers since it reduces vasoconstriction and oxidative stress and improves endothelial physiology.

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Introduction

Epidemiological studies suggest that cardiovascular diseases account for over one-third of deaths of cigarette smokers (Argacha et al. 2008; Balakumar et al. 2008). For smoking-induced damage at the cardiovascular level, it is possible to distinguish immediate effects (acute) and those that appear later (chronic). The acute effects are observed immediately after smoking and are caused mainly by nicotine, while the chronic changes are correlated with a series of complex hemorheological modifications, as well as alterations of the vascular wall (Nicita-Mauro et al. 2008).

Nicotine is a natural active alkaloid present in tobacco that is oxidized to its metabolite cotinine and rapidly absorbed into the circulatory system (Balakumar et al. 2008). It produces structural and functional changes in endothelial and vascular smooth muscle cells, which are considered as early events in the pathogenesis of cardiovascular disease (Luo et al. 2006).

A number of studies suggest that exposure to cigarette smoke leads to morphological alterations of the endothelium, which appears irregular with membrane disturbances (Pittilo 2000). Studies also provide evidence that cigarette smoke alters vascular endothelium not only morphologically, but also functionally, affecting endothelium-dependent arterial dilatation (Bernhard et al. 2005; Rahman et al. 2007). Moreover, it is known that the vasoconstrictor proteins as well as the endothelial dysfunction mentioned above as well as the production of reactive oxygen species (ROS) are the manifestation of the acute smoke effects induced by nicotine (Sener et al. 2005; Cicha et al. 2008). The exact mechanisms by which nicotine induces these alterations are not well understood. From a physiologic perspective, endothelin-1 (ET-1) and nitric oxide (NO) have been reported to be the main mediators of vascular tone, while heat shock proteins (Hsp) protect against cytotoxic agents (Geetanjali et al. 2002). Other markers of cellular injury are the radical scavenging enzymes including catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx). A decrease on the activities of these enzymes...
leads to the excessive availability of both ROS and reactive nitrogen species (RNS) which cause cellular injury (Sener et al. 2005; Baykan et al. 2008).

Melatonin, an endogenously produced indoleamine, is a remarkably functionally pleiotropic molecule (Reiter et al. 2010) which functions as a highly effective antioxidant and free radical scavenger (Paulis and Simko 2007; Gitto et al. 2009; Hardeland et al. 2009; Paradies et al. 2010; Korkmaz et al. 2009). Endogenously produced and exogenously administered melatonin has beneficial actions on the cardiovascular system (Reiter et al. 2009; Domínguez-Rodríguez et al. 2010).

Recently, our group showed that melatonin is a powerful preventive treatment to tissue injury induced by cigarette smoke suggesting that melatonin may be useful for preventing structural and functional alterations in the vasculature (Rodella et al. 2010). Generally, however, there is a paucity of information on melatonin effects resulting from nicotine-induced injury. Nicotine causes both psychological and physical dependence (Nicita-Mauro et al. 2008) and currently numerous strategies, both behavioural or pharmacological, are used to break the dependence but none have been totally successful.

The aim of the present study was to evaluate the effects of melatonin on nicotine-induced damage in rats considering some of the primary vascular parameters previously reported. Moreover, we tested the hypothesis that ET-1, in parallel with the attenuation of endothelium-dependent vasodilatation by elevated eNOS and reduced iNOS, also modulates the production of ROS. A reduction in ROS could induce, in turn, a drop in SOD and Hsp70.

**Materials and methods**

**Animal care and experimental treatments**

Forty male Wistar rats (Harlan Laboratories s.r.l., Udine, Italy) weighing 200–250 g were housed in an animal experimental unit with 12 h alternating light–dark cycle and constant temperature. The animal facility humidity was controlled and light–night was completely eliminated during the 12-h dark-phase period.

Protocols were approved by the Italian Ministry of Health and complied with *Guiding Principles in the Use of Animals in Toxicology*, which were adopted by the Society of Toxicology in 1989.

