

## Pharmacological activation of endogenous protective pathways against oxidative stress under conditions of sepsis

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### Key words

Antioxidants; endothelial cells; sepsis; oxidative stress

### Abstract

#### Background

Mitochondrial oxidative stress has a role in sepsis-induced organ dysfunction. The endogenous mechanisms to initiate protective pathways are controlled by peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC1 $\alpha$ ) and nuclear factor erythroid 2-like 2 (NFE2L2). Activation of these pathways are potential therapeutic targets in sepsis. We used pharmacological activators to determine the effects on markers of mitochondrial damage and inflammation in human endothelial cells under conditions of sepsis.

#### Methods

Human endothelial cells were exposed to lipopolysaccharide plus peptidoglycan G to mimic a sepsis environment, with a range of concentrations of a selective synthetic agonist of silent information regulator-1 (SIRT-1) which activates PGC1 $\alpha$ , or bis(2-hydroxy-benzylidene) acetone (2HBA) which activates NFE2L2, with and without inhibitors of these pathways. Cells were cultured for up to 7d and we measured mitochondrial membrane potential and metabolic activity, and mitochondrial density as a marker of biogenesis, interleukin-6 to reflect inflammation and glutathione as a measure of antioxidant status.

#### Results

Under conditions mimicking sepsis, activation of the PGC1 $\alpha$  and NFE2L2 pathways protected cells from LPS/PepG-induced loss of mitochondrial membrane potential ( $p=0.0002$  and  $p=0.0009$  respectively) and metabolic activity ( $p=0.05$  and  $p<0.0001$  respectively) and dampened interleukin-6 responses ( $p=0.003$  and  $p=0.0001$  respectively). Mitochondrial biogenesis (both  $p=0.0001$ ) and glutathione (both  $p<0.0001$ ) were also increased. These effects were blunted by the respective inhibitors.

#### Conclusions

The development of organ dysfunction during human sepsis is linked to mitochondrial dysfunction and so activation of PGC1 $\alpha$ /NFE2L2 is likely to be beneficial. These pathways are attractive therapeutic targets for sepsis.

Sepsis is essentially a systemic, dysregulated and highly exaggerated inflammatory response to infection, accompanied by oxidative stress and mitochondrial dysfunction. In the developed world the incidence of sepsis continues to rise by around 10% annually and now claims more lives than breast and lung cancers combined. Mitochondria are the major physiological producers of reactive oxygen species (ROS) and during sepsis, mitochondrial ROS production exceeds antioxidant defences, leading to a state of oxidative stress which fuels inflammation and causes direct mitochondrial damage.<sup>1</sup> The resulting mitochondrial dysfunction leads to further ROS release and initiates the same phenomenon, known as ROS-induced-ROS release, in neighbouring mitochondria. This self-perpetuating mechanism resulting in widespread mitochondrial dysfunction and

subsequent bioenergetic failure, is suggested to play a central role in sepsis-induced organ dysfunction<sup>2 3</sup> and so therapeutic strategies to protect mitochondria during sepsis have been recognised as being important.<sup>3-5</sup>

Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) co-activator 1-alpha (PGC1 $\alpha$ ) is a co-activator of a number of transcription factors responsible for controlling cellular metabolism.<sup>6 7</sup> Further transcription factors are under the control of PGC1 $\alpha$ , such as nuclear factor erythroid-derived 2-like-2 (NFE2L2)<sup>7</sup> which regulates the expression of a number of protective mechanisms against oxidative stress, and GA binding protein transcription factor alpha (GABPA), which promotes activation of key transcription factors which control mitochondrial biogenesis. These events result in activation of protective cascades with generation of new mitochondria. A simple representation of the key pathways and the points of action of the agonists and inhibitors used is provided in Figure 1.

PGC1 $\alpha$  is regulated at several levels including transcriptionally and post-translationally.<sup>6 8</sup> One of the various post-translational modifications which PGC-1 $\alpha$  is capable of undergoing is deacetylation, catalysed by the enzyme silent information regulator-1 (SIRT-1).<sup>8</sup> As cellular energy levels decrease, SIRT-1 increases the activity of PGC-1 $\alpha$  by removing acetyl groups. Under normal circumstances, SIRT-1 activity is regulated by the energy status of cells, but it can be also increased by synthetic agonists.<sup>9</sup>

NFE2L2 is present constitutively in the cell cytoplasm bound to a repressor protein called Kelch-like ECH-associated protein 1 (KEAP-1), and activation occurs when oxidant species react with cysteine in the KEAP-1 molecule. This then allows translocation of NFE2L2 into the cell nucleus where it binds to antioxidant response elements (ARE) to induce upregulation of key antioxidant enzymes. Agonists which act on the repressor protein KEAP can be used to activate NFE2L2.<sup>10</sup>

Activation of the PGC1 $\alpha$ -NFE2L2 pathways are attractive potential therapeutic targets in sepsis and so the aim of this study was to pharmacologically activate the PGC1 $\alpha$  and NFE2L2 pathways using two different agonists and to determine the effects of these interventions on markers of mitochondrial damage and inflammation in human endothelial cells under conditions which mimic sepsis. In addition the effect of inhibitors of these pathways were also studied.

