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Running title: Mitochondrial TRX-2 and GSH in sepsis

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ABSTRACT

Oxidative stress and mitochondrial dysfunction are common features in patients with sepsis and organ failure. Within mitochondria, superoxide is converted to hydrogen peroxide by manganese containing superoxide dismutase (MnSOD) which is then detoxified by either the mitochondrial glutathione system, using the enzymes mitochondrial glutathione peroxidase-1 (mGPX-1), glutathione reductase (GRD) and the mitochondrial pool of glutathione (mGSH), or the thioredoxin-2 system, which uses the enzymes peroxiredoxin-3 (PRX-3) and thioredoxin reductase-2 (TRX-2R) and thioredoxin-2 (TRX-2). We investigated the relative contribution of these two systems, using selective inhibitors, in relation to mitochondrial dysfunction in endothelial cells cultured with lipopolysaccharide (LPS) and peptidoglycan (PepG). Specific inhibition of both the TRX-2 and mGSH systems increased intracellular total radical production (p<0.05) and reduced mitochondrial membrane potentials (p<0.05). Inhibition of TRX-2 system, but not mGSH, resulted in lower ATP production (p<0.001) with high metabolic activity (p<0.001), low oxygen consumption (p<0.001) and increased lactate production (p<0.001) and caspase 3/7 activation (p<0.05). Collectively these data show that the TRX-2 system appears to have a more important role in preventing mitochondrial dysfunction than the mGSH system in endothelial cells under conditions that mimic a septic insult.

Sepsis, oxidative stress, endothelium, inflammation, peroxiredoxin, thioredoxin

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INTRODUCTION

Sepsis is a severe infection causing dysregulation of inflammatory responses and may result in multiple organ dysfunction syndrome (MODS), which has a mortality rate of around 70% [1,2]. Oxidative stress and mitochondrial dysfunction are associated with sepsis and organ failure in animal models [3-7] and with poorer outcome in patients with sepsis [8-11].

The impact of mitochondrial functional capacity and the mechanisms related to mitochondrial dysfunction during sepsis and MODS are still not fully understood. During sepsis. leucocytes and mitochondria produce elevated amounts of reactive oxygen species (ROS), reviewed in [12]. Enhanced mitochondrial generation of the superoxide anion radical is observed in non-surviving groups in various models of sepsis and may be a starting point of mitochondrial oxidative stress [4,13,14]. Improved outcomes in animal models of sepsis have been achieved by us and others utilising mitochondrial targeted antioxidants (extensively reviewed [15-17]). Superoxide does not readily cross the mitochondrial membrane and is catalysed to hydrogen peroxide within the mitochondria by manganese-containing superoxide dismutase (MnSOD). Hydrogen peroxide can diffuse out of the mitochondrion and is metabolised by catalase in the peroxisome, but is primarily removed within the mitochondrion by oxidation of reduced mitochondrial glutathione (mGSH) catalysed by mitochondrial glutathione peroxidase-1 (mGPx-1) with recycling back to reduced glutathione catalysed by glutathione reductase (GRD) [18]. It is now known that oxidation of mitochondrial thioredoxin-2 (TRX-2) in the presence of peroxiredoxin-3 (PRX-3) with subsequent recycling via thioredoxin reductase-2 (TRX-2R) is also important [19]. This TRX-2 system has been reported to be more efficient at maintaining mitochondrial proteins in a reduced state compared to the mGSH system [20], although under conditions of low level oxidative stress, both systems keep hydrogen peroxide levels in check and have definitive roles in cell signalling [20]. However under conditions of more severe oxidative stress, the TRX-2 system may become more important [15].

The relative importance of the mGSH and TRX-2 systems and mitochondrial dysfunction during the cellular events of sepsis are not known. Endothelial cells recognise and respond to invading pathogens [21] and loss of endothelial integrity contributes to the morbidity and mortality associated with sepsis. Increased mitochondrial generation of hydrogen peroxide and pro-inflammatory cytokine production in response to bacterial cell wall components and inflammatory mediators by endothelial cells, has been well documented [22,23]. Therefore, the aim of our study was to examine the consequences of inhibiting either mGPX-1 with thiomalic acid or TRX-2R with the gold compound auranofin, on oxidative stress and relative mitochondrial function in human endothelial cells cultured under conditions which mimic a mixed Gram negative/Gram positive septic insult.

