



Original article

Melatonin attenuates thiocyanate-induced vasoconstriction in aortic rings

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ABSTRACT

Cigarette smoking not only has a carcinogenic effect but also leads to an increase in arterial blood pressure. Besides its main components, i.e. nicotine, tar, and carbon monoxide, cigarette smoke also contains thiocyanate. Thiocyanate anions (SCN^-) arise from the detoxification of hydrogen cyanide and its plasma concentrations were found to correlate significantly with cigarette consumption. There is also evidence that atherosclerotic disease progression is much more rapid when serum SCN^- levels are increased. Melatonin, a non-toxic indolamine with various physiologic functions, is believed to protect against inflammatory processes and oxidative stress. It has been demonstrated that melatonin serves as free radical scavenger and represents a potent antioxidant. Therefore, it is believed that melatonin with its atheroprotective effects may be useful either as a sole therapy or in conjunction with others. The aim of this study was to quantify the thiocyanate-induced vasomotor response in aortic tissue and further to examine the potential of melatonin in affecting the generated vasoreactivity. Aortic rings of adult male normotensive Wistar rats were cut into 4-mm rings. Following the administration of thiocyanate in various concentrations, vasomotor response of aortic vessel segments was measured. To assess the effect of melatonin on vasomotor activity, organ bath concentrations were modulated from 60 to 360 pM, which corresponds to physiologic plasma up to the levels of patients with regular oral intake of 3 mg of melatonin as a supplement. Thirty-six rat aortic rings were studied. When exposed to thiocyanate, vessel segments revealed vasoconstriction in a concentration-dependent manner. In rings which were preincubated with melatonin at a concentration of 360 pM, a 56.5% reduction of effect size could be achieved (4.09 ± 1.22 mN versus 9.41 ± 1.74 mN, $P < 0.0001$). Additionally, administration of 360 pM melatonin at a norepinephrine concentration of 80 mM resulted in a relaxation of $10.9 \pm 2.2\%$. The vasodilatory effect of melatonin was significantly reduced to $1.3 \pm 0.5\%$ when concentration of norepinephrine was doubled ($P < 0.002$). This study indicates that vessel segments that were exposed to thiocyanate responded with a dose-dependent vasoconstriction. The effect could be markedly attenuated in segments preincubated in melatonin.

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Abbreviation: SCN^- , thiocyanate.

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1. Introduction

The main components of cigarette smoke are nicotine, tar, carbon monoxide, nitrogen oxides, and hydrogen cyanide. Cigarette smoking is known to be a major risk factor for airway pathologies like chronic obstructive pulmonary disease and emphysema. Furthermore, it has been recognized to have a carcinogenic effect, being responsible not only for malignancies of the respiratory tract (particularly lung and larynx cancer) but also for bladder and pancreatic cancer. Moreover, it also affects the cardiovascular system as it promotes and accelerates atherosclerosis, especially in coronary arteries, aorta, carotid arteries, and arteries of the lower

extremity leading to an increased risk of coronary artery disease, aneurysm enlargement, stroke, and peripheral arterial occlusive disease (Kilaru et al., 2001). Several studies have demonstrated that cigarette smoking leads to an acute increase in heart rate (Tachmes et al., 1978; Koch et al., 1980) and arterial blood pressure (Grassi et al., 1992; Rhee et al., 2007) induced by systemic vasoconstriction (Zhu and Parmley, 1995; Benowitz, 2003). Vasomotor dysfunction and impairment of vasodilatation are the earliest manifestations of atherosclerotic changes in a vessel (Ambrose and Barua, 2004).

Thiocyanate anions (SCN^-), a component of cigarette smoke, arise from the detoxification of hydrogen cyanide (Spagnolo et al., 1988) and these plasma SCN^- concentrations were found to correlate significantly with self-reported daily cigarette consumption (Vesey et al., 1982). The level of hydrogen cyanide in mainstream cigarette smoke ranges from 10 to 400 μg per cigarette in US brands (Fiksel et al., 1981; Mahernia et al., 2015) whereas in non-filtered cigarettes the levels were between 400 and 500 μg (Hoffmann and Hoffmann, 2001). In contrast, the amount of nicotine per cigarette ranges from 1000 to 3000 μg (Hoffmann and Hoffmann, 2001). There is also evidence that atherosclerotic disease progression in the aorta is much more rapid when serum SCN^- levels are increased (Scanlon et al., 1996; Botti et al., 1996). Because of the link between elevated serum SCN^- concentrations and atherosclerotic disease, the effect of SCN^- has been of particular interest (Li et al., 1999).