## Materials and methods

The agonists used in this study were 2-amino-N-cyclopentyl-1-(3-methoxypropyl)-1H-pyrrolo [2,3-quinoxaline]-3-carboxamide also known as SIRT-1-activator-3, a selective synthetic agonist of SIRT-1 which increases deacetylation of PGC1 $\alpha$ ,<sup>9</sup> and bis(2-hydroxybenzylidene) acetone (2HBA) which is structurally related to curcumin<sup>10</sup> and acts as an agonist of NFE2L2 via effects on the KEAP-1 repressor protein. In addition, inhibitors were used to block NFE2L2 and SIRT-1 activation: trigonelline hydrochloride<sup>11</sup> and an indole derivative, 6-chloro-2,3,4,9-tetrahydro-1H-carbazole-1-carboxamide (EX527) respectively.<sup>12</sup>

### Cell studies

All experiments were carried out using the human umbilical vein endothelial cell line, HUVEC-C (ATCC/LGC Standards Ltd., Middlesex, UK). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 100mg L<sup>-1</sup> glucose and without L-glutamine (Invitrogen, Paisley, UK), and supplemented with 10% heat activated foetal calf serum, 50 g mL<sup>-1</sup> gentamicin, and 250  $\mu$ g mL<sup>-1</sup> amphotericin B, at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide.<sup>13</sup> For experimentation cells were cultured in the presence of 2  $\mu$ g mL<sup>-1</sup> lipopolysaccharide (LPS, *Escherichia coli* 0111:B7, Sigma-Aldrich Ltd., Poole, Dorset, UK) plus 20  $\mu$ g mL<sup>-1</sup> peptidoglycan G (PepG), prepared as described previously,<sup>13</sup> to simulate sepsis, plus a range of concentrations of either 2HBA, or SIRT 1-activator-3. In some experiments 20  $\mu$ M trigonelline hydrochloride or 1 $\mu$ M EX527 were included. Some drugs were prepared

initially in ethanol to aid solubility, before diluting in DMEM to 1% (v/v). Control cells were treated with a vehicle control containing 1% ethanol where appropriate.

#### *Acid phosphatase activity*

Acid phosphatase activity was used to assess effects of agonists and inhibitors on cell viability.<sup>14</sup> Cells were grown in 96-well plates and treated as described above for up to 7d then washed twice with phosphate buffered saline (PBS). Acid phosphatase solution containing 0.1M sodium acetate, 1% v/v Triton X-100 and 5mM p-nitrophenyl in distilled water (pH 5.0) was added to each well and cells were incubated in the dark for 1h at 37°C. Sodium hydroxide (0.25M) was added to stop the reaction and the absorbance measured. Viability was calculated relative to vehicle control treated cells.

#### *Interleukin-6 (IL-6)*

Accumulation of IL-6 in cell culture medium was used as a measure of the inflammatory response. Cells were grown in 96 well plates and incubated as above for 24h. IL-6 was measured in cell supernatants using enzyme immunoassay according to the manufacturer's instructions (R&D Systems, Oxford, UK) and as we have previously reported.<sup>13</sup>

#### *Mitochondrial membrane potential*

Mitochondrial membrane potential was analyzed in intact cells using the fluorescent probe JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolcarbocyanine iodide, Invitrogen, Paisley, UK), a lipophilic cation which accumulates within the negatively charged matrix of intact energized mitochondria, as we have reported previously.<sup>13</sup> JC-1 fluoresces green in low concentrations, but at high concentrations forms so called 'J-aggregates' which fluoresce red. Mitochondrial membrane potential is proportional to red/green fluorescence ratio.<sup>15</sup> After 7d treatments as described above, cells were washed with PBS and then incubated for 30 min with JC-1 in PBS at 37°C, in the dark. Following incubation, cells were washed again with PBS and the red/green fluorescence ratio was measured immediately at 37°C. A decrease in the ratio of red/green fluorescence indicates loss of mitochondrial membrane potential.

#### *Metabolic activity*

Metabolic activity was analyzed by measuring the rate of reduction of AlamarBlue™ in intact cells after 7d treatment as above.<sup>16</sup> Briefly, following cell treatments, AlamarBlue™ was added to each well and fluorescence was measured every 10 min for 2 h at 37°C. Metabolic activity was determined as the rate of change in fluorescence over time at 37°C.

#### *Mitochondrial density*

MitoTracker green FM is a dye which localises to mitochondria independently of mitochondrial membrane potential and so can be used to determine mitochondrial density as a surrogate for the number of mitochondria and hence increases reflect biogenesis.<sup>17</sup> Cells were grown in 96-well plates and treated as before for 7d. After incubation, cells were washed twice with PBS then 0.5µM MitoTracker Green FM (Invitrogen) in PBS was added and cells were incubated in the dark, for 30 min at 37°C. Excess dye was removed by washing with PBS then the fluorescence was measured at 37°C.

#### *Total reduced glutathione*

Glutathione was measured as an indicator of mitochondrial antioxidant levels. The lipophilic compound monochlorobimane (Sigma-Aldrich) binds to glutathione via the action of the enzyme glutathione-S-transferase. The fluorescence of the resulting conjugate is proportional to the reduced glutathione concentration.<sup>18</sup> Cells were treated for 7d as previously described, washed in PBS, then monochlorobimane solution added. After incubation at 37 °C in the dark for 15 min, glutathione levels were analysed by measuring fluorescence.

#### *Statistical analysis*

Six independent experiments with 4 technical replicates were undertaken (n=6). Data are presented as percentage of median control value without LPS to allow direct comparisons between cell treatments. No assumptions were made about data distribution and data are shown as median, interquartile and full range. Statistical analysis was undertaken on raw data, using Analyse-It Statistical Add-in for Microsoft

Excel. Comparisons between vehicle control and LPS/PepG treated cells without agonists and between cells treated with LPS/PepG plus agonist with and without the relevant inhibitor were undertaken using Wilcoxon-Mann Whitney testing. Effects of the different concentrations of the agonists on LPS/PepG treated cells was assessed initially using Kruskal Wallis analysis then Wilcoxon-Mann Whitney *post hoc* testing as appropriate. A p value of <0.05 was taken as significant.