EXPERIMENTAL

Materials

Auranofin was obtained from Alexis Biochemicals, AXXORA Ltd, Nottingham, UK; 5-(6)carboxy-2,7'-dichlorodihydro-fluorescein-diacetate and the Molecular ProbesTM ATP determination kit were from Invitrogen, Paisley, UK. Caspase-Glo® was from Promega, Southampton, UK; protease /phosphatase inhibitor cocktail was from Roche, Hertfordshire, UK; Bradford reagent was from Bio-Rad, Hertfordshire, UK and antibodies for western blotting were from Abcam, Cambridge, UK. All other reagents were from Sigma-Aldrich, Poole, Dorset, UK.

Cell culture and treatment

The human umbilical vein endothelial cell line (HUVEC C) was obtained from ATCC.org and were used at passages 5-14. This *in vitro* model of sepsis has been described previously in detail [16]. For experimentation, cells were grown in 96 well plates or on coverslips (see experimental detail below) in the presence of 2µg/ml lipopolysaccharide (LPS, from E. coli strain 0111:134) plus 20µg/ml peptidoglycan G (PepG, from S. Aureus strain 6571), prepared as described [24], plus either thiomalic acid or auranofin for up to 7d to allow mitochondrial dysfunction to develop. LPS/PepG are toll like receptor (TLR) 4 and TLR2 agonists respectively that play a key role in the innate immune system. We have shown previously that HUVEC increase their mitochondrial generation of hydrogen peroxide and pro-inflammatory cytokine production maximally in response to these concentrations of LPS/PepG [16]. Auranofin is a co-ordinated gold(I) compound that can react with selenol-containing residues and is a potent inhibitor of purified thioredoxin reductase protein. Thiomalic acid is a glutathione mimetic that can bind to active site -SH groups. Evidence suggests that the mitochondrial isoforms if TRX-R and GPx are more sensitive to inhibition than the cytosolic isoforms. To determine the selective inhibition of mitochondrial isoforms of the enzymes by auranofin and thiomalic acid we exposed cells to the inhibitors at a range of concentrations in preliminary experiments, and measured enzyme activities of mitochondrial and cytosolic isoforms of TRX-R and GPx (see below.) As a result of these experiements, 4mM thiomalic acid or 2µM auranofin were used in subsequent experiments. Some cells were treated with solvent only (untreated) or solvent plus inhibitor only, or solvent plus 50µM hydrogen peroxide or 25µM t-butyl-hydroperoxide as positive controls. Cell viability was assessed using acid phosphatase activity [25].

TRX-R and GPx activity

TRX-R and GPx activity was determined in cytosolic and mitochondrial fractions using Sigma® thioredoxin reductase assay and glutathione peroxidase activity assay kits respectively. Briefly, cytosolic or mitochondrial lysates were added to the appropriate assay buffers containing NADPH and enzyme mixes in a 1ml cuvette. Reactions were started by the addition of 5,5'-dithiobis(2-nitrobenzoic) acid or t-butyl-hydroperoxide for TRX-R or GPx activity respectively. TRX-R activity was determined by following the increase in absorbance at 412nm after a 2min delay and GPx activity by following the increase in absorbance at 430nm after a 15s delay, using a Helios beta single beam spectrophotometer.

Cytokine production

Cells were grown to confluence in 96 well plates and treated as above for 24h. Levels of interleukin (IL) -6 and IL-8 were measured in culture medium using a commercially available enzyme immunoassay kit.

