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Endothelial and vascular smooth muscle cell dysfunction mediated by cyclophylin A and the atheroprotective effects of melatonin

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ABSTRACT

Aims: This study evaluated the role of cyclophilin A (CyPA) in early phase of atherosclerosis and also examined the atheroprotective effects of melatonin due to its antioxidant properties.
Main methods: APOE null mice at 6 and 15 weeks of age were treated with melatonin at a dose of 0.1 mg/kg/day or 10 mg/kg/day. We evaluated both histopathological alterations in endothelial and vascular smooth muscle cells by CyPA and rolling mononuclear cell expression during the early phase of atherosclerosis development.
Key findings: Our study showed that CyPA expression increases and may modulate inflammatory cell adhesion and interleukin-6 expression inducing vascular smooth muscle cell migration and inflammatory cell extravasation in a time-dependent manner. Moreover, we observed an indirect atheroprotective effect of melatonin on vascular injury; it inhibited CyPA mediated inflammatory cell extravasation and oxidative stress.
Significance: The melatonin treatment may represent a new atheroprotective approach that contributes to reducing the early phase of atherosclerosis involving the rolling of monocytes, their passage to subendothelial space and inhibition of CyPA expression.

Introduction

Atherosclerosis is a chronic disease of the arterial wall, and a leading cause of death and loss of productive life worldwide. Research into the disease has led to many compelling hypotheses about the pathophysiology of atherosclerotic lesion formation and of complications such as myocardial infarction and stroke (Libby et al., 2011).

Normally, atherosclerosis is considered a chronic inflammatory disease with monocyte extravasation into the arterial wall being the critical step in its pathogenesis (Ross, 1999). Initially, endothelial cells (ECs), which normally resist attachment of the white blood cells streaming past them, express chemokines and adhesion molecules leading to leukocyte recruitment. Moreover, there are several changes in endothelial permeability and the composition of underlying extracellular matrix beneath tunica intima that induce the entry and the retention of cholesterol-containing low-density lipoprotein particles in the arterial wall (Tabas et al., 2007). These components can promote leukocyte adhesion that increases intracellular cholesterol accumulation. Many chemoattractants direct the migration of the bound leukocytes into the intima and cause maturation of monocytes, which are the most cells recruited, into macrophages. This step determines the formation of foam cells. It is known that the atheromatous lesions have a specific distribution in the arterial tree and this is due to turbulence and different hemodynamic flow patterns and to the ability of normal laminar shear stresses to elicit an antheroprotective program of gene expression by the endothelium (Majesky, 2007). Atheroma formation also involves the recruitment of vascular smooth muscle cells (VSMCs) from tunica media into tunica intima. In the intima, VSMCs produce extracellular matrix molecules and form a fibrous cap that covers the plaque. The plaques cause clinical manifestations by producing flow-limiting stenosis that leads to tissue ischemia or by producing thrombi thereby interrupting blood flow.

The last several decades have witnessed a burgeoning growth of our understanding of the molecular pathways involved in atherosclerosis, lesion progression and the mechanisms involved. Yet, despite these advances, there is no definitive evidence to identify the markers involved in early atherosclerotic processes. So, there is again considerable interest in the monitoring of these pathways as potential biomarkers for atherosclerosis and cardiovascular diseases. Although the risk can be reduced by lowering lipid levels, the equally important contribution of inflammation and reactive species oxygen (ROS) to the development of cardiovascular disease is not adequately addressed by existing therapies (Charo and Taub, 2011). Therefore, identifying the markers warrants continued investigation.

Recently, a number of proteins, defined as secreted oxidative stress induced factors (SOXF), have been suggested as the bridge between inflammation and atherosclerosis. In this regard, cyclophilin A (CyPA), a ubiquitously expressed protein belonging to the cyclophilin family has been highlighted as a major SOXF in atherosclerosis (Yuan et al., 2010). It has been demonstrated with biochemical studies that this protein can be secreted from monocytes/macrophages, ECs and

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VSMCs in response to a variety of inflammatory stimuli (Payeli et al., 2008). In inflammatory diseases such as atherosclerosis, CyPA works as a proinflammatory cytokine and activates ECs and VSMCs to produce inflammatory mediators, including of E-selectin and vascular cell adhesion molecule-1 (VCAM-1) (Satoh et al., 2008). Furthermore, it has been proposed that secreted CyPA stimulates the extracellular signal-regulated kinase 1/2 (ERK1/2) and JAK/STAT pathways in vitro, thereby increasing DNA synthesis in VSMCs (Jin et al., 2000). In addition, to effects on vascular cells, CyPA has been shown to be a chemoattractant for inflammatory cells and promotes activation of matrix metalloproteinases (Satoh et al., 2010). However, the mechanisms that regulate CyPA secretion are still unknown (Suzuki et al., 2006).

As reported above, numerous basic and clinical studies have identified that ROS have a major role in the development of cardiovascular diseases. However, there is no strong therapeutic strategy for clinical benefits of antioxidant administration.

Given the functions of CyPA and the beneficial role of melatonin, an indolamine, mainly secreted by the pineal gland (Stehle et al., 2011), as antioxidant (Galano et al., 2011; Reiter et al., 2010), we hypothesized that CyPA could be a pivotal target for slow down/inhibit the atherosclerotic process using this antioxidant molecule. It is important to note that the amounts of total gastrointestinal melatonin are several hundred times more than those found in the pineal gland (Huether, 1994) and the actions of melatonin are not restricted to its role in the neuroendocrine physiology since it acts as both a direct free radical scavenger (Reiter et al., 2007) and as an indirect antioxidant through the upregulation of antioxidant enzymes and the downregulation of pro-oxidant enzymes (Hardeland, 2005). Moreover, its antioxidant properties were demonstrated in experimental hypertension (Rezzani et al., 2010).