## Results

### *Cell viability*

None of the concentrations of the agonists or the inhibitors had a detrimental effect on cell viability after 7d exposure with or without LPS/PepG at the concentrations used, although treatments above this range did have a marked effect on viability in the presence of LPS/PepG. Viability of cells was over 90% after 7d exposure to any of the treatments in all experiments. Full viability data are shown in the supplementary file.

### *Interleukin-6*

IL-6 was significantly higher in culture supernatants after 24h treatment of endothelial cells with LPS/PepG compared to vehicle control cells, as expected (Figure 2). Treatment of cells with LPS/PepG plus SIRT-1-activator-3 resulted in significant suppression of LPS/PepG-induced IL-6 release ( $p=0.003$ , Figure 2A) with the most marked effect at the highest concentration. Inclusion of the inhibitor EX527 blunted the effect of SIRT-1 activation on IL-6 concentrations ( $p=0.002$ , Figure 2A). Treatment of cells with LPS/PepG plus 2HBA resulted in markedly lower IL-6 at all concentrations of 2HBA such that levels were similar to control values at the highest concentration of 2HBA ( $p<0.0001$ , Figure 2B). When cells were also exposed to trigonelline the IL-6 concentration was significantly higher than without the inhibitor, but remained lower than with LPS/PepG alone ( $p=0.0007$ , Figure 2B). In the absence of LPS/PepG, basal IL-6 levels were also significantly decreased by 2HBA but not SIRT-act-3 (see supplementary file).

### *Mitochondrial assays*

Significantly lower mitochondrial membrane potential, metabolic activity and mitochondrial volume were seen in cells exposed to LPS/PepG for 7d compared to control cells (Figure 2A-2F). Treatment of cells with SIRT-1-activator-3 or 2HBA plus LPS/PepG resulted in restoration of membrane potential at the highest concentrations of both the agonists, and this effect was blocked by the relevant inhibitor (Figures 3A and 3D). The effect of SIRT-1 activator-3 on metabolic activity just failed to reach statistical significance ( $p=0.05$ ) although co-treatment with the inhibitor resulted in significantly higher metabolic activity than with the agonist alone ( $p=0.0001$ , Figure 3B). In cells treated with LPS/PepG plus 2HBA, metabolic activity was significantly higher ( $p=0.0001$ , Figure 3E) such that at the highest concentrations of 2HBA, levels were similar to that of control cells. Again the inhibitor prevented this increase ( $p=0.0001$ , Figure 3E). Mitochondrial density was significantly increased in SIRT-1-activator-3 treated cells at the highest concentration only and there was no significant effect of the inhibitor (Figure 3C). In contrast, mitochondrial density was lower in those cells treated with LPS/PepG plus 2HBA ( $p=0.0001$ ), an effect which was ameliorated by trigonelline ( $p=0.002$ , Figure 3F). In the absence of LPS/PepG, SIRT-1-activator-3 had no effect on any mitochondrial assay, but metabolic activity was lower in the presence of EX527 alone (see supplementary file). 2HBA also did not affect any of the mitochondrial assays but trigonelline treatment alone resulted in higher mitochondrial metabolic activity and lower density (see supplementary file).

### *Glutathione*

Seven days exposure to LPS/PepG had minor effects on endothelial cell glutathione levels (Figure 4A and 4B). Despite this, SIRT1-activator-3 had marked dose dependent effects on glutathione, resulting in large increases ( $p<0.0001$ , Figure 4A). This effect was partially blocked by the antagonist ( $p=0.0004$ , Figure 4A). In cells treated with LPS/PepG plus 2HBA, glutathione levels were modestly but significantly higher than with LPS/PepG alone only at the highest 2HBA concentration (Figure 4B) and co-treatment with inhibitor resulted in glutathione levels below that of the control cells ( $p<0.0001$ ,

Figure 4B). Even in the absence of LPS/PepG a similar pattern of effect on glutathione by SIRT-1-activator-3 and EX527 was seen but neither 2HBA nor trigonelline had any effect (see supplementary file).

## Discussion

In this study we showed that, under conditions mimicking sepsis, activation of the PGC1 $\alpha$  and NFE2L2 pathways using pharmacological approaches protected human endothelial cells from mitochondrial damage, and dampened the inflammatory response. Activation of NFE2L2 markedly suppressed LPS-PepG induced IL-6 responses, improved mitochondrial membrane permeability and metabolic activity, but did not promote mitochondrial biogenesis or increase glutathione levels. Activation of PGC1 $\alpha$  had minor yet significant effects on IL-6, but protected against loss of mitochondrial membrane potential and metabolic activity and resulted in increased mitochondrial density (biogenesis) in addition to markedly increasing glutathione levels. Inhibitors blunted these effects. These data show that activation of these interacting pathways impacts upon inflammatory responses and mitochondrial changes induced by an environment mimicking sepsis and may suggest future novel therapeutic targets.

Some confusion has arisen in previous literature since the same alias (Nrf2) has been commonly used for both nuclear factor-erythroid-derived 2-like 2 (official abbreviation = NFE2L2) and GA binding protein transcription factor alpha (official abbreviation = GABPA, but also known as nuclear respiratory factor 2).<sup>19</sup> It is now clear that PGC1 $\alpha$  participates in the signalling pathways of both these transcription factors, which are nevertheless distinct. PGC1 $\alpha$  co-activates both NFE2L2 and GABPA, notably under conditions of redox imbalance;<sup>20</sup> NFE2L2 regulates the expression of antioxidant mechanisms, whilst GABPA promotes translocation of transcription factors into mitochondria which ultimately upregulate mitochondrial biogenesis (Figure 1).

