Melatonin and structurally similar compounds have differing effects on inflammation and mitochondrial function in endothelial cells under conditions mimicking sepsis

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Editor’s key points
• Melatonin is known for its antioxidant properties.
• In this study, melatonin and other structurally similar compounds were investigated for their anti-inflammatory and antioxidant effects.
• In a cell-culture model of sepsis, the study compounds protected endothelial cells against oxidative damage.
• The study offers important suggestions for future novel therapeutic strategies in sepsis.

Background. Development of organ dysfunction associated with sepsis is due in part to oxidative damage to mitochondria. Melatonin regulates the sleep–wake cycle and also has potent antioxidant activity. The aim of this study was to determine the effects of melatonin and other structurally related compounds on mitochondrial function, endogenous glutathione (GSH), and control of cytokine expression under conditions mimicking sepsis.

Methods. Human endothelial cells were treated with lipopolysaccharide (LPS) plus peptidoglycan G (PepG) to simulate sepsis, in the presence of melatonin, 6-hydroxymelatonin, tryptamine, or indole-3-carboxylic acid. Nuclear factor κB (NFκB) activation, interleukin (IL)-6 and IL-8, total glutathione, mitochondrial membrane potential, and metabolic activity were measured.

Results. LPS and PepG treatment resulted in elevated IL-6 and IL-8 levels preceded by activation of NFκB (all \( P < 0.0001 \)). Treatment with all four compounds resulted in lower IL-6 and IL-8 levels, and lower NFκB activation (\( P < 0.0001 \)). Loss of mitochondrial membrane potential and endogenous glutathione was seen when cells were exposed to LPS/PepG, but these were maintained in cells co-treated with melatonin, tryptamine, or 6-hydroxymelatonin (\( P < 0.05 \)), but not indole-3-carboxylic acid. Metabolic activity decreased after exposure to LPS/PepG and was maintained by melatonin and 6-hydroxymelatonin at the highest concentrations only.

Conclusions. We have shown that in addition to melatonin, other structurally related indoleamine compounds have effects on NFκB activation and cytokine expression, GSH, mitochondrial membrane potential, and metabolic activity in endothelial cells cultured under conditions mimicking sepsis. Further work is needed to determine whether these compounds represent therapeutic approaches for disrupting the oxidative stress-inflammatory response signalling pathway in sepsis.

Keywords: glutathione; indoleamines; melatonin; mitochondria; oxidative stress; sepsis; tryptamine

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Sepsis is a major cause of death in critical care units worldwide.1 2 It is characterized by a systemic dysregulated inflammatory response and oxidative stress, often leading to organ failure and death. Development of organ dysfunction associated with sepsis is now accepted to be due, at least in part, to oxidative damage to mitochondria.2 Consistent with this mitochondrial oxidative damage in sepsis, we have reported oxidative stress in patients with sepsis.3–7 Furthermore, mitochondrial dysfunction has been described in several animal models of sepsis,8 9 and in livers from patients who had died of severe sepsis, mitochondrial damage and reduced complex I and complex IV activity were observed.10 We have also shown mitochondrial dysfunction in endothelial cells cultured under conditions of sepsis, which was abrogated by antioxidants targeted to mitochondria and in a rat model of acute organ failure, treatment with antioxidants targeted to mitochondria decreased organ dysfunction and reduced circulating interleukin (IL)-6 levels.11

Melatonin is a product of the amino acid tryptophan. After its uptake into cells, tryptophan is first converted to serotonin (5-hydroxytryptamine) and in some cells, serotonin is able to be converted to N-acetylseryotonin and then N-acetyl-5-methoxytryptamine (melatonin). The melatonin is enzymatically degraded in the liver to 6-hydroxymelatonin.
Melatonin has profound antioxidant activity, reacting with both oxygen- and nitrogen-derived reactive species.\textsuperscript{12, 13} Through the process of scavenging reactive species, other metabolites are generated which also have antioxidant activity.\textsuperscript{14} For example, cyclic 3-hydroxymelatonin is formed through the reaction of melatonin with hydroxyl radicals, and 6-hydroxymelatonin, the same degradation product enzymatically produced in the liver, is formed from the reaction of this with peroxynitrous acid.

Melatonin is highly lipophilic and can reach all cellular compartments and although concentrations vary in cellular subcompartments, the highest levels are found within mitochondria, where melatonin is able to interact with lipid membranes and stabilize the mitochondrial inner membrane.\textsuperscript{15} The aim of this study was to determine the effects of melatonin compared with other structurally related tryptamines/indoleamines, on mitochondrial function, endogenous glutathione (GSH), and control of cytokine expression under conditions mimicking sepsis.

