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Vascular endothelial cells and dysfunctions: role of melatonin

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1. ABSTRACT

Several pathological conditions, including hypertension, atherosclerosis, diabetes, ischemia/reperfusion injury and nicotine-induced vasculopathy, are associated with vascular endothelial dysfunction characterized by altered secretory output of endothelial cells. Therefore there is a search for molecules and interventions that could restore endothelial function, in particular augmenting NO production, reducing the generation of free radicals and vasoconstrictors and preventing undesired inflammation. The pineal hormone melatonin exhibits several endothelium protective properties: it scavenges free radicals, activates antioxidant defence enzymes, normalizes lipid and blood pressure profile and increases NO bioavailability. Melatonin improved vascular function in experimental hypertension, reducing intimal infiltration and restoring NO production. Melatonin improved the NO pathway also in animal models for the study of diabetes and prevented NO down-regulation and adhesive molecules up-regulation in nicotine-induced vasculopathy. The protection against endothelial damage, vasoconstriction, platelet aggregation and leukocyte infiltration might contribute to the beneficial effects against ischemia-reperfusion injury by melatonin. Therefore, melatonin administration has endothelium-protective potential in several pathological conditions. Nevertheless, it still needs to be established, whether melatonin is able to revert already established endothelial dysfunction in these conditions.

2. INTRODUCTION

2.1. Vascular endothelium

The view on the endothelium has shifted from the early concept of inert cellophane-like membrane through an idea of selective but static physical barrier to the current view of the endothelium as a dynamic, heterogeneous, disseminated organ with secretory, synthetic, metabolic and immunologic functions (1, 2). It is actively involved in the control and regulation of vascular tone, fluid and solute exchange, haemostasis, coagulation and inflammatory responses and therefore the conditions of endothelial cells (ECs) modulate many aspects of the vascular function (3).

Endothelium synthesizes several peptides and biologically active molecules such as nitric oxide (NO), endothelium-derived hyperpolarizing factors (EDHFs), cytochrome P-450, monooxygenase, epoxyeicosatrienoic acids, endocannabinoids (4, 5) and prostacyclin (PGI2) as well as vasoconstrictors such as endothelin-1 (ET-1), endothelium-derived contracting factors (EDCFs), endoperoxides and thromboxane (6, 7). It also produces growth factors, coagulation protein (3, 8) and mitogenic factors (6).

Given the critical role of these mechanisms, endothelial dysfunction is a feature of hypertension, atherosclerosis, diabetes and other unfavourable cardiovascular conditions (9, 10).

2.2. Melatonin

Melatonin (N-acetyl-5-methoxy-tryptamine) was first isolated in 1958 from the bovine pineal gland by Lerner's group (11). Its production is controlled by the suprachiasmatic nucleus (SCN), the central circadian pacemaker (12). It is synthesized in several organs, including the pineal gland, retina, Harder's glands, gastro-enteric mucous membrane, megakaryocytes and platelets, at rates and in quantities, modalities and conditions that vary in relation to the respective organs (13). Melatonin displays high lipid and water solubility which facilitates passage across cell membranes (14). Melatonin is present in several fluids, tissues and cellular compartments (saliva, urine, cerebrospinal fluid, pre-ovulatory follicle, seminal fluid, amniotic fluid and milk). The peak of pineal secretion occurs at night, with maximum plasma levels around 03:00–04:00 a.m., whereas levels during the day period are low or even undetectable (15).

In mammals, melatonin represents the biological signal of darkness, because the duration of its release is proportional to night length. It acts as sleep regulator (16), inhibits dopamine release in the hypothalamus and retina (17), it is involved in the aging process (18) and pubertal development (19), blood pressure control (20), free-radical scavenging (21) and regulation of the immune response (22).

Melatonin has receptor-mediated and receptor-independent effects. Three distinct melatonin receptor subtypes, termed MT1, MT2, and MT3 receptors, have been identified and shown to mediate vascular tone interacting with melatonin (23; 24).Besides its classical endocrine effects, melatonin has autocrine and paracrine actions and also functions as a direct scavenger of free radicals (25).