Oxidative stress, inflammation, antioxidant depletion and mitochondrial damage and dysfunction have been consistently described in sepsis<sup>1 3 21 22</sup> and so restoration of endogenous antioxidant levels and preventing and/or restoring mitochondrial energetic function is likely to be beneficial in sepsis.<sup>3-5</sup> In this context, the PGC1 $\alpha$ -NFE2L2 pathway has emerged as a potential target due to its control of mitochondrial biogenesis, metabolism, inflammatory mediators and endogenous antioxidants.<sup>6</sup> In this study we pharmacologically activated SIRT-1 with SIRT-1-activator-3, a compound which has been shown to specifically interact with and activate SIRT1, but not other SIRT isoforms, resulting in deacetylation and activation of PGC1 $\alpha$ ,<sup>9</sup> whilst EX527 is a potent and selective inhibitor of SIRT1 activity.<sup>12</sup> We used 2HBA to activate NFE2L2 via modifying its repressor KEAP-1,<sup>10</sup> and trigonelline, which inhibits NFE2L2 by blocking its translocation into the nucleus.<sup>11</sup>

Activation of NFE2L2 by 2HBA acts by directly modifying cysteine sulphydryl groups in KEAP-1, causing a conformational change, allowing release and translocation of NFE2L2 into the nucleus. This upregulates expression of targets with ARE in their upstream promoters and results in transcription of several protective pathways such as enzymes which control the synthesis and metabolism of glutathione (Figure 1).<sup>7 10</sup> Thus 2HBA is an indirect inhibitor of the interaction between NFE2L2 and KEAP-1; such inhibitors can be grouped according to their structures and the way in which they interact with cysteine sulphydryl groups. So called 'Michael acceptors' are olefins or acetylenes conjugated with electron-withdrawing carbonyl groups and include both curcumin and 2HBA.<sup>23 24</sup> Trigonelline (*N*-methylnicotinic acid) is one of the major alkaloids in raw coffee beans, and has been shown to inhibit the nuclear translocation of NFE2L2.<sup>11</sup> Treatment of cells with 2HBA plus trigonelline would therefore promote the dissociation of NFE2L2 from its repressor but translocation would be inhibited and NFE2L2 would be functionally inactive.

Treatment of endothelial cells with LPS/PepG as used here has previously been shown to result in oxidative stress with alterations to mitochondria and consumption of

glutathione, along with inflammatory responses associated with activation of the transcription factor nuclear factor kappa B (NF $\kappa$ B).<sup>13 25</sup> We have also shown previously that antioxidants which specifically protect mitochondria ameliorate such LPS/PepG mediated effects both *in vitro* and in whole animal models.<sup>13 25 26</sup> In this current study we used pharmacological agonists to promote the endogenous signalling mechanisms which protect and replenish damaged mitochondria. Activation of the PGC1 $\alpha$ -NFE2L2 pathway under conditions mimicking sepsis was able to ameliorate the reduction in mitochondrial membrane potential and metabolic activity mediated by LPS/PepG treatment in a similar way to exogenous antioxidants which act in mitochondria such as melatonin or mitoQ.<sup>13 25 15</sup> Indeed, melatonin may exert some of its protective effects via SIRT-1.<sup>27</sup>

Reactive oxygen species produced by mitochondria (mtROS) may drive inflammatory cytokine production including IL-6<sup>3 28</sup> and early relative levels of IL-6 and the anti-inflammatory cytokine IL-10 are important in terms of the severity of sepsis.<sup>29</sup> Evidence of mitochondrial damage and dysfunction plus increased NF $\kappa$ B activation and elevated biomarkers of inflammation are also seen in patients with sepsis.<sup>1 3-5</sup> Previous studies have described a role for NFE2L2 signalling in down-regulation of inflammation<sup>19 30</sup> and we showed here that activation of NFE2L2 by 2HBA had profound effects on IL-6, with almost complete suppression of the IL-6 response under conditions of sepsis, an effect also seen in cells without LPS/PepG. This is likely to be via effects on NF $\kappa$ B since disruption of NFE2L2 signalling in knockout mice treated with LPS had enhanced NF $\kappa$ B activation with increased inflammatory cytokine expression and higher mortality.<sup>30</sup> Others have also reported that PGC1 $\alpha$  knockout mice have decreased NFE2L2 with increased levels of inflammatory cytokines.<sup>31</sup> We found a definite dampening effect of PGC1 $\alpha$  activation on IL-6 levels, but the effect was much less than that seen when NFE2L2 was activated. IL-6 has important roles in various aspects of the immune response during sepsis and contributes to acute phase and inflammatory responses. High IL-6 concentrations have been shown previously to be associated with increased morbidity and mortality in sepsis.<sup>32</sup> We have reported elevated IL-6 levels in conjunction with decreased antioxidant defences and biomarkers of oxidative stress and acute phase inflammation, in patients with sepsis.<sup>22</sup> The agonist 2HBA, as well as facilitating nuclear translocation of NFE2L2, may also activate SIRT1 and/or cAMP response element binding protein (CREB), thus promoting activation of PGC1 $\alpha$  which then in turn co-activates NFE2L2; this may explain the large effects on IL-6.<sup>19 33</sup> It is interesting that the complete reversal of the effect of 2HBA on the mitochondrial assays and glutathione by trigonelline was not reflected in the effect of trigonelline on IL-6. Although the 2HBA-mediated decrease in IL-6 release was not completely reversed by trigonelline, it is important to note the effect of the inhibitor did result in IL-6 levels above that seen with 2HBA alone with no overlap of the ranges, although still well below the level with LPS/PepG alone. IL-6 was measured after 24h since this is the time frame for the response to LPS/PepG exposure and this may impact on the difference between the effects of trigonelline on IL-6 compared to other measures which were after 7d exposure to treatments. IL-6 is regulated mainly via NF $\kappa$ B and we have assumed the effects of 2HBA on IL-6 are mediated by NFE2L2/NF $\kappa$ B interactions. The effects of 2HBA on mitochondrial function are not regulated via NF $\kappa$ B.