**Methods**

**Cell culture and treatment**

The human umbilical vein endothelial cell line (HUVEC-C) was used as described previously in detail.\textsuperscript{11} For experimentation, cells were cultured in 96- or 6-well plates in the presence of 2 µg ml\(^{-1}\) lipopolysaccharide (LPS) plus 20 µg ml\(^{-1}\) peptidoglycan G (PepG) (Sigma, Poole, Dorset, UK) plus either 0, 0.1, 1.0, 10, 100, and 500 µM melatonin, 6-hydroxymelatonin, tryptamine, or indole-3-carboxylic acid. Untreated control cells were exposed to solvents only. Cell viability was assessed using acid phosphatase activity.\textsuperscript{16} Appropriate time points were chosen after preliminary experiments to determine optimum activation (data not shown).

**Nuclear factor κB and IL-6 and IL-8**

For assessment of nuclear factor κB (NFκB) activation, cells were treated with LPS/PepG as above for 4 h and nuclear extracts were prepared using the Novagen\textsuperscript{®} Nucbuster\textsuperscript{TM} protein extraction kit (Merk Chemicals Ltd, Nottingham, UK). NFκB activation was measured as the amount of the p65 subunit present in the nucleus using the Novagen\textsuperscript{®} NoShift\textsuperscript{TM} transcription factor assay kit. The time point of 4 h was chosen after preliminary experiments revealed this as the time point of maximal activation in response to LPS/PepG. To measure the effect on cytokines, cells were cultured as described above for 24 h and IL-6 and IL-8 were measured in culture supernatants using an enzyme immunoassay (R&D Systems Europe, Abingdon, Oxon, UK).

**Fig 1** NFκB in nuclear extracts of endothelial cells treated for 4 h with 2 µg ml\(^{-1}\) LPS plus 20 µg ml\(^{-1}\) PepG plus either (A) melatonin, (B) 6-hydroxymelatonin, (C) tryptamine, or (D) indole-3-carboxylic acid. Box and whisker plots show median, IQR, and full range (n=6). P-value is Kruskal–Wallis. *Significantly different from untreated cells (P<0.05); #significantly different from cells treated with LPS/PepG only (P<0.05).
Total GSH determination (adapted from ref. 17)

Cells were incubated in 96-well plates for 7 days as described above. After incubation, cells were washed with phosphate-buffered saline (PBS, pH 7.4) and incubated at 37 °C with 20 μM monobromobimane in PBS for 15 min at 37 °C. Cells were then washed twice with PBS and fluorescence was measured (excitation 355 nm, emission 520 nm).

Mitochondrial membrane potential

Cells were incubated in 96-well plates for 7 days as described above. Mitochondrial membrane potential was analysed in intact cells using the fluorescent probe JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarboxyline iodide, Invitrogen, Paisley, UK). Briefly, after treatments as described above, cells were washed with PBS and then incubated for 30 min with 10 μM JC-1 in PBS at 37 °C in the dark. After incubation, cells were washed again with PBS and the red/green fluorescence ratio was measured immediately.

Metabolic activity

Metabolic activity was analysed by measuring the rate of reduction of AlamarBlue™ in intact cells after 7 days treatment as described above. Alamar Blue™ (Invitrogen) is a novel redox indicator that exhibits both fluorescent and colourimetric changes in response to changes in metabolic activity via oxidative metabolism. Briefly, after cell treatments, Alamar Blue™ was added to each well and fluorescence was measured every 15 min for 2 h at 37 °C. Metabolic activity was determined as the rate of change in fluorescence over time.

Statistical analysis

Six independent experiments with three technical replicates were undertaken (n=6). No assumptions were made about data distribution and data are presented as median, interquartile (IQR), and full range. Statistical analysis was undertaken using the Kruskal–Wallis analysis of variance with the Mann–Whitney U post hoc testing as appropriate. A P-value of <0.05 was taken as significant.

Results

Acid phosphatase levels were similar in all cells after up to 7 days incubation, indicating that cell viability was not affected by any compound at the doses studied (data not shown).
Nuclear factor κB, IL-6, and IL-8

NFκB activation was measured as the amount of the p65 subunit of NFκB in nuclear extracts after 4 h exposure to LPS/PepG since only active p65 is present in nuclei. NFκB was undetectable in nuclear extracts from untreated control cells and was significantly increased in cells treated with LPS/PepG (Fig. 1). In cells treated with LPS/PepG plus either melatonin, tryptamine, 6-hydroxymelatonin, or indole-3-carboxylic acid, NFκB was significantly and dose dependently lower in nuclear extracts (Fig. 1).

