Morphological Alterations and NO-Synthase Expression in the Heart after Continuous Light Exposure of Rats

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Running title: Continuous light effects on heart morphology and NO-synthase

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Summary

Although exposure to continuous light is associated with hypertension and modulates the outcome of ischemia-reperfusion injury, less attention has been devoted to its effects on cardiac morphology. We investigated whether 4-week exposure of experimental rats to continuous 24 h/day light can modify cardiac morphology, with focus on heart weight, fibrosis and collagen I/III ratio in correlation with NO-synthase expression. Two groups of male adult Wistar rats were studied: controls exposed to normal light/dark cycle (12 h/day light, 12 h/day dark) and rats exposed to continuous light. After 4 weeks of treatment the heart weight and the relative heart weight were determined and myocardial fibrosis and collagen type I/III ratio was evaluated using picrosirius red staining. Endothelial and inducible NO-synthase expression was detected immunohistochemically. The exposure of rats to continuous light resulted in an increase of body weight with proportionally increased heart weight. Myocardial fibrosis remained unaffected but collagen I/III ratio increased. Neither endothelial nor inducible NO-synthase expression was altered in light-exposed rats. We conclude, that the loss of structural homogeneity of the myocardium in favour of collagen type I might increase myocardial stiffness and contribute to functional alterations after continuous light exposure.

Key words: myocardium, melatonin, fibrosis, collagen I/III, nitric oxide synthase

Introduction

Melatonin, the product of the pineal gland, is involved in the regulation of many organs, including cardiovascular system (Važan *et al.* 2004). Moreover, decreased melatonin levels were reported in various pathological conditions including ischemic heart disease (Brugger *et al.* 1995) or hypertension with non-dipper pattern (Jonas *et al.* 2003).

Deficit of melatonin may be experimentally induced by surgical pinealectomy (removal of epiphysis), which decreases day- and night-time melatonin levels (Brown *et al.* 1991). Pinealectomy is associated with temporarily increased blood pressure (Zanoboni and Zanoboni-Muciaccia 1967) and myocardial fibrosis (Mizrak *et al.* 2004). Another approach to decrease melatonin levels is the long-time exposure to continuous 24 h/day light, which abolishes nocturnal rise of melatonin serum levels (Brown *et al.* 1991). Our recent reports have shown that exposure of rats to continuous light modified the susceptibility of rat hearts to ischemia-reperfusion and attenuated cardiac NO-synthase activity (Paulis *et al.* 2005). Since NO was shown to inhibit proliferation (Garg and Hassid 1989) and fibrosis (Kolpakov *et al.* 1995) in the smooth muscle cells culture, continuous light may induce morphological changes in the myocardium, which could participate in the modulation of cardiac response to ischemia-reperfusion injury.

In the present study, we investigated whether four-week exposure of experimental rats to continuous 24 h/day light can modify heart weight, fibrosis, collagen type I/III ratio and NO-synthase expression.

Materials and methods

Experimental animals

The experiments were performed on adult male Wistar rats (body weight 250-320 g), in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Rats were housed under standard laboratory conditions and fed with standard pellet diet and tap water *ad libitum*. The room for rats was windowless with automatic regulation of light/dark cycle. Controls (n=6) were housed under standard 12 h/day light, 12 h/day dark cycle and animals exposed to continuous light (n=6) under 24 h/day light (450 lux) for 4 weeks. In ether anaesthesia the hearts were removed weighted and the relative heart weight was calculated (heart weight/body weight).

Myocardial fibrosis

Samples from the left ventricle were fixed in 4% formaldehyde (Sigma Chemie, Germany) for 24 h, embedded in paraffin, cut in 5 μm slices and stained routinely with hematoxylin and eosin. Modified picrosirius red staining technique (Dolber and Spach 1993) was used to determine myocardial fibrosis. After deparaffinisation in xylene and rehydration in distilled water, the slides were incubated for 5 minutes in 0.2 % phosphomolybdic acid (Sigma Chemie, Germany) and stained with 0.1 % sirius red F3BA in saturated picric acid solution (Sigma Chemie, Germany) for 90 minutes. Finally slides were washed 2 minutes in 0.01 mol/l hydrochlorid acid. Sections were evaluated by microscopy in polarized light at the 40x magnification and myocardial fibrosis was measured by histomorphometry in 10 microscopic fields using the ImageJ morphometric software v.1.33 (National Institutes of Health, USA).

Collagen types I/III ratio

The slices stained with picrosirius red were used for collagen types I/III ratio analysis. The areas rich for collagen type I (red colour in polarized light) and collagen type III (green colour in polarized light) were analyzed by digital colour subtraction using ImageJ software and collagen type I/III ratio was calculated.