Intracellular total radical production

Briefly, cells were grown to confluence in 96 well plates and loaded with 50μ M oxidation sensitive 5-(6)-carboxy-2,7'- dichlorodihydrofluorescein diacetate (CDHFD) or 50μ M oxidation-insensitive 5,(6)-carboxy-2,7-dichlorofluorescein (CDF) for 60min in the dark. Cells were then washed twice with phosphate buffered saline (PBS) before being treated immediately as described above. The rate (positive) of total radical formation was determined fluorimetrically as a result of oxidation of 5-(6)-carboxy-2,7'-dichlorofluorescein (CDF) as a continuous

recording until saturation of CDHF was reached, at a temperature of $37^{\circ}C$ at an excitation wavelength of 485 nm and emission wavelength 530nm. MitoQ (1µM) was used as a ROS scavenger to demonstrate the selectivity and reproducibility of CDHF.

Mitochondria isolation

Mitochondria were isolated following treatment, for analysis of GSH concentration and TRX-2, PRX-3 and mGPX-1 protein expression. Cells were typsinised, pelleted and washed with icecold PBS before being resuspended in 5vol homogenization buffer containing 0.25M sucrose, 20 mM HEPES-KOH pH7.5, 10mM KCl, 1.5mM MgCl₂, 1mM EDTA, 1mM EGTA, 1mM dithiotreitol and protease/phosphatase inhibitor cocktail for 10min on ice. The cells were then homogenised with a Dounce borosilicate glass homogeniser before being centrifuged at 300g to remove cell debris and nuclei. Cytosolic and mitochondrial fractions were obtained by centrifugation of the cleared lysate for 10min at 13,000g. Protein concentrations of the cytosolic and mitochondrial fractions were determined by the Bradford protein assay procedure.

Mitochondrial and total GSH: GSSH ratio

The ratio of reduced:oxidized glutathione (GSH:GSSG) in isolated mitochondria and whole cell lysates was used to indicate mitochondrial glutathione system activity (mitochondrial oxidative stress) and total glutathione metabolism. Isolated mitochondria or cell lysate was added to reaction buffer containing 0.1%v/v Triton-X 100 and 0.1M potassium phosphate, pH 6.5. GSH was measured by adding 20µM mono-bromobimane and 0.3mg/ml glutathione-S-transferase and incubated at 37°C in the dark for 15min. To measure oxidised glutathione, GSSG was reduced to GSH in separate lysed mitochondria or whole cell lysate samples with 2.22mM diethylene-triamine-penta-acetic acid and 2mM dithiothreitol in 1M HEPES buffer, pH8.5 for 30min at 37°C and measuring GSH as before. Fluorescence was determined at room temperature (excitation 355nm, emission 520nm) and normalized to µg mitochondrial protein.

Mitochondrial TRx-2, PRX-3 and mGPX-1 protein expression

Mitochondrial expression of TRX-2 protein and the enzyme PRX-3 were measured using redox western blotting techniques to determine the redox (oxidation) states of TRX-2 and PRX-3 [26,27]. This was achieved by modifying any reduced protein with small molecules that are capable of covalently modifying reduced active sites. For redox western blotting of TRX-2, mitochondria were resuspended in 20mM Tris buffer, pH8.0, and incubated with 15mM 4acetoamido-4'-maleimidvlstilbere-2,2'-disulphonic acid (AMS) for 3h at 37°C. For redox determination of PRX-3, mitochondria were incubated with 100mM N-ethylmaleimide, 40mM HEPES pH7.4, 50mM NaCl, 1mM EDTA, 1mM EGTA and 10µg/ml catalase for 15min at room temperature before addition of 5%w/v 3-(3-cholamidopropyl) dimethylammonio)-1-propanesulphonate hydrate (CHAPS). mGPX-1 protein expression was measured using standard western blotting techniques without redox modification. All proteins were separated by 12-15% polyacrylamide gel electrophoresis and transferred to Immobilon-PTM transfer membrane using standard techniques. Blocked membranes were probed with primary polyclonal antibodies using rabbit anti-human PRX-3, sheep anti-human TRX-2 and rabbit anti-human GPX-1 (with appropriate horseradish peroxidase-linked secondary antibodies). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as loading control. Bands were visualized by



enhanced chemiluminescence and quantified using GeneTools[™] (SynGene, Synoptics Ltd, Cambridge, UK).