Cytokine levels were negligible in culture supernatants from untreated control cells after 24 h (Figs 2 and 3). Supernatants from cells treated with LPS/PepG had markedly higher IL-6 and IL-8 concentrations when compared with control cells (P<0.0004, Figs 2 and 3) and were markedly lower in cells treated with any of the indoleamine compounds plus LPS/PepG (all P<0.0001). Most notably, tryptamine almost completely obliterated LPS/PepG-induced cytokine production even at 0.1 μM (P<0.0001, Figs 2c and 3c).

Total GSH determination

We determined the effects of the four compounds on intracellular GSH. After 7 days treatment, cells treated with LPS/PepG had significantly lower GSH levels than untreated control cells (P<0.0001, Fig. 4). In cells treated with LPS/PepG plus either melatonin, tryptamine, or 6-hydroxymelatonin, GSH was lower than in control cells but was significantly higher than cells treated with LPS/PepG alone (P=0.0043, Fig. 4a–c). In cells treated with LPS/PepG plus indole-3-carboxylic acid, however, GSH was similar to LPS/PepG alone (Fig. 4d).

Mitochondrial membrane potential

Mitochondrial membrane potential was determined by the ratio of red/green JC-1 fluorescence. After 7 days, mitochondrial membrane potential was lower in cells treated with LPS/PepG compared with control cells (P=0.001, Fig. 5). The lower mitochondrial membrane potential was attenuated when cells were treated with melatonin or tryptamine in addition to LPS/PepG (P=0.0043, Fig. 5a–c) and was even lower in cells also treated with 6-hydroxymelatonin (P=0.0007, Fig. 2a). In cells treated with LPS/PepG plus indole-3-carboxylic acid, membrane potential was similar to that seen in those treated with LPS/PepG alone (Fig. 5d).

Metabolic activity

Investigation of the functional capacity of mitochondria was achieved by measuring the metabolic activity as determined by the rate of reduction of Alamar BlueTM by mitochondrial reducing equivalents and respiratory complex activity. After
7 days treatment, metabolic activity was significantly lower in LPS/PepG-treated cells compared with control cells ($P$, 0.05, Fig. 6). Little effect was seen when cells were also exposed to any of the indoleamines (Fig. 6).

**Discussion**

We have shown that melatonin and other structurally related indoleamine compounds have profound but differing effects on NFκB activation and cytokine expression, GSH, mitochondrial membrane potential, and metabolic activity in endothelial cells cultured under conditions mimicking sepsis. Structurally related indoleamines have been shown to be more effective antioxidants than melatonin,14 but there is little information about the effects of these other compounds on inflammatory mediators, mitochondrial function, and endogenous antioxidant enzymes under conditions mimicking sepsis. We used human endothelial cells cultured with LPS and PepG to simulate an inflammatory septic environment.

In vivo, endothelial activation leads to the production of various inflammatory mediators and ultimately cellular injury, with implications for sepsis. Organ failure, severity of illness scores, and mortality are higher in patients with elevated IL-6 levels20 and high circulating IL-8 concentrations correlate with fatal outcome in patients with sepsis.21 We found that melatonin, tryptamine, 6-hydroxymelatonin, and indole-3-carboxylic acid were all able to almost completely obliterate LPS/PepG-induced IL-6 and IL-8 release by endothelial cells, and that this effect was preceded by decreased NFκB activation, suggesting an important role for NFκB in this process.

NFκB is a redox-sensitive transcription factor and its activation levels in cells from patients with sepsis are linked to increased mortality.22 23 In unstimulated cells, the NFκB is kept in an inactive state within the cytoplasm by virtue of binding with inhibitors of NFκB (IκB) which mask a nuclear localization sequence via ankyrin repeats. When NFκB is activated, the IκB dissociates, the nuclear localization signal is revealed, and the active NFκB moves into the nucleus where it binds to targeted DNA and up-regulates expression of a variety of inflammatory mediators. Thus, NFκB in nuclear extracts is active. Cytokine responses are regulated by the redox state of the cell at several levels, including activation of oxidant- and redox-sensitive transcription factors such as NFκB. However, supplementation of patients with several different combinations of antioxidants, excluding melatonin, has not been shown to have beneficial effects on cytokine responses in a variety of conditions.
inflammatory conditions, including rheumatoid arthritis and diabetes.

Melatonin has been shown to reduce cytokine levels in rodent models of inflammation, including sepsis. Studies in patients are limited; Gitto and colleagues' studies on neonates reported lower plasma IL-6 levels in babies treated with melatonin than in untreated babies and in children with Duchenne’s muscular dystrophy, low-dose melatonin treatment for 9 months resulted in marked reductions in IL-6 and other cytokines.