NO-synthase expression

Nitric oxide synthase expression was analyzed by immunohistochemistry using mouse monoclonal antibodies directed against endothelial and inducible NO-synthase (BD Transduction Laboratories, USA). Deparaffinized sections were rehydrated in phosphate-buffered physiological salt solution (PBS) with 0.5 % bovine serum albumin, 0.05 % NaN_3 and 0.005 % Tween 80, pH 7.2. After 1 h incubation with primary antibodies (1:100) at room temperature and 3 rinsing steps of 5 min each in PBS, sections were incubated for 15 min with the streptavidin-peroxidase complex (Dako, Carpinteria CA, USA); peroxidase activity was visualized with diaminobenzidine (Zymed, San Francisco CA, USA) as substrate and sections were counterstained with hematoxyllin.

Statistics

Data are presented as means ± standard error of mean (SEM). Results were considered significant when $p<0.05$. Two-tailed unpaired Student t-test was used. Normality of distribution was tested according to Kolmogorov and Smirnov and the difference in standard deviations was tested by Barlett´s test.

Results

Heart weight, body weight and relative heart weight

Heart weight of control rats was 0.89 ± 0.02 g, the exposure of rats to continuous light increased the heart weight by 21 % ($p<0.05$) (Figure 1A). Body weight of control rats was 265 ± 4.3 g, the exposure of rats to continuous light increased body weight by 18 % ($p<0.05$). The relative heart weight (heart weight/body weight) was 3.36 ± 0.06 g/kg in controls and the exposure of rats to continuous light had no significant effect on the relative heart weight (Figure 1B).

Myocardial fibrosis

The total level of myocardial fibrosis of control rats was 2.26 ± 0.23 ‰, the exposure of rats to continuous light had no significant effect on myocardial fibrosis (Figure 2A).

Collagen types I/III ratio

The collagen type I/III ratio of control rats was 3.39±018, the exposure of rats to continuous light increased the type I/III ratio by 37 % ($p<0.001$) (Figure 2B).

NO-synthase expression

Both types (endothelial and inducible) of NO-synthase were present in myocardial cells showing cytoplasmatic granular positivity. One third of cardiomyocytes did not show any NO-synthase positivity. No differences in the localization and expression of NO-synthase (neither isoform) between the experimental groups were observed.

Discussion

We investigated the effect of 4-week continuous light exposure of experimental rats on morphological changes and NO-synthase expression in the myocardium. The light-exposure increased heart weight and collagen type I/III ratio but the relative heart weight, level of fibrosis and NO-synthase expression remained unaffected.

The exposure to continuous 24 h/day light, which abolishes the nocturnal rise of melatonin levels (Brown *et al.* 1991), represents a well established model of ´functional pinealectomy´ (Barrett *et al.* 2000, Delibas *et al.* 2002, Briaud *et al.* 2004). Light exposure suppressed circadian rhythms of blood pressure and heart rate after already 3 weeks (Witte *et al.* 1998) and enhanced systolic and diastolic blood pressure (Briaud *et al.* 2004). Recently we have reported that the exposure of rats to continuous light has altered cardiac susceptibility to ischemia- reperfusion and has decreased cardiac NO synthase activity (Paulis *et al.* 2005). However, limited data are available on the effects of continuous light on morphological alterations in the heart, potentially accompanying the functional changes.

The ´functional pinealectomy´ in our study was associated with increased heart weight similarly to surgical pinealectomy (Mizrak *et al.* 2004). It was supposed that cardiac hypertrophy represented an adaptive response to increased haemodynamic load reported after pinealectomy (Mizrak *et al.* 2004). Nevertheless, the increased heart weight after surgical pinealectomy (Sahna *et al.* 2002a, 2002b) as well as in our study using ´functional pinealectomy´ was proportional to the increase in body weight. Therefore the increase in cardiac mass is likely to be a result of body growth in conditions of increased food efficiency, insulin and leptin levels and decreased locomotor activity, which could be expected in melatonin-deficient animals (Wolden-

Hanson *et al.* 2000). Moreover, the gain in heart weight in surgical pinealectomy did not correlate with blood pressure rise (Sahna *et al.* 2002a, 2002b).