3. THE ENDOTHELIUM PROTECTIVE EFFECT OF MELATONIN

Melatonin and its metabolites have been shown to scavenge different types of free radicals in *in vitro* systems as well as *in vivo*, both in body fluids and in cells (21, 26, 27, 28). Furthermore, melatonin plays an important role in activating antioxidant defences such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase (29, 30), and protects cells from oxidative load and apoptosis induced by mitochondrial DNA deletion (31).

Both effects allow melatonin to reduce the extent of reactive oxygen species (ROS), improving the outcomes of oxidative related pathologies such as hypertension (32), atherosclerosis (33), cancer (34), ischemia (35) or neurodegenerative diseases (36) and preventing aging (37, 38).

ECs, which play an important role in vasorelaxation, in particular via NO formation, play an important role in cardiovascular conditions including hypertension, diabetes and atherosclerosis (39, 40). Thus, cardioprotective interventions are more effective if they concomitantly improve endothelial function (41, 42).

The antioxidant action of melatonin and its possible interaction with EDHFs, may have contributed to blood pressure lowering effect of melatonin, which was observed even when the NO pathway was inhibited (43).

The effects of melatonin interaction with its receptors and their action on vascular tone are complex: few studies demonstrated that melatonin other than vasodilatative effect in certain arteries, showed also a vasoconstriction effect in others (44).

MT2 receptors were coupled to phosphoinositol hydrolysis with a subsequent increase in cytosolic calcium (45): this, in VSMCs, leads to vasoconstriction, effect that has been overridden by the activation of MT2 receptors in ECs (46), resulting in cytosolic Ca2+ increase in ECs (47, 48).

Tunstall *et al*. (2010) demonstrated that activation of MT2 receptors by melatonin inhibits NO-induced increases in cyclic GMP as well as the ability of the smooth muscle to relax in response to NO (44). The finding that melatonin also inhibits relaxation induced by sodium nitroprusside, which is independent of endothelial nitric oxide synthase (eNOS) (49), suggests a site of action for melatonin other than, or in addition to, eNOS. A likely possibility is that melatonin acts directly on the vascular smooth muscle cells, which express MT2 receptors and are the primary site of action for the vasorelaxing effect of NO. The primary mechanism by which NO relaxes vascular smooth muscle is by increasing intracellular cyclic GMP levels, followed by activation of protein kinase G and the subsequent phosphorylation of several regulatory proteins (50, 51).

In humans, melatonin production not only diminishes with age, but it is also significantly lower in many age-related diseases, including various cardiovascular diseases (52, 53).

The purpose of the following paragraphs will be to provide a summary of the studies about the beneficial effects of melatonin against vascular ECs dysfunction linked to cardiovascular diseases. Nevertheless, these protective effects of melatonin are actually not completely understood and demonstrated. The effect of melatonin on endothelial dysfunction in experimental and clinical hypertension. Hypertension is associated with endothelial dysfunction.

Hypertension is a major risk factor of severe cardiovascular complications (42, 54) and it is clearly associated with endothelial dysfunction (39, 55-57), augmented oxidative load and vascular inflammation (58, 59). The impaired endothelium-dependent vasodilatation in hypertension is characterized by an imbalance between EC-derived vasodilatant and vasoconstrictor factors (56, 57, 60). ROS may be important in the development and maintenance of hypertension, in term of excess production

of oxidants, decreased NO bioavailability and decreased antioxidant capacity in the vasculature (61). Several studies demonstrated that hypertension may be beneficially affected by antioxidants treatment (62, 63) and that these substances may improve vascular functions and structure, prevent target organ damage and reduce blood pressure both in animal model and in human hypertension (62-64).

At this regard, reductions in both vascular oxidative stress and inflammation have been shown to reverse endothelial dysfunction through the administration of antioxidant, such as melatonin or pycnogenol, in an experimental model of genetic hypertension (65, 66).

3.1. The effects of melatonin on endothelium in hypertension

Many experimental and clinical reports have supported the concept that melatonin has anti-hypertensive actions, influencing BP, regulating arterial vasodilatation and exerting protection against oxidative vascular disorders (47, 54, 67, 68-72).