We carried out a series of experiments using APOE null mice (APOE −/−) at 6 and 15 weeks of age; this mouse strain spontaneously develops hypercholesterolemia and atherosclerotic lesions in the aorta in a time-dependent manner (Rodella et al., 2007) and we treated them with the antioxidant melatonin. For this aim, we evaluated both CyPA expression and its signaling pathways, which are involved in atherosclerosis development and the potential atheroprotective effects obtained by melatonin treatment.

Materials and methods

APOE −/− are a murine model that lacks the gene encoding apolipoprotein E (ApoE) and that spontaneously develops hypercholesterolemia and atherosclerotic lesions similar to those found in human (Cola et al., 2010). Whereas C57BL/6 mice have undetectable production of melatonin in the pineal gland and very low-to-undetectable concentrations in plasma (Akel et al., 2009; Otálora et al., 2008; Slominski et al., 2003; Uz et al., 2004), these inbred mice are a natural knockdown for the arylalkylamine N-acetyltransferase (AANAT) gene, that results in the predominant transcription of aberrant isoforms encoding protein(s) without enzymatic activities. However, Vivien-Roels et al. (1998) observed a short-term (30 min) nocturnal melatonin peak in these mice when blood samples were taken every 15 min (Vivien-Roels et al., 1998).

Eighty male mice (Charles River Laboratories S.r.l., Lecco, Italy and Harlan Laboratories S.r.l., Udine, Italy) were housed in an animal experimental unit with 12 h alternating light–dark cycle and constant temperature and fed with standard food and water ad libitum. 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. Mice were randomly divided into eight groups (ten animals each): group I, C57BL/6 mice without treatment and 15 weeks old at sacrifice; group II, C57BL/6 mice without treatment and 6 weeks old at sacrifice; group III, C57BL/6 mice treated with melatonin ARMONIA RETARD 0.1 mg/kg/day from the 6th to 15th week of life; group IV, C57BL/6 mice treated with melatonin ARMONIA RETARD 10 mg/kg/day from the 6th to 15th week of life; group V, APOE −/− without treatment and 6 weeks old at sacrifice (APOE −/− 6w); group VI, APOE −/− without treatment and 15 weeks old at sacrifice (APOE −/− 15w); group VII, APOE −/− treated from the 6th to 15th week of life with melatonin ARMONIA RETARD 0.1 mg/kg/day and group VIII, APOE −/− treated from 6th to 15th week of life with melatonin ARMONIA RETARD 10 mg/kg/day.

The melatonin (kindly provided by Nathura S.r.l., Reggio Emilia, Italy) was administered orally mimic ARMONIA RETARD (RETARD) formulations. RETARD was formulated to release a low dose of melatonin rapidly and a higher dose over a longer period of time (these dose are in a ratio of 1 to 3). In this case, the administration was available in the drinking water every night (from 18.00 to 06.00 h). The melatonin-containing water bottles were removed at 06.00 h and were replaced with bottles of water until noon. From noon to 18.00 h, no drinking water was given to stimulate mice to drink more during the dark period (Rodella et al., 2011).

At the end of the study, all the animals were killed by dislocation and aortic arches were collected. The aortic arch was used since it is the area of greatest plaque formation according to Coleman et al. (2006). Samples were fixed in 4% paraformaldehyde and embedded in paraffin wax according to standard procedures. Serial sections were cut using a microtome (7 μm thickness) for the analyses with light and confocal microscopies. In particular, 20 slices for each group were used both for morphological analysis (hematoxylin–eosin) and stained according to standard procedures and for immunostaining.

For the transmission electron microscopy (TEM) evaluation, the samples were rapidly immersed in 2.5% glutaraldehyde, diluted in cacodilate buffer 0.1 M pH 7.4, for 3 h at 4 °C, post-fixed in 1% (v/v) osmium tetroxide, dehydrated in ethanol and propylene oxide and then embedded in Araldite 502 resin (Serva, Germany). Both semithin (1 μm thickness) and ultrathin sections (800 nm thickness) were obtained by an ultramicrotome (Ultracut E, Reichert-Jung, Germany) using glass or diamond blades (Microstar, USA).

Semithin sections were stained by toluidine blue and observed using a light microscopy (Olympus, Germany). Whereas, ultrathin sections were stained by uranyl acetate and lead citrate solutions and observed using a TEM (FEI, Tcnai G^2 Spirit TEM) set at 85 kV.

Immunofluorescence analyses

Alternate sections were deparaffinized, rehydrated and incubated in 1% bovine serum albumin (BSA) for 1 h at room temperature and then overnight at 4 °C with rabbit polyoclonal antibody against VCAM-1 (1:100; Santa Cruz Biotechnology, CA), or with goat polyclonal antibody against interleukin-6 (IL-6) (1:100; Santa Cruz Biotechnology, CA) or with the mix of rabbit polyclonal antibody against CypA (1:200; Abcam, Cambridge, UK), used also alone, and mouse monoclonal antibody against CD14 (1:100; Beckman Coulter, Milan, Italy). Thereafter, the sections were labeled using anti-goat Alexa Fluor 488, or anti-rabbit Alexa Fluor 546 or anti-rabbit Alexa Fluor 488 conjugated secondary antibodies (1:200, Invitrogen, UK). Finally, the samples were counter-stained with DAPI, mounted and observed with a confocal microscope (510 Meta Zeiss, Germany). The control for immunofluorescence method was performed by omitting the primary antibody and in the presence of isotype matched total IgGs.

Semiquantitative analysis

Two blinded observers evaluated all the slices of each experimental group analyzed by light and confocal microscopies. The results were expressed following a range of intensity/graveness of the vessel damage: no vascular alteration (−); weak vascular