The rats were treated with oral administration of nicotine or melatonin in the drinking water. Nicotine (Sigma Aldrich, Milan, Italy) was orally administered at a dose of 100 μg/ml/day according to Lindenblatt et al. (2007), while the melatonin (kindly provided by Nathura s.r.l., Reggio Emilia, Italy) was given at a dose of 5 mg/kg/day. The rats were randomly divided into four groups (each of 10 animals): group I served as control (CTR) without treatment with either molecule (for 56 days); group II was treated only with melatonin for the first 28 days; group III was treated only with nicotine for the first 28 days and group IV was given nicotine for 28 days and thereafter given melatonin for additional 28 days. At the end of the study, the animals were killed by decapitation and the abdominal and thoracic aorta including the aortic arch were removed. The tissues were fixed in 10% neutral formalin for 48 h and then embedded in paraffin wax using a standard protocol. Twenty serial sections (7 μm thick) of each biopsy were cut using a microtome and used for both the histological and immunohistochemical analyses.

**Histological analyses**

Alternate sections were processed for hematoxylin-eosin and Verhoeff–Van Gieson stainings respectively according to Wilson and Gamble (2002) and Verhoeff (1908). Then, we examined these sections with an optical light microscope (Olympus, Hamburg, Germany) at a final magnification of 20×.

**Immunofluorescence analysis**

Alternate sections were deparaffinized, rehydrated and incubated in 1% bovine serum albumin (Sigma Aldrich, St. Louis, MO, US) for 2 h at room temperature. To obtain a double staining, the sections were incubated simultaneously with polyclonal goat antibody against ET-1 (diluted 1:100; N8: sc-21625; Santa Cruz Biotechnology, Santa Cruz, CA, US) and with polyclonal rabbit antibody against eNOS (diluted 1:70; AnaSpec, San Jose, CA, US) or with rabbit polyclonal antibody against inducible NOS (diluted 1:40; N-20: sc-651; Santa Cruz Biotechnology, Santa Cruz, CA, US) and with mouse monoclonal antibody against Hsp70/Hsp72 (diluted 1:40; C92F3A-5; Stressgen, Ann Arbor, MI, US) for 1 h at room temperature and overnight at 4 °C. Thereafter, sections were labeled using anti-mouse Alexa Fluor 546 and anti-rabbit Alexa Fluor 488 conjugated secondary antibodies (1:200, Invitrogen, UK). Finally, the samples were counterstained with DAPI, mounted and observed with a confocal microscope (LSM 510 Zeiss, Germany) at a final magnification of 40×. The immunofluorescent control was performed by omitting the primary antibody and in the presence of isotype-matched IgGs.

**Immunohistochemical analyses**

Alternate sections were deparaffinized in xylene, rehydrated in descending concentration of ethanol solutions and subjected to antigen retrieval in 0.05 M sodium citrate buffer (pH 6.0) in a microwave oven for 2 cycles of 5 min at 650 W and 1 cycle of 3 min at 400 W (Rodella et al. 2006). Endogenous peroxidase activity was blocked by incubation with a solution of 3% hydrogen peroxide in methanol for 30 min. The sections were then incubated with appropriate normal serum (Vector Labs., Burlingame, CA, US) for 1 h and successively with mouse monoclonal antibody against Hsp70/Hsp72 (diluted 1:40; C92F3A-5; Stressgen, Ann Arbor, MI, US) and rabbit polyclonal antibody against SOD (diluted 1:500; FL-154: sc-11407; Santa Cruz Biotechnology, Santa Cruz, CA, US) for 1 h at room temperature and overnight at 4 °C. After incubation in primary antibodies, the sections were sequentially incubated in appropriate biotinylated immunoglobulins, avidin–biotin peroxidase complex (Vector Labs., Burlingame, CA, US) and in a solution of 0.05% 3-3-diaminobenzidine tetrahydrochloride (Sigma Aldrich, St. Louis, MO, US) and 0.33% hydrogen peroxide. All sections were finally counterstained with hematoxylin, dehydrated and mounted. Control reactions were performed by omitting the primary antibody and with isotype-matched irrelevant IgGs as the negative control.

**Statistical analyses**

The data related to Hsp70 and SOD-1 were evaluated using an optical light microscope (Olympus, Hamburg, Germany) at a final magnification of 20× with the investigator blinded to the treatment. Digitally fixed images of slices were analysed using an image analyser (Image Pro-Plus, Milan, Italy) and were calculated as percentage of area in 20 random fields with the same arbitrary area for each section.

The data were pooled to give a mean value ± standard deviation (SD) and a statistical analysis was applied to compare the results obtained from the different samples. All data were analysed by ANOVA and Bonferroni’s test. A P-value less than 0.05 was considered statistically significant.