Melatonin is a tryptophan-derived, multifunctional, non-toxic indolamine that is mainly produced by the pineal gland, but also by other extrapineal organs. It is released from the pineal gland in a circadian manner resulting in low circulation levels during the day and high concentrations during the night (Dominguez-Rodriguez, 2012). Melatonin regulates a variety of physiological functions (Reiter et al., 2007) and has been attributed to be uncommonly effective in reducing oxidative stress (Reiter et al., 2016). One of its pleiotropic activities is the capability of scavenging not only oxygen- but also nitrogen-based reactants (Korkmaz et al., 2009) leading to a reduction in oxidative damage (Manchester et al., 2015). Therefore, Tan and associates (2015) entitled melatonin a potent and inducible endogenously-occurring antioxidant. Besides its antioxidative properties, melatonin also has an anti-inflammatory potential (Mauriz et al., 2013). As oxidative stress and inflammation are important causative factors in atherosclerosis formation (Favero et al., 2014), it is believed that melatonin with its atheroprotective effects may be useful either as a sole therapy or in conjunction with others (Korkmaz et al., 2009). In addition, melatonin is also involved in vasomotor control (Hardeland et al., 2011) as it has the potential to decrease the aortic contractile responses to phenylephrine (Ilhan et al., 2015). In the aorta with its elastic properties, changes in the aortic wall structure can lead to increased aortic stiffness which is known to be associated with several vascular diseases and predictive of increased cardiovascular mortality (Amar et al., 2001). Apart from the aforementioned abilities, melatonin develops its beneficial effects also against toxic agents including nicotine (El-Sokkary et al., 2007; Esteban-Zubero et al., 2016). Sener and coworkers (2004) reported that melatonin administration to nicotine-treated rats caused a marked reduction in the microscopic damage of the aortic tissues compared to those of the untreated group. Rodella et al. (2010a) described beneficial effects of melatonin in nicotine-induced vasculopathy by blocking the activation of ERK. The same group also reported about the reduction of vasoconstriction and improvement of endothelial physiology by melatonin (Rodella et al., 2010b). In a recent study, the protective role of melatonin supplementation against nicotine-induced liver damage in mice has been reported (Mercan and Eren, 2013). The presented study was thus undertaken to quantify the

thiocyanate-induced vasoconstriction in rat aortic segments and further to examine the potential of melatonin in attenuating the generated vasomotor response.,

2. Methods and materials

2.1. Animals and preparation of vessel segments

Experiments were carried out in sixteen adult male normotensive Wistar rats grown to 200 g and approximately 6 weeks of age. These rats were housed in a room with constant temperature of 24 °C and kept under light from 08:00 to 20:00 during the day. Rats had access to food and water ad libitum. After about one week of housing they were sacrificed by decapitation, the abdominal aorta was carefully removed. Collected aortas were dissected and freed from adhering fat and surrounding connective tissue. The abdominal aortas were cut into small segments of 4 mm in lengths. Special care was taken to avoid damage of the endothelium. Material from each rat was split, to use for its own control, in order to decrease the chance that differences could be caused by investigating different animals. Investigations were designed in accordance with the guidelines of the local ethics committee.

2.2. Organ bath procedure and vasomotor responses

Each vessel segment was mounted in a temperature-controlled 15 mL tissue bath (37 °C) containing a modified Krebs–Henseleit buffer solution (118 mmol/L NaCl, 20 mmol/L NaHCO_3 , 4 mmol/L KCl, 1.25 mmol/L CaCl_2 , 1.2 mmol/L NaH_2PO_4 , 1.2 mmol/L MgSO_4 , 4.5 mmol/L glucose, and 10 U/L insulin) as described and used previously (Plass et al., 2012a,b). The bath was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 , giving a pH of 7.40. The buffer solution was continuously renewed from a reservoir using a peristaltic pump (Ismatec REGLO Digital, IDEX Germany) at a steady-state flow of 1.5 mL/min, while excess volume was drained. To measure the isometric circular wall tension of the vessels, each segment was suspended between 2 L-shaped metal pins (0.4 mm in diameter) in a myograph, as initially described by Mulvany and Halpern (1976). To achieve maximal active force development, the vessels were initially stretched to equalize 90% of L100 (L100 = the distance between the pins if the vessel is exposed to a passive transmural pressure of 13.3 kPa). After approximately one hour, the vessels were maximally contracted with norepinephrine at a steady-state concentration of 50 μM . Only vessel segments responding with a reproducible contraction after wash-out with the normal buffer solution were used for further investigation. Vessel segments were precontracted to approximately 50% of their maximum contractile capacity with norepinephrine. This contraction, induced by norepinephrine, was set arbitrarily to 100% and used as an internal standard with which the relaxant/constrictant response in the same vessel segment was compared. Maximum endothelium-dependent relaxation was regularly achieved by bolus application of 10 nmol/L of substance P directly into the organ bath in order to verify preserved endothelial responsiveness. Afterwards, the rings were exposed to SCN^- , ranging from final concentrations in the organ bath of 10^{-6} to 10^{-1} M (Hill et al., 1983) using a peristaltic dosing pump. To assess the effect of melatonin on vasomotor activity, organ bath concentrations were modulated from 60 to 360 pM, which corresponds to physiologic plasma up to the levels of patients with regular oral intake of melatonin as a supplement (Kotlarczyk et al., 2012). In accordance to their study, we also used 3 mg of melatonin in order to achieve an equivalent effect. Experiments were also conducted after preincubation with melatonin (360 pM) and exposure to SCN^- shortly thereafter. Furthermore, the effect of melatonin on aortic rings pre-

