Melatonin Reduces Cardiac Injury Induced by Lipopolysaccharides in Rats

Authors

Ola M. Tork¹, Shaimaa N. Amin¹, Laila A.Rashed²

¹MD, Physiology Department, Faculty of Medicine, Cairo University
²MD, Biochemistry Department, Faculty of Medicine, Cairo University

Corresponding Author

Shaimaa N. Amin
Department of Medical Physiology-Faculty of Medicine-Cairo University, 11451
Email: shaimaa599@yahoo.com

ABSTRACT

Lipopolysaccharide (LPS) is known to cause inflammation and switch myocardial fuel similar to that of the failing heart: reduced mitochondrial substrate flux and myocyte lipid accumulation. The nuclear receptor: peroxisome proliferator activated receptor-alpha (PPARα) and PPAR-gamma coactivator-1alpha (PGC-1α) play important roles in transcriptional regulation of myocardial metabolism and may contribute to the changes that accompanied cardiomyopathic alterations in chronic inflammation. Melatonin is claimed to have anti-inflammatory activity in animal models. Our work was designed to assess the modulatory effect of melatonin on LPS-induced lipotoxic effect in heart of male albino rats for 6weeks. A systemic vehicle or LPS alone or combined with melatonin was administered. Serum cardiac enzymes, cardiac lipids and 3-nitrotyrosin (3-NT) and the gene expression of cardiac nuclear factor-kappa B (NF-κB), PPARα and PGC-1α were measured. Melatonin treatment showed a significant improvement of cardiac enzymes together with reduced cardiac lipid accumulation, 3-NT and NF-κB and PPARα that paralleled enhancement of PGC-1α gene expression compared with the LPS rats. We conclude that cardioprotection of melatonin in chronic inflammatory condition is related to restoration of PGC-1α expression in addition to its antioxidant and anti-inflammatory capacity.

Keywords: Lipopolysaccharide, melatonin, 3-nitrotyrosin; peroxisome proliferator–activated receptor-γ coactivator-1α; peroxisome proliferator activated receptor-alpha
INTRODUCTION

To date, the links between inflammatory signaling and myocardial fuel and energy metabolism remain unclear. Altered heart metabolism was considered a byproduct of heart failure. However, emerging evidence, including observations of the phenotypic expression of genetic defects in humans and animals, supports the notion that derangements in mitochondrial energy metabolism contribute to cardiac dysfunction (Finck and Kelly, 2007).

Peroxisome proliferator activated receptor gamma coactivator-1 (PGC-1) proteins enhance the transcriptional activity of transcription factors through direct protein-protein interactions. Unlike most known transcriptional coactivators; PGC-1α and PGC-1β expression are enriched in tissues with high capacity mitochondrial systems (Puigserver et al., 1998). The expression of PGC-1α is induced by conditions known to increase the demand for ATP production such as cold, exercise, and fasting (Baar et al., 2002).

Because PGC-1 proteins cannot bind DNA directly, they exert their effects through interactions with transcription factors bound to specific DNA elements in the promoter region of genes. DNA bound transcription factors interact with complexes of coactivator and corepressor proteins (Roeder, 2005). PGC-1α functions as an adaptor or scaffold to recruit other coactivator proteins that remodel chromatin. PGC-1α also docks with a protein called ménage-à-trois 1, which is a component of the cyclin-dependent kinase 7 complex that phosphorylates RNA polymerase II and selectively modulates its activity (Sano et al., 2005). PGC-1 interacts with many members of transcription factors to control of diverse cellular energy metabolic pathways (Finck and Kelly, 2006).

The first cardiac PGC-1α target identified was peroxisome proliferator-activated receptor-α (PPARα) (Vega et al., 2000). PPARα is a ligand-activated transcription factor involved in the regulation of inflammation, immunity, nutrient metabolism and energy homeostasis (Rakhshandehroo et al., 2010).

High expression levels of PPARα are found in organs with elevated rate of fatty acid catabolism (Marx et al., 2002 and Bookout et al., 2006). Evidence suggests that mice and humans share similar PPARα tissue expression profiles (Bookout et al., 2006). Natural ligands of PPARα include a variety of fatty acids as well as numerous fatty acid derivatives (Khan and Vanden 2003).