**Results**

The morphological analysis did not show differences between aorta and aortic arch in any groups. Thus, we merely identified aorta and aortic arch with the term “aorta”. Moreover, there were no differences between the aortas obtained from the control and melatonin-only treated rats.
**Fig. 1.** Photomicrographs showing hematoxylin–eosin staining in rat aortas: (A) nicotine-treated animals; (B) control group; (C) nicotine+melatonin treatment, after nicotine. L=lumen; M=media; (bar=20 μm). The arrows indicate endothelial cell detachment in the tunica intima of nicotine-treated animals.

**Fig. 2.** Photomicrographs showing staining of Hsp70 expression (brown staining) in rat aortas: (A) nicotine-treated animals; (B) control group; (C) nicotine+melatonin treatment, after nicotine; (D) graphic showing the statistical analysis (expressed as arbitrary unit, AU) of Hsp70 immunostaining in the aortas in the various groups. L=lumen; M=media (bar = 20 μm); CTR = control; NIC = nicotine; MEL = melatonin. *P<0.05 vs CTR; # vs NIC.
Histological results

Histological examination of the aorta in nicotine-treated rats revealed marked vascular damage with endothelial cell detachment in the tunica intima (Fig. 1A). On the contrary, in control groups and in the nicotine+melatonin treated rats, tunica intima showed a normal morphology (Fig. 1B and C).

Verhoeff–Van Gieson staining showed that nicotine treatment reduced the elastic fibers in the tunica media of the aorta. The fibers appeared fragmented, discontinuous and visibly thinner compared to thick and regular elastic fibers seen in the control rats. In contrast, the aorta of the nicotine+melatonin treated rats were morphologically similar to those of the controls (data not showed).

Hsp 70 and SOD immunohistochemistry and statistical analysis

Hsp70 immunohistochemistry documented that nicotine administration induced high levels of this protein in the tunica media, whereas minimal levels were seen in the aortas from the control rats and those given both nicotine and melatonin (Fig. 2A, B, and C). Statistical analysis of Hsp70 is shown as histogram in Fig. 2D.

As depicted in Fig. 3, immunohistochemical staining and statistical analysis for SOD revealed a reduction in the tunica intima and media in the aortas from the nicotine-treated animals compared to those in the other groups.

ET-1/eNOS double immunofluorescence

As depicted in Fig. 4A, B, and C, the red staining identifies eNOS expression, whereas ET-1 is stained green. Immunofluorescence analysis revealed that nicotine induced an increase in ET-1 expression in some endothelial cells of the tunica intima as well as in the tunica media and tunica adventitia (Fig. 4A). In regard to eNOS expression, in nicotine-treated animals a reduction of this protein in tunica intima was apparent (Fig. 4A). Images from control and nicotine+melatonin treated aortas did not show any positive staining in tunica media for ET-1 and a moderate positivity in tunica intima for eNOS (Fig. 4B and C).

Fig. 3. Photomicrographs showing SOD expression (brown staining) in rat aortas: nicotine-treated animals (A), control group (B); nicotine+melatonin treatment after nicotine (C); (D) graphic showing the statistical analysis (expressed as arbitrary unit, AU) of SOD immunostaining in the aortas in the various groups. L=lumen; M=media; (bar=20 μm); CTR=control; NIC=nicotine; MEL=melatonin. *P<0.05 vs CTR; # vs NIC.
In Fig. 4D, E, and F, the red stain identifies Hsp70 expression, whereas the green positivity indicates iNOS. Immunofluorescence analysis showed that nicotine induced a rise in Hsp70 and iNOS expression. In particular, we found that the Hsp70 was elevated in the tunica media, whereas iNOS levels were found both in some endothelial cells and in the tunica media (Fig. 4D). Aortas from control rats and those given both nicotine and melatonin could not be distinguished from each other (Fig. 4E, and F). The expression of these proteins was very weak in these latter groups.

The data related to Hsp70 immunofluorescence was also confirmed by the immunohistochemical analysis, as mentioned above.

**Discussion**

Nicotine exposure due to chronic or “chain” cigarette smoking is a known cause of cardiovascular disorders in most industrialized nations. Indeed, vascular endothelial dysfunction is a hallmark of the various cardiovascular disorders (Balakumar and Kaur 2009). Our results confirm and extend the previous data showing that the administration of nicotine for 28 days induces injury in the major vessels, i.e., aorta and aortic arch of rats. These alterations include changes in the tunica intima and media with increased levels of ET-1 and reduced expression of eNOS and SOD. These data are consistent with the results of others (Noma et al. 2005) suggesting that in smokers a marked reduction of the endothelium-dependent vasodilatation occurs.