contracted with 80 mM, as well as 160 mM, norepinephrine was investigated. Unchanged sensitivity of the smooth muscle preparations to external nitric oxide was secured by sodium nitroprusside mediated vasodilatatory response curves after use. To prevent tachyphylaxis, only one concentration-response experiment was allowed on each artery segment. All reagents were dissolved in ultra-pure water. Melatonin preparations were made freshly and restricted from ambient light. All chemicals were obtained from Sigma-Aldrich (Vienna, Austria).

2.3. Data analysis and statistics

Vessel responses are given in millinewtons (mN) for contraction experiments, and in percentages of the maximal contraction induced by norepinephrine for relaxation experiments. Results are given as mean \pm standard error of the mean (n), where n is the number of vessels, each vessel from a different animal. Statistical analysis was performed by Levene's test for equality of variances followed by an independent-sample t test for equality of means. All statistical analyses were performed using the IBM SPSS Statistic 20 software package (SPSS, Chicago, IL). P-values <0.05 were considered statistically significant.

3. Results

Rat aortic rings with a length of 4 mm with no evidence of macroscopic atheromatous plaque formations (n = 36) removed from twelve rats were analyzed. After being precontracted with norepinephrine, the rings revealed an endothelium-dependent vasorelaxation which was induced by administration of substance P. Subjection of these experimentally proven physiologically intact vessel segments to SCN^- resulted in a vasoconstriction. This effect could be markedly attenuated (56.5% reduction of effect size) in vessel segments that were preincubated with melatonin at a concentration of 360 pM. A representative original trace record is depicted in Fig. 1. In addition, SCN^- -induced contractile reaction also showed a concentration-dependent manner. Vasoconstriction of rat aortic rings in the presence (n = 12) or absence of melatonin (n = 12) was significantly reduced (4.09 ± 1.22 mN versus 9.41 ± 1.74 mN, $P < 0.0001$). Respective curves are given in Fig. 2.

Additionally, a total of twelve rings prepared out of four rat aortas were investigated to determine the vasomotor reactivity of melatonin in vessel segments precontracted with norepinephrine. At a norepinephrine concentration of 80 mM, administration of

increasing concentrations of melatonin resulted in a vessel relaxation of $10.9 \pm 2.2\%$ (n = 6) when up-regulated from 60 pM to 360 pM melatonin. The vasodilatatory effect of melatonin was significantly reduced to $1.3 \pm 0.5\%$ (n = 6) when concentration of norepinephrine was doubled to 160 mM ($P < 0.002$). Fig. 3 illustrates respective vasorelaxation curves.

4. Discussion

In a study performed by Robertson and coworkers (1987) measuring SCN^- concentrations in office workers as a means of validating smoking histories and assessing passive exposure to cigarette smoke, a significant rise in plasma SCN^- corresponding with increasing smoking was obtained. All smoking groups showed mean concentrations greater than non-smokers, except those smoking five or fewer cigarettes a day. They reported SCN^- levels of around 40 $\mu\text{mol/l}$ in non-smokers, whereas smokers consuming more than 25 cigarettes per day had concentrations approaching 160 $\mu\text{mol/l}$. In case of cessation of smoking, increased concentrations of SCN^- return to normal quite slowly, usually after 14 days.