We investigated the effect of activating the PGC1 $\alpha$ /NFE2L2 pathways on glutathione levels under conditions of sepsis. We did not find altered total cellular glutathione although we have previously found a decreased ratio of reduced:oxidised glutathione after 7d of LPS/PepG treatment using the same model, indicating consumption of reduced glutathione and oxidative stress.<sup>13</sup> Strikingly however, treatment of cells with SIRT-1-activator-3 resulted in profound dose-dependent increases in glutathione levels, which were decreased slightly by the EX527 inhibitor; similar effects were seen even in the absence of LPS/PepG. Treatment of cells with 2HBA plus LPS/PepG also resulted in increased glutathione levels, but the effects were much less marked than the effects of SIRT1 activation. Trigonelline reversed the effect of 2HBA on glutathione. We have shown previously that the oxidative stress induced by LPS/PepG in the endothelial cell

model used here results in mitochondrial oxidative stress.<sup>34</sup> Superoxide generated inside mitochondria is primarily converted to hydrogen peroxide within the mitochondria by superoxide dismutase. The hydrogen peroxide formed is then removed mainly by oxidation and reduction of mitochondrial glutathione by the actions of mitochondrial glutathione peroxidase-1 and glutathione reductase. Oxidation of mitochondrial thioredoxin-2 (TRX-2) also has an important role in detoxifying hydrogen peroxide and we have shown previously in the same cell model that the TRX-2 system may be more important for protection against mitochondrial dysfunction induced by LPS/PepG than the glutathione system.<sup>34</sup> Most glutathione is present in the reduced form (GSH) and the large increases seen in response to SIRT1 activation are likely to be due to increased synthesis. Synthesis of  $\gamma$ -L-glutamyl-L-cysteinyl-glycine, the tripeptide known as GSH, occurs in a two step process and it is the first step involving the enzyme glutamate-cysteine ligase (GCL, previously known as  $\gamma$ -glutamylcysteine synthase), which is rate limiting. Expression of GCL has been shown to be controlled via NFE2L2<sup>19</sup> and so it was somewhat surprising to see that SIRT1-activator-3 had a much larger effect on glutathione than 2HBA. However it has been suggested that in addition to PGC1 $\alpha$  co-activating NFE2L2, the converse is also true.<sup>19</sup> In addition glutathione synthesis can also be affected by substrate availability and feedback inhibition.

The production of structurally and functionally intact mitochondria occurs via biogenesis, a process involving growth and division of existing mitochondria and requires the coordinated response of both mitochondrial and nuclear transcription factors which direct transcription and replication of mitochondrial DNA. Only a small number of the proteins required for biogenesis are actually encoded by mitochondrial genes. In fact biogenesis requires the co-ordinated synthesis and importation of around a thousand proteins into the mitochondria, along with post-translational assembly processes in the mitochondrial membrane. In addition biogenesis also requires sequential fusion and fission processes.<sup>6</sup> The master regulator of this complex process is PGC1 $\alpha$ . Although biogenesis is a physiological process needed for replacement of damaged mitochondria and can be triggered by exercise, exposure to cold, and other environmental stressors, under conditions of inflammation including sepsis, biogenesis is crucial for production of adequate numbers of functionally intact mitochondria to cope with the increased energy demand. Studies in animals have shown that recovery after sepsis is associated with recovery of mitochondrial number and increased expression of PGC1 $\alpha$ <sup>35</sup> and animals genetically deficient in PGC1 $\alpha$  did not mount this response.<sup>36</sup> These PGC1 $\alpha$ <sup>+/-</sup> mice also had decreased nuclear NFE2L2 expression, confirming the role of PGC-1 $\alpha$  as a co-activator of NFE2L2.

Here, we investigated the scenario of conditions of sepsis with oxidative stress but in the presence of pharmacological PGC1 $\alpha$  or NFE2L2 activation. We found that only SIRT1-activator-3 (ie activation of PGC1 $\alpha$ ) was able to promote biogenesis. This fits with the role of PGC1 $\alpha$  in co-activation of GABPA which then promotes the activation pathways required for biogenesis to occur.<sup>6</sup><sup>19</sup> However PGC1 $\alpha$  activation is complex and can occur via a large number of routes, such as calcium and second messenger pathways, hormones, cyclin dependent kinases, energy dependent pathways and post-translational modifications including phosphorylation and deacetylation.<sup>6</sup> EX527 did not have a full reversal effect on the increase in mitochondrial density in cells co-treated with LPS/PepG plus SIRT-1-activator-3. The action of EX527 has been shown to be via a unique NAD-dependent mechanism with partial formation of a product<sup>37</sup> such that EX527 inhibits the action of the activator rather than PGC1 $\alpha$  activation per se. It is possible to speculate that this may have some impact on our findings. Direction of cellular responses towards biogenesis, antioxidant mechanisms or anti-inflammatory responses is also likely to depend on the exact mechanism of activation. The decreased/increased biogenesis seen with 2HBA alone/2HBA plus trigonelline respectively suggests that direction of the cellular response pathways towards antioxidant/anti-inflammatory responses occurs in preference to biogenesis pathways when NFE2L2 is activated by 2HBA. Inclusion of