One ubiquitous transcription factor of particular importance in immune and inflammatory responses is nuclear factor-kappa B (NFκB). NFκB considered as an amplifying mechanism that can exaggerate the disease-specific inflammatory process through the coordinated activation of several inflammatory genes (Barnes and Karin 1997; Pandzic, 2010). Specifically, it has been shown that activated PPARα binds to c-Jun and to the p65 subunit of NFκB, thereby inhibiting NFκB- mediated signaling. Additionally, PPARα induces the inhibitory protein IκBa, which normally retains
NF-κB in a nonactive form (Rakhshandehrooet al., 2010).

PPARα also governs inflammation by downregulating gene expression via a mechanism called transrepression. The first clue towards anti-inflammatory effects of PPARα came from the observation that PPARα−/− mice exhibit a prolonged inflammatory response in the ear swelling test (Devchand et al., 1996).

The major biological role of the PPAR/PGC-1α complex in the myocardium appears to be the transcriptional control of enzymes involved in fatty acid uptake and oxidation (Finck and Kelly, 2007).

PGC-1α activates mitochondrial biogenesis (Baar et al., 2002) and induces the expression of reactive oxygen species (ROS) scavengers (St-Pierre et al., 2006).

Melatonin (N-acetyl-5-methoxytryptamine) is secreted by the pineal gland of all mammals, including man. A quite number of different organs have the capability to synthesize melatonin (Pandi-Perumal et al., 2013). Melatonin participates in diverse functions of the body including sleep and circadian rhythm regulation and immunoregulation (Pandi-Perumal et al., 2006). Melatonin seems to have cardioprotective properties via its direct free radical scavenger and its indirect antioxidant activity (Galano et al., 2011).

Increased expression and activity of inducible isoform of nitric oxide synthase (iNOS) was demonstrated in the myocardium of animals and patients with heart failure (HF) (Dai et al., 2004). The combination of superoxide and NO yields peroxynitrite that has been demonstrated in various HF both in animals and human subjects (Turk and Murad, 2002).

Peroxynitrite is known to inhibit mitochondrial respiration, contractile function of cardiac myocytes (Xie and Wolin, 1996). Therefore, the generation of peroxynitrite by the septic heart may contribute to its dysfunction (Khadouret al., 2002). Nitrotyrosine is a useful estimate of tissue peroxynitrite formation (Xie and Wolin, 1996).

This study was designed to investigate the link between LPS-induced overexpression of inflammatory mediators in the lipotoxic induced cardiac injury and the effect of early melatonin treatment.

**MATERIALS AND METHODS**

The study protocol was approved by ethical and scientific committee of Physiology Department, Kasr Al Ainy- Faculty of Medicine.

24 adult male albino rats initially weighing 150-200g were used in this study. The rats were obtained from the Animal House Unit of Kasr Al-Aini- Faculty of Medicine, Cairo University. They were housed in cages (4/cage). They were kept at room temperature and normal dark-light cycles with free access to laboratory rat chow and tap water throughout the study.

**Animal Groups (8 rats/group)**

**Group I:** control treated with vehicle.

**Group II:** (LPS group): received LPS (Sigma, st. Louis, MO, USA) 80μg/100g i.p.once (Khairallah et al., 2014).
**Group III**: melatonin +LPS co-treatment group, melatonin (Sigma, st. Louis, MO, USA), 10 mg/kg i.p. (Mungru et al., 2002) at 17:00 hr. (Prunet et al., 2003) for 6 weeks - 5 days / week) started just after the single LPS injection (80μg/100g i.p.). The increased effect of melatonin administration before the end of the light period related to increase density of melatonin receptor (Vanecek, 1998).

At the end of the experimental period, blood samples were taken for the determination of serum cardiac enzymes. The rats were sacrificed and hearts were excised, rapidly frozen at -80°C, for the determination of the level of cardiac lipids, 3-NT, and the gene expression of NFκB, PPAR α and PGC-1α transcription factors.

**Biochemical Measurements**

**Measurement of serum lactate dehydrogenase (LDH) & creatinine phosphokinase (CPK):**

CPK and LDH were detected according to (Rosalki, 1967).

**Detection of Cardiac Lipids**

Part of the heart was homogenized and extracted with isopropanol (1 ml/50 mg) (Oakes et al., 2001). After centrifugation, 5μl aliquots of supernatant were added to 300 μl of reagent for enzymatic colorimetric determination of total cholesterol and triglyceride contents using commercial available kit.

**Detection of 3-NT**

It was detected using ELISA kit supplied by Abcam, USA according to manufacturer’s instruction.