Regarding the vasoreactive potential of SCN^- , it has been reported that SCN^- substitution for chloride in the extracellular environment induces a contractile response in vascular smooth muscle of isolated rat aortas. After changing of the incubation medium from normal chloride-containing to a SCN^- -modified Krebs-Ringer bicarbonate solution, resting tension increased dramatically. This effect could not be inhibited or attenuated by specific adrenergic, cholinergic, histaminergic, serotonergic or cyclooxygenase inhibitors (Zhang et al., 1990). The described SCN^- -induced contraction of vascular smooth muscle was also observed in rat portal veins (Zhang et al., 1991). However, incubation in the absence of extracellular Ca^{2+} abolished the SCN^- -induced contractile responses (Standley et al., 1996; Li et al., 1999). Engström and Sehlín (1996) observed similar vasoconstrictory effects of SCN^- in their rat tail artery perfusion model.

On the other hand, melatonin is known to serve as a modulator in a variety of cytokines and, when administered exogenously, is quickly distributed throughout the organism. Therefore, its potential clinical applicability and possible role as a disease-modulating therapeutic agent in different pathological conditions (Rodella et al., 2010a,b) was proposed. Recently, the beneficial effects of melatonin due to its free radical scavenging capability, anti-inflammatory properties, and positive influence on the activity of

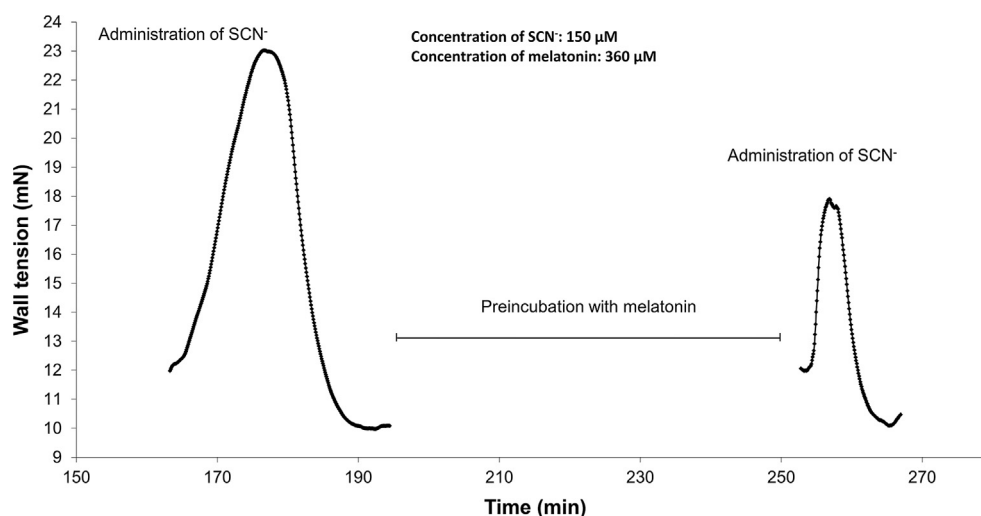


Fig. 1. Original trace of vessel segments exposed to thiocyanate (SCN^-) with and without preincubation of melatonin.

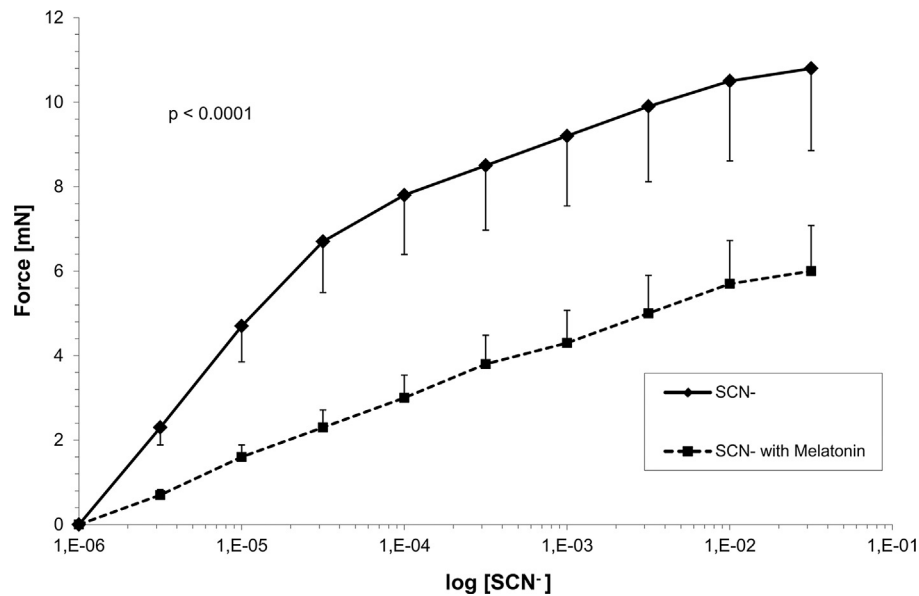


Fig. 2. Dose-dependent constriction of vessel segments exposed to thiocyanate (SCN^-) in the presence or absence of melatonin.