trigonelline prevents nuclear translocation of NFE2L2 but activation of PGC1 $\alpha$  by 2HBA may still occur. Our data suggest that dissection of the relative contribution of activation of PGC1 $\alpha$  or NFE2L2 is complex, with reciprocal co-activation and engagement of multiple pathways as a result of exposure to the septic insult in addition to the pharmacological agonists.

The development of organ dysfunction during human sepsis is linked to mitochondrial dysfunction and cellular energetic failure and so activation of PGC1 $\alpha$ /NFE2L2 is likely to be beneficial. Although genetic techniques such as the use of small interfering RNA (siRNA) can offer exact specificity, the pharmacological approach used here has perhaps more potential for translation to clinical use in the future. Other compounds which can activate the PGC1 $\alpha$  pathway and have been given safely to humans include curcumin analogues, resveratrol and a synthetic SIRT-1 activator SRT2104.<sup>38</sup> A mixed response to pre-treatment with oral SRT2104 in healthy men given a dose of LPS was recently reported, with dampening effects on IL-6 and IL-8 but no effect on clinical symptoms or markers of cell activation.<sup>39</sup> However SRT2104 levels after oral dosing were variable and oral bioavailability of SRT2104 was low.<sup>38 39</sup> Resveratrol has also been hampered by similar oral bioavailability issues although improvements using nanotechnology are encouraging.

In summary we have shown that pharmacological activation of the PGC1 $\alpha$  and NFE2L2 pathways in an endothelial cell model under conditions mimicking sepsis has protective effects on mitochondria, the glutathione system and IL-6. Although cell studies are only models for research purposes endothelial cells do have vital roles in host defence and inflammation during sepsis. Approaches which augment the body's own endogenous responses to combat oxidative damage are likely to be the most promising treatment strategies for sepsis in the future and the PGC1 $\alpha$ -NFE2L2 pathway is a potential target.

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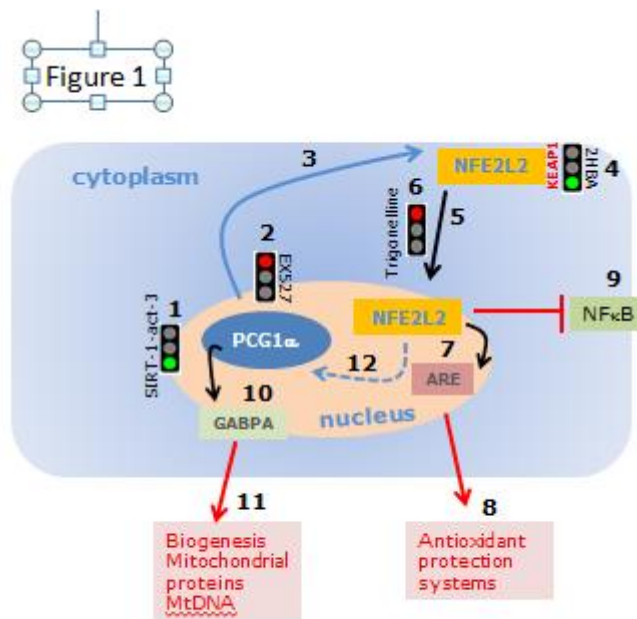
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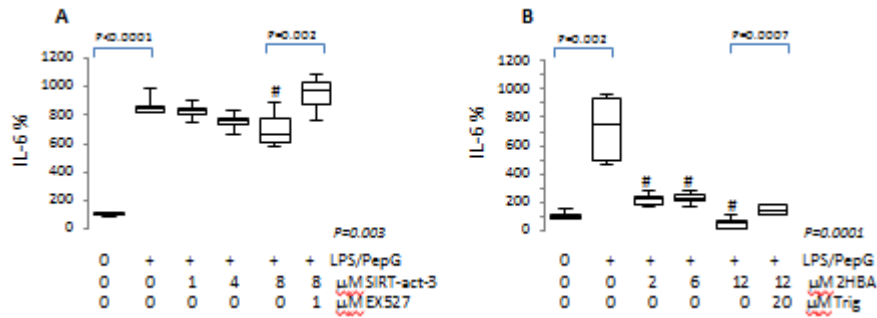
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## Figures