Measurement of caspase 3/7 activity

Apoptosis was assessed by measuring the activation of caspase-3 and caspase-7 using the Caspase-Glo® 3/7 assay. Briefly, cells were grown to confluence in 96 well plates and treated as described above for 7d, then an equal volume of Caspase-Glo® 3/7 substrate solution was added to the culture medium and mixed gently for 30s on a vibrating platform. Cells were then incubated for 1h at room temperature in the dark. Luminescence was measured immediately using a fluorimeter set to detect luminescence.

Mitochondrial membrane potential

Mitochondrial membrane potential was analysed in intact cells using the fluorescent probe 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazol-carbo-cyanine iodice (JC-1). Briefly, following cell treatments in 96 well plates for 7d, cells were washed twice with PBS and then incubated for 30min with $10\mu g/ml$ JC-1 at 37° C in the dark. Following incubation, cells were washed twice with PBS and the orange/red and green fluorescence was measured immediately at 37° C (excitation 490 nm, emission 590/520 nm). As a positive control, cells were incubated with the uncoupling agent, rotenone (1mM) for 4h prior to JC-1 analysis. In intact mitochondria J aggregates form and JC-1 fluoresces red. When the mitochondrial membrane potential drops the JC-1 assumes a monomeric form and fluoresces green (see online supplementary figure).

Metabolic activity

Metabolic activity was analysed by measuring the rate of reduction of AlamarBlueTM in intact cells after treatment for 7d. Alamar Blue is a novel redox indicator that exhibits both fluorescent and colourimetric changes in response to changes in metabolic activity (oxidative metabolism) [28]. Briefly, following cell treatments, 10µl AlamarBlueTM in 100µl culture medium was added to wells. Fluorescence was measured every 15min for 2h at 37°C at an excitation wavelength of 530nm and emission wavelength 590nm.

ATP: ADP ratio

Cellular ATP: ADP ratio was determined after 7d treatment using the Molecular ProbesTM ATP determination kit. Briefly, following cell treatments in 96 well plates, 1×10^6 cells were washed with ice cold PBS before the addition of 50µl ice cold lysis buffer (25mM HEPES pH7.8, 5mM MgCl₂, 140mM NaCl and 1x detergent (somatic cell ATP-releasing reagent). Immediately following lysis, 10µl cell lysate was added to 100µl ATP determination buffer containing 1mM dithiothreitol, 0.5mM D-luciferin and 12pg firefly luciferase. To determine ADP levels, samples were treated with ADP converting solution, which contains ATP sulphurylase to remove endogenous ATP, and pyruvate kinase and phosphoenolpyruvate to convert ADP to ATP. ATP was then measured as above. Luminescence was read immediately.

Oxygen consumption

Cellular oxygen consumption was measured following 7d treatments, using a Clark-type oxygen electrode. Briefly, cells were grown on poly-L-lysine coated coverslips and, following cell

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treatments, were added to a 7ml oxygen electrode containing normal growth media maintained at 37°C. Oxygen consumption was measured for 15min.

Lactate production

Lactate was measured in culture medium after 7d treatment in 96 well plates, using the Abcam® lactate fluorometric assay kit. Briefly, conditioned culture media was removed and diluted in lactate assay buffer before being added to 96 well plates containing the lactate enzyme mix and probe. Appropriate calibration standards were also made and added to the plate. The reaction was allowed to proceed for 30min in the dark before fluorescence was measured at an excitation wavelength of 535nm and emission wavelength 590nm.

Statistical analysis

Six separate independent experiments were undertaken using 6 different cultures on different days including western blotting (n=6). No assumptions were made about the distribution of the data and non-parametric testing was performed using Kruskal Wallis analysis of variance and Mann Whitney *post hoc* testing as appropriate. A p value of <0.05 was taken to be significant.

RESULTS

TRX-R and **GPx** activity

The concentrations of the inhibitors chosen for this study were determined from initial experiments where maximal inhibition of the mitochondrial isoforms with no significant loss in cytosolic enzyme activity was seen. Our results showed that mitochondrial TRX-R and GPx enzyme activity decreased maximally at 2µM and 4mM respectively (p=0.004 and 0.024 respectively, Fig. 1A and 1C), with no change in cytosolic enzyme activity (Fig. 1B and 1D). Higher concentrations reduced mitochondrial isoform enzyme activities a little further, but with loss of mitochondrial selectivity such that cytosolic enzyme activities were also decreased.