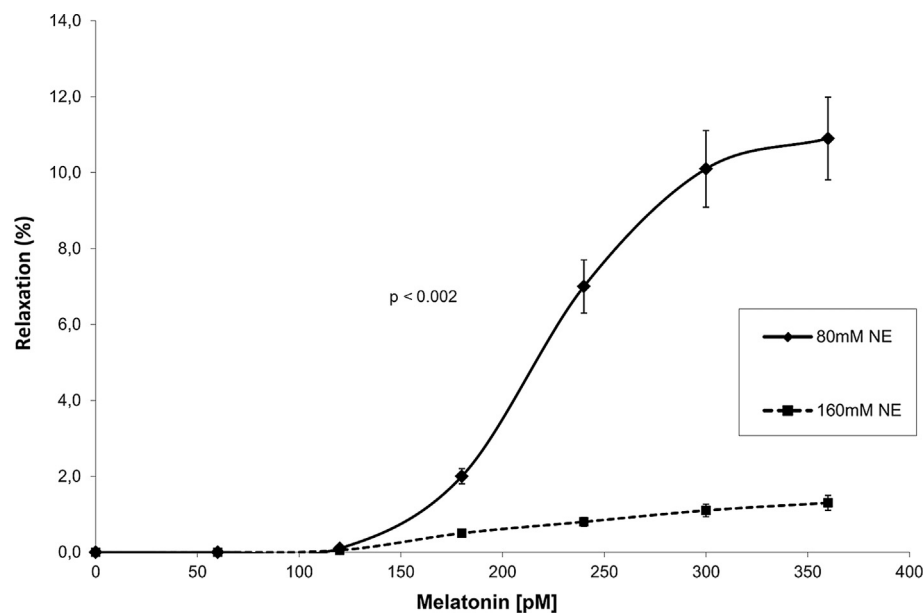


Fig. 3. Increasing vasodilatory effect of melatonin in vessel segments precontracted with norepinephrine (NE) at different concentrations.

antioxidative enzymes have been shown in clinical settings for several chronic diseases, such as rheumatoid arthritis (Forrest et al., 2007) and diabetes (Peschke, 2008). In addition, an antihypertensive and lipid normalizing effect of melatonin could be demonstrated in patients with metabolic syndrome (Koziróg et al., 2011). Furthermore, it has been reported that melatonin positively influences neurobehavioral (Di Paolo et al., 2014) and neurochemical changes (Allagui et al., 2014) in the central nervous system. In the presented study, we elucidate that melatonin also has a functional vasoreactive effect as it significantly influences SCN^- -induced vasomotor response in aortic rings. In detail, melatonin attenuates the vasoconstrictory effect of SCN^- by more than 50% when its concentration reaches plasma levels of patients with regular intake of a licensed product. The underlying biochemical pathways are already investigated and described in detail elsewhere (Rodella et al., 2010a,b).

Being an effective antioxidant, melatonin has also proven to have a significant anti-inflammatory potential on the cardiovascular system (Singh and Jadhav, 2014). Its beneficial effect by alleviating the inflammation as well as oxidative stress has been reported in hypoxia-induced vascular injury (Hung et al., 2013) and nicotine-induced vasculopathy (Rodella et al., 2010a,b). However, it is known that the production of melatonin experiences an age-related decline. In addition, Reyes-Toso and coworkers (2007) reported that advanced age and the absence of an intact endothelium augmented the contractility response of aortic rings to vasoconstrictory substances. Therefore, elderly individuals, and especially individuals of advanced age with atherosclerotic diseases and who smoke or have a history of smoking may benefit from elevation of their reduced melatonin levels. This hypothesis is supported by data from Hung et al. (2013) who concluded that melatonin is protective against hypertension and endothelial dys-

function via an antioxidant and anti-inflammatory mechanism. In addition, recently published data suggest that melatonin could prevent the cigarette smoke-induced restenosis in rat carotid arteries after balloon injury and the mechanism of its protective effect may be the inhibition of the inflammatory reaction. This also implies melatonin has potential therapeutic applicability in the prevention of restenosis following vascular angioplasty in smokers (Yang et al., 2014). The presented data, together with the low toxicity and high safety of melatonin, warrant further investigations in this area of vascular pathology.

Author contribution

Alexander M. Prusa and Christian A. Plass contributed to the study design, conducted experiments, performed data analysis, and wrote the manuscript.

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Conflicts of interest

None.

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