**Figure 1**

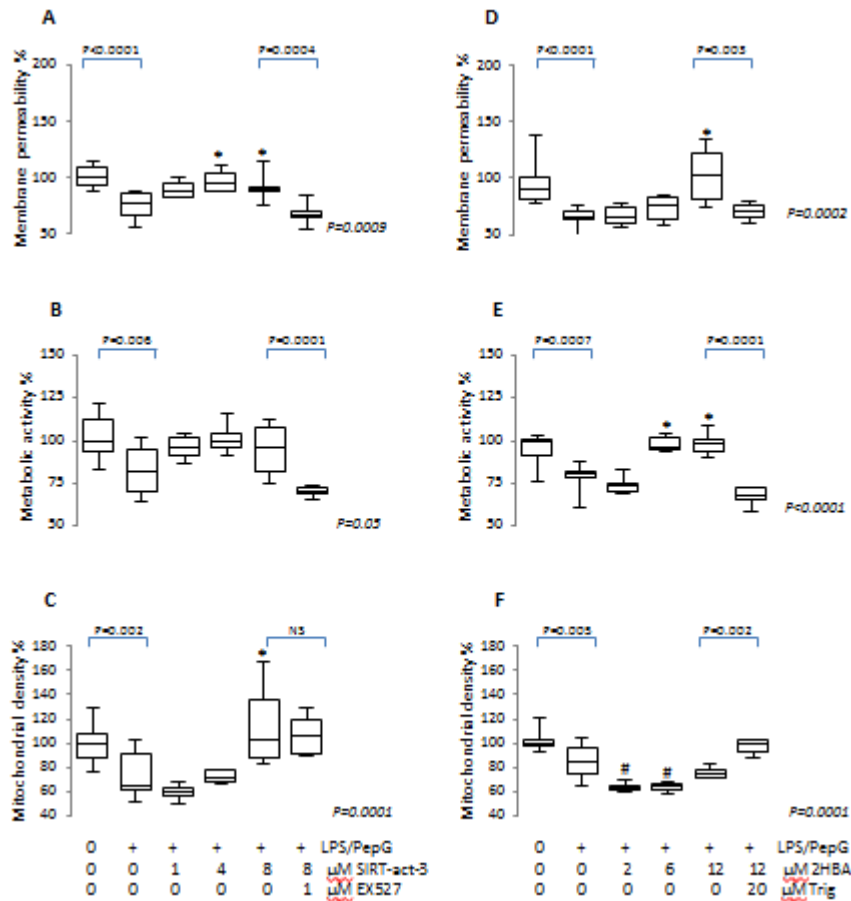
Very simplified depiction of the pathways described in this paper. PGC1 $\alpha$  can be regulated post-translationally via SIRT-activator-3 (1) and this is inhibited by EX527 (2). Activation of PGC $\alpha$  co-activates NFE2L2 (3), which can also be activated by 2HBA via actions on its repressor protein KEAP-1 (4). When activated, NFE2L2 translocates into the nucleus (5) and this can be blocked by trigonelline (6). In the nucleus NFE2L2 activates ARE (7) which leads to upregulation of protective antioxidant pathways (8), and inhibits NF $\kappa$ B (9). PGC $\alpha$  also co-activates GABPA (10) leading to transcription of genes needed for biogenesis and synthesis of key mitochondrial proteins. There is also the suggestion that NFE2L2 may in turn activate PGC $\alpha$  (12). For abbreviations see main text. Green traffic light indicates activator; red traffic light indicates inhibitor.



**Figure 2**

Effect of A. activation of PGC $\alpha$  by SIRT-1-activator-3 with and without and the inhibitor EX527 and B. activation of NFE2L2 by 2HBA with and without the inhibitor trigonelline, on IL-6 concentrations in culture medium from endothelial cells treated with LPS and PepG for 24h. Box and whisker plots show median, interquartile and full range as percentage of cells treated with vehicle control alone (n=6). P value in italics is Kruskal Wallis across LPS/PepG plus activator groups. Other p values are from Mann Whitney-Wilcoxon test.

# = significantly lower than with LPS/PepG alone (p<0.05).

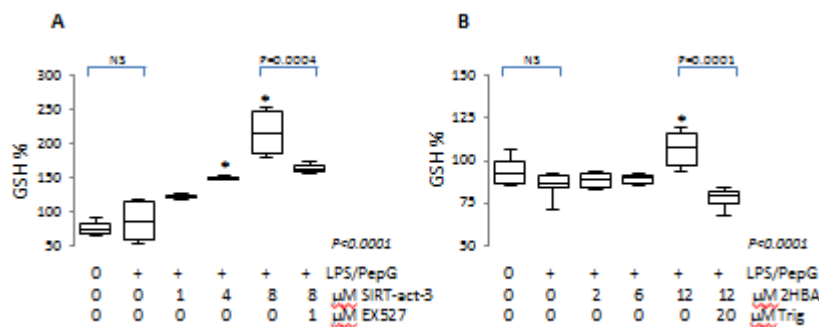


**Figure 3**

Effect of activation of PGC $\alpha$  by SIRT-1-activator-3 with and without the inhibitor EX527 on A. mitochondrial membrane potential B. mitochondrial metabolic activity C. mitochondrial volume. Effect of activation of NFE2L2 by 2HBA with and without the inhibitor trigonelline on D. mitochondrial membrane potential E. mitochondrial metabolic activity and F. mitochondrial volume, in intact endothelial cells treated with LPS and PepG for 7d.

Box and whisker plots show median, interquartile and full range as percentage of cells treated with vehicle control alone (n=6). P value in italics is Kruskal Wallis across LPS/PepG plus activator groups. Other p values are from Mann Whitney-Wilcoxon test.

\*= significantly higher and # = significantly lower than with LPS/PepG alone (p<0.05).



**Figure 4** Effect of A. activation of PGC $\alpha$  by SIRT-1-activator-3 with and without the inhibitor EX527 and B. activation of NFE2L2 by 2HBA with and without the inhibitor trigonelline on glutathione (GSH) content in intact endothelial cells treated with LPS and PepG for 7d. Box and whisker plots show median, interquartile and full range as percentage of cells treated with vehicle control alone (n=6). P value in italics is Kruskal Wallis across LPS/PepG plus activator groups. Other p values are from Mann Whitney-Wilcoxon test. \*= significantly higher than with LPS/PepG alone (p<0.05).