**Detection of NFκB, PPAR α and PGC-1α gene expression using quantitative real time Polymerase chain reaction (qRT-PCR)**

**RNA Isolation and Reverse Transcription**

RNA was extracted from the heart tissue homogenate using the RNeasy plus mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturer’s instructions. Genomic DNA was eliminated by a DNase-on-column treatment supplied with the kit. The RNA concentration was determined spectrophotometrically at 260 nm using the NanoDrop ND-1000 spectrophotometer (ThermoFisher scientific, Waltham, USA). cDNA synthesis reaction was performed using the Reverse Transcription System (Promega, Leiden, The Netherlands). Total RNA was incubated at 70°C for 10 min to prevent secondary structures. The RNA was supplemented with MgCl2 (25mM), RTase buffer (10X), dNTP mixture (10mM), oligo d(t) primers, RNase inhibitor (20 U) and AMV reverse transcriptase (20 U/μl). This mixture was incubated at 42°C for 1 h.

**qRT-PCR**

It was performed in an optical 96-well plate with an ABI PRISM 7500 fast sequence detection system (Applied Biosystems, Carlsbad, California) and universal cycling conditions (min 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C).
Each 10 µl reaction contained 5 µl SYBR Green Master Mix (Applied Biosystems), 0.3 µl gene-specific forward and reverse primers (10 µM), 2.5 µl cDNA and 1.9 µl nuclease-free water. The sequences of PCR primer pairs used for each gene are shown in Table 1. Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) genes (Kenneth et al., 2001).

Table 1. Primer sequences used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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</table>
| NFKB | Forward primer: 5'-GCTAGGATGCGAGGACAGCT-3'  
Reverse primer: 5'-TTAGGATGCCAGGGATATG-3' |
| PPARα | Forward primer: 5'-ACTAGCGTCCTGTTCCCGG-3'  
Reverse primer: 5'-CCGGAGAGCAACCTTGCA-3' |
| PGC-1α | Forward primer: 5'-TGTTGACTTGTGGAGAGAC-3'  
Reverse primer: 5'-TGGTGGACTTGTGGAGAGAC-3' |
| GAPDH | Forward primer: 5'-CTCCCTTCACACCTTGG-3'  
Reverse primer: 5'-CTGCTCTAGATCTCCCGC-3' |

Data Treatment and Statistics

The results were analyzed using SPSS computer software package, version 21 (IBM, USA). Data were presented as mean±SD. Comparison of quantitative variables between the studied groups was done using Kruskal Wallis test with Wilcoxon signed rank test according to the result of the Shapiro-Wilk test for normality of distribution. Correlations were calculated by Spearman’s test. Results were considered statistically significant at p≤0.05 (Altman, 2005).

RESULTS

As Revealed from Table 2

CPK and LDH serum levels showed significant increase in LPS group (p<0.05) compared to control. Melatonin treatment resulted in significant decrease in serum CPK and LDH (p<0.05) compared to LPS group.

Table 2: Biochemical Parameters in the studied groups (n=8 rats/group)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control group</th>
<th>LPS group</th>
<th>LPS +melatonin</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPK (U/l)</td>
<td>107.6±12.9</td>
<td>203.9±16.5</td>
<td>161.9±16.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDH (U/l)</td>
<td>79.5±16.5</td>
<td>100.3±16.6</td>
<td>80.7±9.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cardiac lipids(µg/mg)</td>
<td>6.0±1.6</td>
<td>20.5±4.6</td>
<td>8.1±1.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Norepinephrine(µg/mg)</td>
<td>0.1±0.1</td>
<td>0.3±0.1</td>
<td>0.2±0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NFKB</td>
<td>1.3±0.2</td>
<td>3.0±1.6</td>
<td>1.6±1.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PPARα</td>
<td>115±24</td>
<td>596±135</td>
<td>294±242</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PGC1α</td>
<td>1.19±1.6</td>
<td>328±25</td>
<td>1.08±0.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Significant compared to control at P-value ≤ 0.05
# Significant compared to LPS group at P-value ≤ 0.05

-Cardiac lipids showed significant increase in LPS group (p<0.05) compared to control. Melatonin treatment with LPS caused significant decrease in cardiac lipids (p<0.05) compared to LPS group.

-3-NT level showed significant increase (p<0.05) in LPS group compared to control. Melatonin treatment with LPS resulted in significant decrease (p<0.05) compared to LPS group.

NFκB expression in cardiac tissue of LPS group showed significant increase (p<0.05) compared to control. Melatonin treatment with LPS caused significant decrease in NFκB expression (p<0.05) compared to LPS group.