The mechanisms of these effects on BP include the promotion of endothelium-dependent vasorelaxation (68), direct hypothalamic effect, decrease of catecholamine levels and its antioxidant properties (59, 66, 73). The melatonin-induced receptor-mediated vasoconstriction on vascular smooth muscle cells might be counterbalanced by augmentation of the receptor-mediated NO release from ECs, which is further enhanced by anti-oxidant properties of melatonin (68, 74, 75). Melatonin seems to increase NO levels either through the stimulation of NO production and/or the prevention of coupling to the superoxide anion radical (68). Nevertheless, the mechanisms of these antihypertensive effects of melatonin are actually not completely understood.

It is known that the presence of free radicals impairs the crucial Ca2+ signaling process in ECs (47). Pogan and collegues (2002) revealed that the incubation of bovine aortic endothelial cells (BAE), primary cultured vascular ECs from Sprague Dawley rats (SDR) and from spontaneously hypertensive rats (SHR) with melatonin improved the mobilization of internal Ca2+ and agonist-evoked Ca2+ entry in aortic ECs from SHR, while reversing the inhibitory effect of free radicals on the internal release of Ca2+ in SDR and BAE cells (47). Increased cytosolic Ca2+ levels in ECs may result in increased NO production via enhancement of eNOS activity, augmented guanylate cyclase levels and decreased intracellular Ca2+ in smooth muscle cells with subsequent vasodilatation (47, 76, 77). In addition melatonin was able to modulate *in vitro* acetylcholine-induced relaxation or phenylephrine-induced vasoconstriction of aortic rings of aging rats (76, 77-80) and New Zealand rabbits respectively in an endothelium-dependent manner (76).

SHRs show EC dysfunction (81) and a more rapid decline of melatonin production with aging than normotensive rats (82). The treatment of SHR with melatonin resulted in graduate decrease in BP, heart rate and plasma renin activity (54), at least partly by EC-dependent reduction of BP (83) and augmented NO (54, 84).

NG-nitro-l-arginine-methyl ester (L-NAME) administration is a well established model of hypertension (54, 85) and endothelial dysfunction (54, 60). L-NAME hypertension was associated with reduced NOS activity, increased oxidative stress, impaired acetylcholine-induced relaxation and augmentation of endothelial-dependent vasoconstrictor factors. Treatment with melatonin completely prevents oxidative stress, reduces EC dysfunction and moderately slows down hypertension development (54, 60).

Experimental pinealectomy results in EC dysfunction, vasoconstriction (68), elimination of circulating melatonin levels induces a temporary rise in arterial BP (68, 86, 87), unchanged cardiac output (88), which can be prevented by melatonin administration (71, 86).

The administration of melatonin was reported to reduce rise in BP (68, 70, 71, 89). Although the mechanisms of its antihypertensive effects are actually studied. Endothelial dysfunction in human patients as well in animal model have been linked to decrease in NO bioavailability, reflecting the impaired generation of NO and/or the enhanced inactivation of NO by ROS (56).

3.2. The effects of melatonin on endothelial dysfunction in atherosclerosis

Injury to endothelium could lead to increased entry of ox-LDL into the intima from the lumen or it could impair other important endothelial functions, such as anti-thrombotic activity and vascular relaxation (90). In states of melatonin deficiency, excessive ox-LDL induced by oxidative stress increases the risk of atherosclerotic development (91).

Melatonin may inhibit endothelium-derived adhesion molecules formation, reduce fatty acids infiltration in the intimal layer (92), neutralize free radicals (59, 71), and prevent electron leakage from mitochondrial respiratory chain (33). Moreover, melatonin has been shown to reduce plasma levels of total cholesterol, enter the lipidic phase of the LDL particles, preventing lipid peroxidation, and augment the endogenous cholesterol clearance (59). Melatonin *in vitro* also prevents ox-LDL-induced activation of myosin light chain kinase (MLCK), a regulator of EC contraction (93) and permeability (93, 94). On the other hand melatonin only partially inhibited the oxidation of LDL in bovine aortic ECs and did not inhibit ox-LDL toxicity toward cultured ECs (90).

Atherosclerosis might be experimentally induced by cholesterol feeding (95), which is associated with endothelial dysfunction (81, 93). Pita *et al.* (2002) showed that long-term melatonin administration modified the fatty acid composition of rat plasma and ameliorated the fatty infiltration in intima induced by cholesterol feeding (92). Wakatsuki *et al.* (2001) showed that melatonin protected against the ox-LDL-induced inhibition of NO production in the human umbilical artery. These findings support the hypothesis that melatonin acts as a scavenger in these cells and could potentially protect against LDL oxidation (59, 96).