### Authors' contributions

Study design/planning: HFG, DAL

Study conduct: MMFS, GM, DAL

Data analysis: MMFS, GM, HFG

Writing paper: HFG, NRW

Revising paper: all authors

GM and MMFS contributed equally to the study.

### Declarations of interest

NRW is Chairman and HFG is an Editor of the British Journal of Anaesthesia. Both NRW and HFG are Members of the Board of Management of the BJA and HFG, NRW and DAL have previously received research funding from the BJA.

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## Effect of activators and inhibitors on cell viability after 7d

**Table 1. SIRT-1-activator-3 and EX527 with and without LPS/PepG**

Concentration $\mu\text{M}$	SIRT-1-activator 3 alone %	SIRT-1-activator 3 + LPS/PepG %	EX527 alone %	EX527 + LPS/PepG %
1	99 [97-102]	99 [95-101]	103 [98-105]	98 [97-105]
2	100 [97-102]	99 [95-103]	103 [95-106]	103 [98-108]
5	100 [100-105]	99 [96-104]	101 [100-105]	100 [96-103]
8	102 [99-104]	98 [86-101]	104 [102-106]	100 [98-103]
10	99 [97-104]	93 [78-96]*	102 [97-106]	98 [95-105]
15	99 [98-102]	92 [78-97]*	102 [98-106]	100 [96-102]
20	70 [53-85]*	55 [41-76]*	95 [92-100]*	93 [89-99]*

Median [range] percentage of vehicle control treated cells (n=6).

\*significantly lower than control (post hoc Mann Whitney U test).

For full details see main manuscript.

**Table 2. 2HBA and trigonelline with and without LPS/PepG**

Concentration $\mu\text{M}$	2HBA alone %	2HBA + LPS/PepG %	Trigonelline alone %	Trigonelline + LPS/PepG %
2	100 [94-102]	101 [100-102]	101 [93-102]	100 [98-103]
4	98 [92-101]	96 [92-100]	101 [102-104]	101 [97-102]
6	92 [90-101]	99 [90-101]	102 [98-103]	100 [99-104]
8	99 [99-100]	99 [95-101]	103 [101-104]	101 [99-102]
12	100 [99-101]	94 [93-99]	103 [99-104]	101 [100-103]
16	71 [70-72]*	82 [80-85]*	100 [96-103]	101 [98-105]
20	18 [18-19]*	18 [19-21]*	102 [98-105]	103 [98-104]

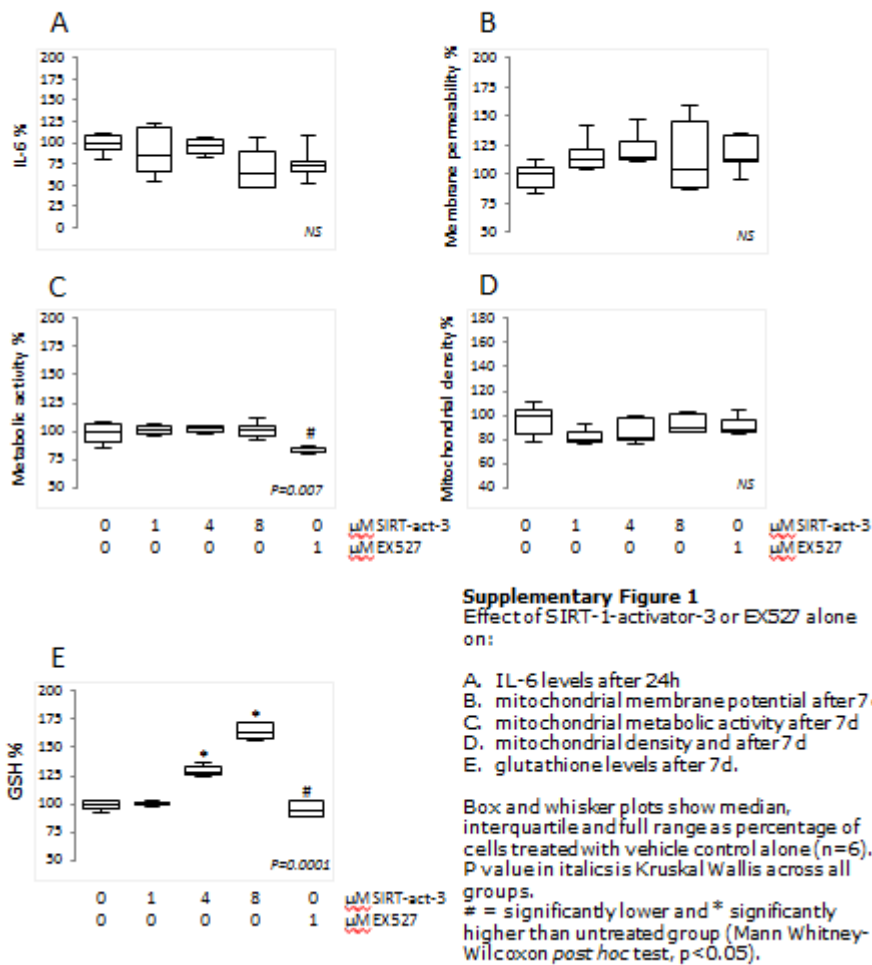
Median [range] percentage of vehicle control treated cells (n=6).

\*significantly lower than control (post hoc Mann Whitney U test).

For full details see main manuscript.

## Effect of activators and inhibitors without LPS/PepG

Figure 1. SIRT-1-activator 3 and EX527





## Effect of activators and inhibitors without LPS/PepG

Figure 2. 2HBA and trigonelline