-PPARα expression showed significant increase (p<0.05) compared to control. Melatonin treatment to LPS group resulted in significant decrease (p<0.05) compared to LPS group.
PGC-1α expression in cardiac tissue significantly decreased in LPS group (p<0.05) compared to control. Melatonin treatment with LPS caused significant increase in PGC-1α expression (p<0.05) compared to LPS group.

Moreover, the current study showed significant negative correlation between cardiac PGC-1α expression and serum CPK, serum LDH, cardiac lipids, cardiac NFκB relative expression and PPARα relative expression (r= -.659, -.591, -.691, -.604, -.765, respectively and P≤ .005 for all).

However, there was significant positive correlation between 3-NT level and serum CPK, serum LDH, cardiac lipids, cardiac NFκB relative expression and cardiac PPAR-α relative expression (r= .848, .804, .823, .859,.782, respectively and P≤ .001 for all). On the other hand, there was significant negative correlation between 3-NT level and PGC-1α expression (r=- .749; P≤ .001).

There was significant positive correlation between PPAR-α gene expression and cardiac lipids (r=.861, P≤ .001).

DISCUSSION

In the present work, we analyze the relationship between a chronic infection triggering inflammation, mimicked by LPS injection, and the development of cardiac injury in rats. We have observed that LPS induced cardiac injury as indicated by significant elevation of serum cardiac enzymes: CPK & LDH. This was accompanied with significant elevation of cardiac lipids in LPS rats compared with controls. The occurrence of cardiac injury was probably related to lipid induced toxicity in the heart (Chiu et al., 2001). So we can suggest that LPS stimulation suppresses cardiac mitochondrial fatty acid oxidation leading to myocyte lipid accumulation which was in accordance with the finding of (Schilling et al., 2011).

Although plasma free fatty acids (FFA) are the primary carbon fuel for the myocardium but, when elevated, can adversely impact the heart. Recent studies showed that elevated plasma FFA is a strong predictor of sudden cardiac death (Jouvenet et al., 2001), this could be due to deleterious effects on mitochondrial function or activation of Na+ or Ca2+ channels (Chiu et al., 2005).

The LPS mediated increase in cardiac enzymes and myocardial lipid accumulation were reduced by melatonin treatment. The effect of melatonin on LPS induced changes in lipid metabolism may be due to altered lipid delivery to the heart as consequence of decreased serum lipids or related to improvement of the mitochondrial function that affect the fatty acid oxidation. But the study of (Schilling et al., 2011) demonstrating that the reduction in myocardial triglyceride is not a consequence of altered lipid delivery to the heart, rather an effect of increased PGC-1.

In the current study 3-NT showed significant increase in LPS group compared to control. It can be formed both by NOS-dependent” and by myeloperoxidase (MPO) -dependent “process (Ricciardolo et al., 2006). In agreement with our
results: Khadour et al., (2002) showed that in LPS treated rats there was increased nitrotyrosine staining. Also Eleuteri and coworkers demonstrated an increased 3-NT plasma level in patients with congestive heart failure (CHF) and suggested a role for MPO and Tumor Necrosis Factor-α (TNFα) in sustaining this increment (Eleuteri et al., 2009). It is to be noted that an increased oxidative/nitrosative stress has been linked to deterioration of heart function in CHF (Colombo et al., 2005).

We demonstrated that melatonin treatment with LPS resulted in significant decrease of 3-NT compared to LPS group (% difference 71.16%). In agreement to our results; Yaman et al., (2010) showed that concomitant administration of melatonin with LPS resulted in decrease in NT level in rat heart.

Melatonin and its metabolites efficiently interact with various reactive oxygen and reactive nitrogen species and also upregulate antioxidant enzymes and downregulate pro-oxidant enzymes. Moreover, melatonin enters all cells and subcellular compartments and crosses morphophysiologic barriers. These findings have implications for the protective effects of melatonin against cardiac diseases induced by oxidative stress (Galano et al., 2011).

In the current work LPS stimulation led to sustained induction of myocardial NFκB as indicated by its significant rise in LPS rats compared with the controls.

There is evidence that NF-kB activity is elevated in the CHF (Anker and von Haehling, 2004). In addition (Tavener and Kubes, 2006) showed that TNFα, interleukine-6 (IL-6), and iNOS are induced by LPS and NFκ_B–dependent manner and have all been implicated in sepsis-induced cardiodepression.