We found that treatment of endothelial cells with LPS and PepG resulted in loss of total GSH and loss of mitochondrial membrane potential, suggesting oxidative damage. We have also shown previously that this model results in increased production of reactive oxygen species. After 7 days, GSH was markedly depleted in LPS/PepG-treated cells and melatonin, tryptamine, and 6-hydroxymelatonin were able to reduce this loss of GSH. Melatonin has been shown to up-regulate activity of antioxidant enzymes in animal models and in addition has been shown to stimulate the activity of the rate-limiting enzyme for GSH synthesis, γ-glutamyl-cysteine synthetase via effects on activator protein-1, suggesting that the effect we describe may be via de novo synthesis of glutathione. The protective effects on GSH mirror previous findings in terms of preventing lipid peroxidation in rat liver mitochondria, with tryptamine having the greatest effect on lipid peroxidation and indole-3-carboxylic acid the least.

We also found that exposure of cells to LPS/PepG resulted in loss of mitochondrial membrane potential which was maintained by melatonin and tryptamine treatment, but, despite the beneficial effects on GSH, 6-hydroxymelatonin had no effect on membrane potential. Indole-3-carboxylic acid had no effect on either GSH or membrane potential. Melatonin protects mitochondrial function via several mechanisms, but the effects of the other compounds are not well studied. Although the compounds have differing effects on cytokines, antioxidants, and mitochondrial function, the reasons remain unclear, although all the compounds had pronounced inhibitory effects on NFκB activation and cytokine responses.

We found that metabolic activity decreased when cells were treated with LPS/PepG and that melatonin and 6-hydroxymelatonin treatment resulted in further decreases at the highest concentrations, but without affecting cell viability. Alamar Blue is an oxidation–reduction indicator that responds to changes in the ability of mitochondria to utilize energy substrates. It is not reduced or affected by...
exogenous oxidants or by compounds in the cell culture medium. A decrease in metabolic activity may indicate damage to either components of the tricarboxylic acid (TCA) cycle, to the mitochondrial oxidative–phosphorylation complexes, or to both. We have shown very recently that in cells exposed to LPS/PepG, large decreases in ATP production were associated with significant increases in metabolic activity presumably because the intra-mitochondrial increase in ADP concentration acting as a molecular switch to increase the activity of the TCA cycle to produce more reducing equivalents. Melatonin has been shown previously to protect against loss of ATP in models of sepsis, but there have been no reports on total metabolic activity.

Melatonin exerts its effects on diurnal rhythms via the G-protein-coupled melatonin receptors MT-1 and MT-2, but may also affect protein kinase A and phospholipase C, several transcription factors in addition to NFκB, and also the ERK–MAPK pathway. Some of the actions of melatonin depend on non-receptor-mediated processes—for example, melatonin can directly activate protein kinase C. Melatonin has effects on many aspects of the immune/inflammatory response in addition to IL-6 and IL-8. The effects of other structurally related indoles on these pathways have not been previously reported.

Circulating endogenous concentrations of melatonin in healthy young subjects peak at night at around 250 pg ml⁻¹ (~1 nM) and in ICU patients peak levels are around 10-fold lower. Oral doses of 3–5 mg, available as over the counter treatments for jet lag in some countries, result in plasma levels up to 100 times higher than the normal night time peak level (i.e. around 100 nM) when ingested by healthy subjects. The levels of melatonin required for antioxidant effects are thought to be considerably higher than doses administered to counteract sleep disturbance. In healthy young men given a single melatonin dose of 80 mg, peak levels were between 400 nM and 10 μM, varying 25-fold between individuals. In critically ill patients, the maximum plasma concentrations were around 10 times higher than expected after a much smaller dose (3 mg), suggesting altered pharmacokinetics in the critically ill. Thus, the levels used in our study roughly cover the range of plasma concentrations expected after oral doses for antioxidant effect.

In summary, we showed that in endothelial cells cultured under conditions mimicking sepsis, melatonin and, in

<table>
<thead>
<tr>
<th>Condition</th>
<th>Metabolic Activity (units min⁻¹)</th>
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<tbody>
<tr>
<td>Untreated</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>LPS PepG</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>10 μM LPS PepG</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>100 μM LPS PepG</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>500 μM LPS PepG</td>
<td>2.0 ± 0.1</td>
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P-value is Kruskal–Wallis. *Significantly different from untreated cells (P < 0.05); #significantly different from cells treated with LPS/PepG only (P < 0.05).

![Fig 6](https://example.com/fig6.png)
addition, structurally related indoleamines, differentially protected against oxidative damage to mitochondria and reduced inflammatory responses. Further work is needed to determine whether any of these compounds could represent future novel therapeutic strategies for disrupting the oxidative stress-inflammatory response signalling pathway in sepsis.

Conflict of interest
N.R.W. is the Chairman and H.F.G. is an Editor of the British Journal of Anaesthesia (BJA). D.A.L., N.R.W., and H.F.G. have received research funding from the BJA.

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