Although eNOS is a constitutively expressed enzyme, its expression is regulated by a number of biophysical, biochemical and hormonal stimuli, both under physiological and pathological conditions. Shear stress, growth factors, hormones including estrogens, insulin, angiotensin II, and ET-1 and other molecules modulate eNOS expression (Li et al. 2002). We found a rise in ET-1 expression in tunica media of nicotine-treated rats with a reduction of eNOS in the intima. Thus, considering the close relationship between ET-1 and eNOS, we suggest that ET-1 is a key factor and an initial step in the development of nicotine-mediated injury. Of special interest, ET-1 is released continuously, mostly from endothelial cells, by a constitutive and regulated pathway and contributes to the maintenance of vascular tone (Thorin and Webb 2010). ET-1 and NO are functionally closely interdependent, with a strong inhibitory effect of ET-1 on NO-mediated dilation, including in human coronary and cerebral arteries (Thorin 2001). We suggest that the main role of melatonin is to reduce ET-1 expression, which in turn, leads to a decrease in NO down-regulating eNOS expression and the increase of iNOS. These data are consistent with Zemse et al.’s paper (Zemse et al. 2008) showing that ET-1 decreases eNOS expression. In contrast to the decrease of eNOS expression, iNOS increases as reported by Koh (Koh 2008) in experimental conditions.

Moreover, we found an elevation of ET-1 expression in tunica adventitia of nicotine-treated rats. This finding is in agreement with emerging evidence suggesting that this layer may be a potent source of vasoactive hormones such as growth factors and ET-1, which may regulate vascular structure and function via autocrine or paracrine signalling mechanisms (Di Wang et al. 2010). There is also evidence...
that ET-1 contributes to increased oxidative stress by stimulating the production of ROS, particularly via membrane-bound NAD(P)H oxidase. The rise in ROS may lead to excess radicals thereby diminishing the levels of active antioxidant species (Skalska et al. 2009). A number of studies demonstrated that, in hypertensive and diabetic patients, higher plasma ET-1 levels are independently associated with lower plasma antioxidant status measured by ferric reducing ability of plasma and decreased vitamin C concentration. These changes would be expected to cause increased oxidative stress that is observed in these diseases.

In light of these observations, we examined SOD immunoreactivity and found a reduction of its expression after nicotine-induced damage and an increase after melatonin treatment; this is consistent with cigarette smoking favouring vascular diseases through elevated oxidative stress. With regard to Hsp70, we found an increase in tunica media after nicotine administration; this indicated an attempt of the physiological balance between ET-1 and antioxidant enzymes that control vasocostruction, blood pressure and vascular remodeling.

**Fig. 5.** A proposed explanation for the damage induced by nicotine: 1) nicotine disrupts the physiological balance between vasocostruction, increasing endothelin-1 (ET-1) and vasodilatation, reducing endothelial nitric oxide synthase (eNOS) and elevating inducible nitric oxide synthase (iNOS); 2) nicotine inhibits superoxide dismutase (SOD) expression which induces reactive oxygen species (ROS) production. Thus, ROS are responsible for heat shock protein 70 (Hsp70) induction which protects against cytotoxic agents and regulates vascular smooth muscle cell homeostasis. Melatonin minimizes the nicotine-induced damages, reestablishes the physiological balance between ET-1 and antioxidant enzymes that control vasocostruction, blood pressure and vascular remodeling.

**Conclusion**

Based on experimental evidence relative to the importance of oxidative stress in vascular damage, there has been enormous interest in developing strategies that target ROS and the other hallmarks in the treatment of hypertension and other cardiovascular diseases, i.e., vascular damage induced by nicotine. Therapeutic approaches that we considered in this study include mechanisms that: 1) decrease ET-1; 2) increase NO bioavailability; 3) reduce ROS generation; and 4) elevate antioxidant capacity and inhibit the stress proteins. Thus, the therapeutic intervention with melatonin against nicotine vascular injury could represent a new strategy for improving endothelial function and highlight future therapeutic strategies.

**Conflict of interest statement**

The Authors declare that there are no conflict of interests.

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**References**