3.3. The effects of melatonin on endothelial injury in diabetes

Vascular complications represent a major cause of morbidity and mortality in diabetic patients (97). Although the specific mechanisms are not completely elucidated, endothelial dysfunction is closely linked to the development of retinopathy, nephropathy, and atherosclerosis in both type 1 (T1D) and type 2 (T2D) diabetes (98). Diabetes-associated endothelial dysfunction is characterized by changes in proliferation, barrier function, adhesion of circulating cells, sensitivity to apoptosis, and altered angiogenic and synthetic properties of ECs (99-101) with impaired endothelium-dependent vasodilatation in various vascular beds of different models of diabetes (102-103) with ROS being involved in the impairment of endothelium-dependent relaxation (78, 104).

Increased plasma glucose levels in diabetes contribute to ECs apoptosis and endothelial dysfunction (105). Insulin resistance causes endothelial dysfunction partly by augmented oxidative load and reduced NO availability (106-108) due to attenuated stimulation of phosphoinositide-3 (PI-3) kinase and subsequent eNOS phosphorylation (109). The endothelial function in diabetic condition is also modified by the augmented oxidative stress, which is linked to diabetes (110, 111). It is implicated in endothelial dysfunction since inhibition of hyperglycemia-induced ROS production prevents the formation of advanced glycation end-products (AGE) (112). The ROS may modify endothelial function by peroxidation of membrane lipids, activation of NF-κB, or by decreasing the NO availability (113). Thus, the restoration of the antioxidant status in diabetic patients should reduce the development of target organ damage. In fact, some antioxidants were shown to reverse EC dysfunction due to oxidative stress (114, 115). The antioxidant action might be responsible for the beneficial effect of melatonin, that normalized NO levels and lipid peroxides in a broad spectrum of diabetic models (104, 116-118).

In insulin resistant mice on high fet diet, 8-week treatment with melatonin oral restored the vascular responsiveness to insulin along with aortic insulin-mediated signaling, such as insulin-induced Akt phosphorylation (119) which leads to eNOS phosphorylation (120) and thus correction of impaired endothelium-dependent vasodilatation in insulin-resistant states (119).
In alloxan-induced diabetes, melatonin reduced the formation of free radicals and β-cell damage in a dose-dependent fashion (121).

In streptozotocin-induced diabetic rats, melatonin started 3 days prior to diabetes induction and continued for another 8 weeks, reduced hyperglycemia and lipid peroxidation (104). On the other hand, melatonin had no effect on diabetic blood glucose levels, when started only after the diabetes induction, while it reduced glucose levels when started few days before streptozotocin injection (115). However, it was still able to attenuate the rise in lipid peroxide levels (122) and to increase antioxidant enzymes (115). It is however still unclear to which extent the vasoactive properties of melatonin (123, 124) might participate in the amelioration of endothelial dysfunction in diabetic rats that display reduced vasodilative responses (78, 125).

In a pancreatectomy model of diabetes, melatonin similarly improved endothelium-dependent vasorelaxations (78, 79). The relaxations were most likely mediated by NO, since they were blunted by NO-synthase inhibition or endothelium removal (78, 79).

3.4. The effects of melatonin on endothelium in ischemia/reperfusion injury

Acute coronary occlusion is the leading cause of morbidity and mortality in the Western world. According to the World Health Organisation, it will be the major cause of death in the world by the year 2020 (126). Melatonin was recently reported to prevent ischemia/reperfusion (I/R) damage in splanchnicand middle cerebral artery occlusion-induced stroke, in traumatic brain injury and in spinal cord injury in rats and mice (127-130).

Cardiomyopathic (CM) Syrian hamsters, a model of dilated congestive cardiomyopathy (131), are characterized by altered coronary microvasculature in which free radicals and NO could cause an increased number of terminal arterioles and a reduced density of capillaries (132). In this model, melatonin reduced leukocyte adhesion, vascular permeability, enhanced capillary perfusion and restored normal arteriolar responsiveness to L-NMMA (a NO-synthase inihibitor), norepinephrine (NE) and angiotensin II (ANG II) (133). Melatonin might reduce vasoconstriction during the ischemic phase via the effects on ROS production and on NO level. It was observed that the vasoconstriction of terminal arterioles was reduced while the length of perfused capillaries was increased. The authors suggested that melatonin can preserve the capillary perfusion through its ability to modulate lipid peroxidative products of ECs leading to deterioration of capillary membrane and function (133).