As regard the effect of melatonin treatment, we showed that NF-kB significantly decreased in melatonin treated rats compared to the LPS group. Supporting our results the finding of Veneroso et al. (2009) who demonstrated that melatonin prevented cardiac inflammation in a rat model of acute exercise; by reducing iNOS expression and blocking the overexpression of inflammatory cytokines possibly through an NFκB-dependent mechanism.

Also the study of Tamura et al., (2009) revealed that; the addition of melatonin prevented LPS-induced iNOS expression in cultured rat endothelial cells and aortic rings by a mechanism that is NFκB-dependent through “reverse remodeling” and this may explain our findings.

To determine the mechanism by which LPS alters myocardial metabolism, we initially focused our investigation on myocardial PPARα, given their ability to “boost” the activity of several transcription factors that regulate mitochondrial FAO (Rakhshandehroo et al., 2010).

Interestingly Burkarte et al., (2007) provided evidence that cardiac-specific overexpression of PPARα increased FAO, elevated serum TG, left ventricular hypertrophy and systolic dysfunction which is exacerbated by high fat feeding. These
results suggest that long-term PPARα activation can be deleterious to the heart.

Although previous works on sepsis revealed that PPARα expression together with PGC-1 were down regulated in LPS treated rats (Schilling et al., 2011), we found that cardiac PPARα expression was highly induced after 6w from LPS administration compared with the controls. This may suggest that cardiac PPARα expression recovered early after LPS. Indeed, there was positive correlation between cardiac PPARα gene expression and cardiac lipids, indicating that its activation could cause cardiac lipotoxicity. While lipotoxic effect of over expressed PPARα may be explained by: its stimulatory role on transcription of FA transporters in a mechanism independent on PGC-1 interaction, which results in more lipid accumulation in the heart than their oxidation. Thus the increased PPARα by LPS treatment in our work and its reduction under melatonin supplementation can’t alone explain the observed metabolic changes in the heart.

Given the regulatory role of PGC-1 coactivators in maintaining mitochondrial system and regulating genes involved in the cellular uptake and mitochondrial oxidation of FAs via direct co-activation of PPARα (Vega et al., 2000; Roeder, 2005) we assessed the effect of LPS on the myocardial gene expression of the PGC-1α. We found that LPS injection led to a sustained downregulation of PGC-1α. We can suppose that elevated 3-NTand NF-κB are required for LPS-mediated suppression of PGC-1α gene expression according to (Schilling et al., 2011). Moreover, it was found that blocking the LPS-NFκB axis prevented the downregulation of PGC-1 expression by LPS directly or indirectly (Alvarez-Guardia et al., 2010).

Surprisingly the expression of PGC-1α, is downregulated in several models of HF (Arany et al., 2006). This accounts for the “fuel shift” in substrate preference from FA to glucose, steadily reported in the HF (Huss and Kelly, 2006). Yet, whether a shift towards glucose utilization is a compensatory or maladaptive event remains to be elucidated (Lehman et al., 2009).

Probably our work is the first to demonstrate the interrelation between melatonin: the systemic orchestrator of circadian rhythm (Bechtold, 2008) and PGC-1α in the heart. We showed that melatonin treatment induced PGC-1α, which is supposed to reduce LPS-triggered lipid accumulation in the heart and cardiac injury. The circadian clock has been reported to regulate metabolism and energy homeostasis in peripheral tissues (Bechtold, 2008). This is achieved by mediating the expression and/or activity of certain metabolic enzymes and transport systems (Kohsaka and Bass, 2007). Thus resetting the circadian clock is another intervention that can lead to cardioprotective effect of melatonin.

CONCLUSION

Data obtained indicate that melatonin protects against heart lipotoxicity associated with chronic inflammation. Impaired production of noxious mediators involved in the inflammatory process
and down-regulation of the NF-κB signal transduction pathway appear to contribute to the beneficial effects of melatonin. In addition, our findings revealed a major involvement of the PGC-1α–PPARα interaction in the protective role of melatonin against LPS-induced cardiac injury. These results suggest that PGC-1α activation by melatonin may be one of its underlying mechanisms for cardioprotection.

Declaration of Interest
There is no conflict of interest

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Author Contributions
Ola M. Tork: wrote the protocol of the work, performed the experimental work, wrote and revised the manuscript.
Shaimaa N. Amin: performed statistical analysis, wrote and revised the manuscript.
Laila A. Rashed: performed the biochemical analysis.

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