The ´functional pinealectomy´ in this study in contrast to surgical pinealectomy (Mizrak *et al.* 2003) has not enhanced the level of myocardial fibrosis (Figure 3). The absence of fibrosis after 4-week light exposure of rats might be explained by the fact that functional pinealectomy suppress only night-time melatonin levels or that a longer period of continuous light is required to promote the development of myocardial fibrosis. Despite the unchanged level of fibrosis we found increased collagen I/III ratio. Types I and III collagens are major structural proteins forming the myocardial collagen matrix. Type I collagen determines the stiffness of the myocardium, while type III collagen contributes to its elasticity (Brilla *et al.* 1993, Yamamoto *et al.* 2002). It has been proposed that collagen type I/III ratio is an important marker for determination of the quality of collagen and prediction of myocardial stiffness (Pathak *et al.* 2001). The quality of collagen in the remodelling of myocardium might be responsible for adverse cardiovascular events (Weber, 2000). Changes in collagen I/III ratio were also suggested to decrease electrical stability of myocardium (Xu *et al.* 2004) and could be involved in the modulation of the outcome of ischemia-reperfusion in functionally/surgically pinealectomized rats. Excessive collagen content was proposed to be a result of increased collagen synthesis and/or inadequate collagen degradation (Varo *et al.* 2000). Both might be possibly modulated by nitric oxide (Rossi *et al.* 2003). Attenuation of NO-synthase activity in the heart in association with continuous light exposure was observed in our previous recent experiments (Paulis *et al.* 2005).

The immunohistochemical study of NO-synthase expression was realized with the aim to elucidate whether previously reported changes in NO-synthase activity are

caused by altered NO-synthase expression. We have observed no changes in the localization or level of NO-synthase (either inducible or endothelial) positivity in cardiomyocytes (Figure 4). Thus we suppose that decreased levels of melatonin in light exposed rats may be insufficient to exert its reported antioxidant effect (Tan *et al.* 2000, Girouard *et al.* 2004, Sahna *et al.* 2005) or inhibition of glucocorticoid formation (Rebuffat *et al.* 1987), which could potentially attenuate NO formation by inhibition of NO-synthase protein activity (Maxwell 2002), without alteration of NO-synthase expression.

We conclude that four-week continuous light exposure of rats has not affected relative heart weight, the level of myocardial fibrosis or NO-synthase expression but increased collagen type I/III ratio. The loss of structural homogeneity of the myocardium in favour of collagen I might increase myocardial stiffness and contribute to functional alterations after continuous light exposure.

Acknowledgements

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Figure legends

Figure 1 Heart weight (A) and relative heart weight (heart weight/body weight) (B) in control group (control) and rats exposed for 4 weeks to continuous 24 hours/day light (light). Data are means ± SEM. *p<0.05 Student t-test.

Figure 2 Myocardial fibrosis (A) and collagen type I/III ratio (B) in control group (control) and rats exposed for 4 weeks to continuous 24 hours/day light (light). Data are means ± SEM. ***p<0.001 Student t-test.

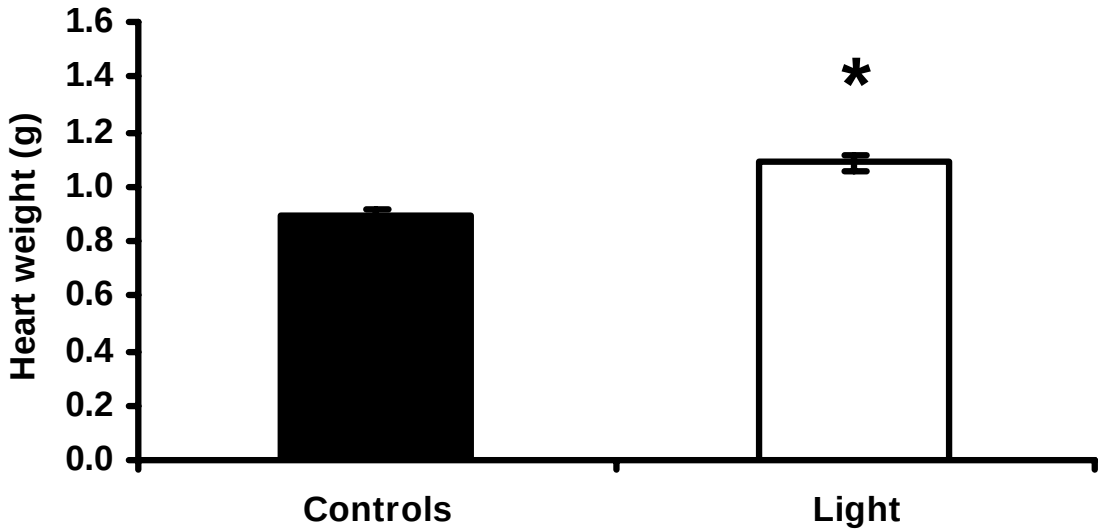
Figure 3 Myocardial fibrosis analyzed in polarized light after picrosirius red staining in control group (control) and rats exposed for 4 weeks to continuous 24 hours/day light (light). The continuous light exposure (light) had no effect on fibrosis level. Final magnification 40x.