Beside the generation of ROS, the I/R injury is associated with exaggerated inflammatory response (134). Activated polymorphonuclear leukocytes (PMNs) aggregate and adhere to endothelium, resulting in capillary plugging, impaired blood flow and development of EC edema (135). It was suggested that melatonin can inhibit neutrophil infiltration during postischemic reperfusion (97). Such inhibitory effect on PMNs infiltration was observed in several studies on I/R (136). Recently, melatonin reduced morphological injury and PMNs infiltration in splanchnic artery occlusion shock along with abolished P-selectin expression and intercellular adhesion molecule (ICAM-1) up-regulation on ECs (129).

3.5. The effects of melatonin on endothelial dysfunction in nicotine-induced vasculopathy

Nicotine exposure is one of the most important risk factors for cardiovascular disease and premature death in developed world (137). The acute effects are observed immediately after smoking and are caused mainly by the

direct toxicity of nicotine, while the chronic changes are correlated with a series of alterations of the vascular wall (138). Nicotine produces structural and functional alterations in the vascular endothelium, inducing intimal thickening (139), cell swelling, cytoplasmic vacuolisation and irregularity of the vessel luminal surface (140), membrane disturbances (137) and functional dysfunction, affecting endothelium-dependent arterial dilation (141). Structural damage, a direct toxic effect, a decreased production or bioavailability of endothelial NO, have been proposed as mechanisms of smoking-induced vascular damage (142). 28-day nicotine administration was associated with altered aortic cytoarchitecture, endothelial injury increased ET-1 levels and reduced eNOS expression (143). The elevated ET-1 levels might have also contributed to enhanced ROS formation (144). Melatonin was demonstrated to prevent tissue injury and structural and functional alterations in the vasculature induced by cigarette smoking (145). It has been suggested that melatonin reduced ET-1 expression and thus prevented the downregulation of eNOS and the up-regulation of adhesive molecules on ECs (146). ET-1 and NO are functionally closely interdependent, with a strong inhibitory effect of ET-1 on NO-mediated dilation (147). The main role of melatonin is to reduce ET-1 expression, which in turn, leads to a decrease in production or bioavailability of endothelial NO (142; 143). Melatonin minimizes the damage induced by nicotine, re-establishes the physiological balance between vasodilatation (increasing eNOS) and vasoconstriction (decreasing ET-1 and antioxidant enzymes that control vasoconstriction), promoting vascular remodelling.

Moreover, ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) molecules, that precede monocyte adhesion to the endothelium (148) were up-regulated in the nicotine-treated animals (149). It has been suggested that nicotine activates extracellular signal regulated kinase (ERK)1,2 through its phosphorylation. This enzyme, in turn, activates NF-kB, that translocates into the nucleus where it stimulates ICAM-1/VCAM-1 transcription. These latter promote cell surface expression of the adhesion molecules. Melatonin can be able to minimize the negative effects of nicotine blocking the activation of ERK and the other signalling pathways in which this enzyme is involved (149).

The use of melatonin against nicotine-induced vascular injury represents a novel field for the therapeutic implication of this molecule.

4. CONCLUSIONS

Endothelial dysfunction is associated with a broad spectrum of cardiovascular and metabolic pathologies such as hypertension, atherosclerosis or diabetes. The hallmarks of these conditions include increased formation of ROS, reduced production of NO and exaggerated inflammatory response. Therefore, the pineal hormone melatonin, with its antioxidant and antinflammatory properties is being expected to act cardioprotective in this setting. Indeed, several animal studies and small clinical trials have indicated that the administration of melatonin might at least partly prevent end-organ damage in hypertension, diabetes, ischemia-reperfusion injury or nicotine-induced injury along with preservation of the endothelial function. However, it still needs to be established, whether treatment with melatonin is able to revert already established organ alterations and endothelial dysfunction in these conditions.

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