Figure 4 Endothelial NO-synthase expression in control group (control) and rats exposed for 4 weeks to continuous 24 hours/day light (light). One third of cardiomyocytes was not showing any NO-synthase positivity. There were no differences in the localization of NO-synthase positivity between the groups. Final magnification 40x, arrow, NO-synthase positivity, **¶, NO-synthase negativity.

Figures

Figure 1

A



B

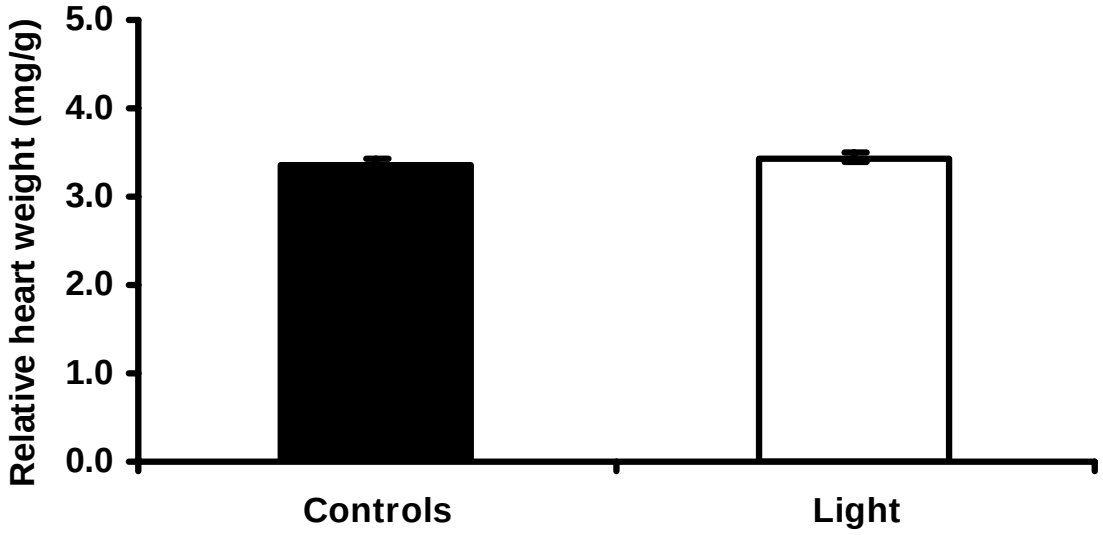
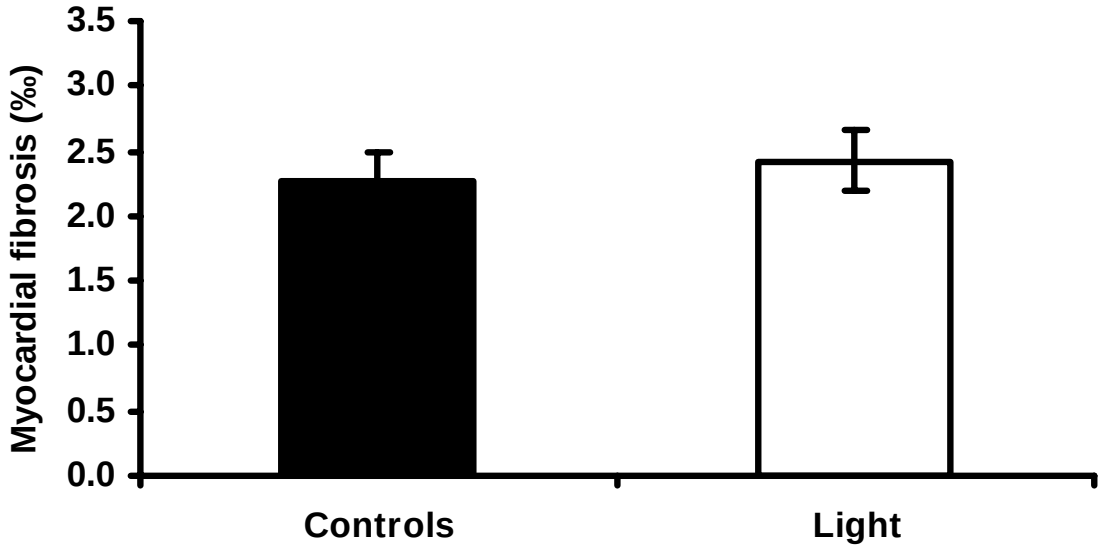


Figure 2

2A



2B

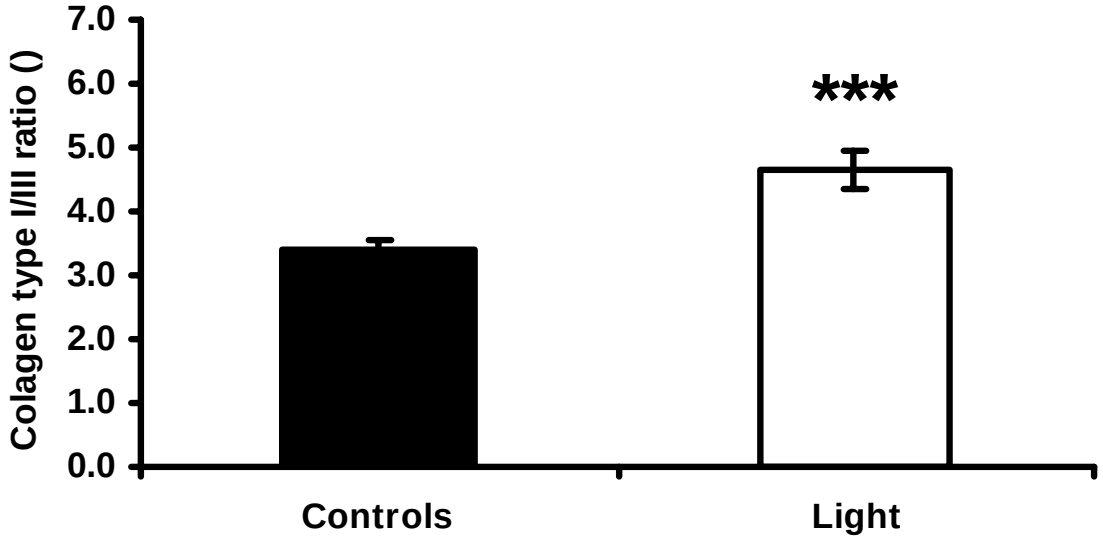


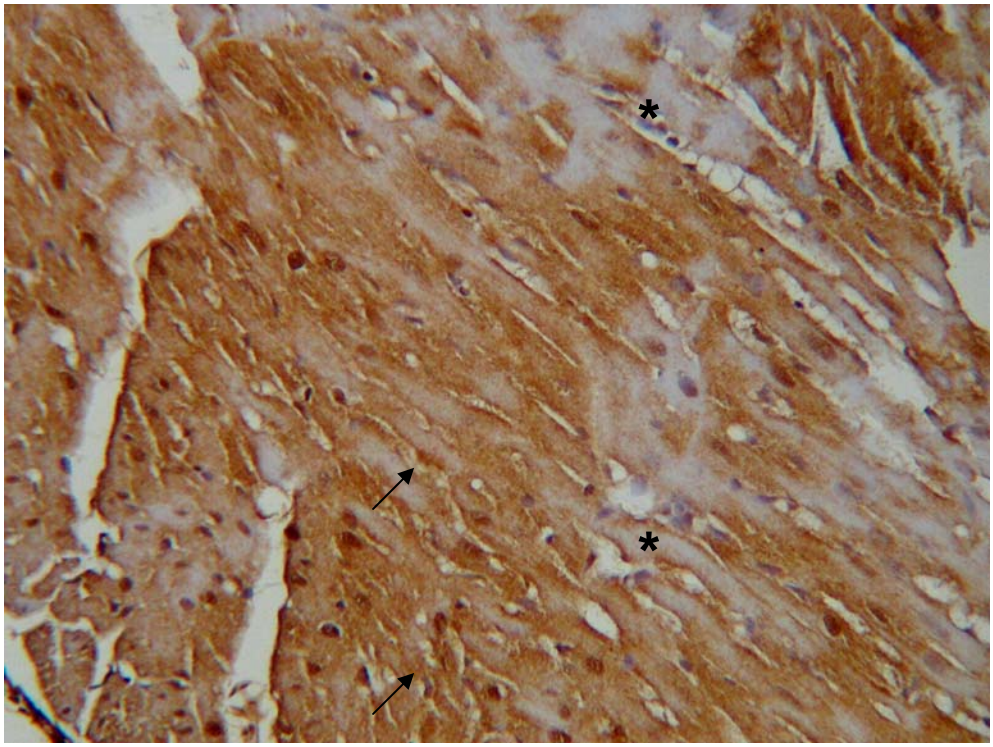
Figure 3

3A

3B

Figure 4

4A



4B