Cell viability

Cell viability was 100% for all cell treatments, measured at 24 hours or 7 days, with the exception of cells exposed to LPS-PepG plus auranofin for 7 days treatment only, where the viability decreased to (median [interquartile range] 69.6 [63-79]% (p=0.0022).

Cytokine production

As expected, IL-6 and IL-8 concentrations were significantly increased in cells exposed to LPS/PepG compared to untreated cells (p<0.0001, Fig. 2). There was no effect of auronofin or thiomalic acid on LPS/PepG induced cytokine production (Fig. 2)

Intracellular total radical production

To assess whether inhibition of either PRX-3 or mGPX-1 under conditions of sepsis would induce a higher rate of intracellular oxidative stress, we incubated cells with LPS/PepG and with auranofin or thiomalic acid. Results showed that the rate of total intracellular radical formation in cells cultured under our conditions of sepsis was significantly greater than in untreated cells (p=0.03, Fig. 3). The rate was higher still in cells exposed to the inhibitors in addition to LPS and



PepG (p=0.03, Fig. 3). Radical production was also slightly higher in cells treated with the inhibitors alone (p=0.041, Fig. 3).

Mitochondrial thioredoxin and glutathione

To evaluate the inhibition of PRX-3 or mGPX with auranofin or thiomalic acid respectively, on overall glutathione and thioredoxin-2 systems, we measured intra-mitochondrial expression of TRX-2 protein and GSH concentrations following 7d septic insult. Mitochondrial TRX-2 protein levels were higher in all LPS and PepG treated cells than in untreated cells (p<0.001, Fig. 4A). However, cells treated with thiomalic acid in addition to LPS and PepG had significantly more TRX-2 expression than other cell treatments (p=0.01, Fig. 4A). In comparison, the intra-mitochondrial GSH:GSSG ratio was lower in cells treated with LPS/PepG (p<0.001, Table 1) and lower still in cells also treated with auranofin (p<0.001). Cells treated with thiomalic acid, with or without LPS-PepG, had similar mitochondrial GSH:GSSG ratios to untreated cells. In addition, all cell treatments resulted in a significant reduction in the total GSH:GSSG ratio (p=0.0159, Table 1).

Expression of PRX-3 and mGPX-1

We determined the effect of inhibition of PRX-3 and mGPX-1 activity on subsequent mitochondrial protein expression of these enzymes after 7d treatment with the inhibitors plus LPS/PepG. In cells treated with either LPS or PepG, or with LPS/PepG and the inhibitor, there was higher PRX-3 expression, which was greatest in cells treated with auranofin (p<0.001, Fig. 4C). Expression of mGPX-1 was higher in all cell treatments compared to untreated cells and was highest in cells treated with auranofin plus LPS/PepG (p<0.05, Fig. 4B).

Caspase 3/7 activation

The effect of inhibition of PRX-3 and mGPX on apoptosis was determined by measuring the activation of caspase 3/caspase 7 following 7d culture. There was no effect of LPS and PepG exposure when compared to untreated cells (p=0.3, Fig. 5A). However, when cells were treated with auranofin, which inhibits TRX2-R, both with and without LPS-PepG, caspase activation was higher than in cells without auranofin (p<0.03, Fig. 5A).

Mitochondrial membrane potential

Mitochondrial membrane potential was determined by the ratio of red/green JC-1 fluorescence after 7d treatments (Fig. 5B). Cells exposed to LPS and PepG had a significantly lower JC-1 fluorescence ratio than untreated cells, indicating loss of mitochondrial membrane potential (p<0.001, Fig. 5B). In those cells treated with LPS-PepG plus auranofin, there was an even greater loss of mitochondrial membrane potential (p=0.01 and <0.001 respectively, Fig. 5B). This was not seen with thiomalic acid treated cells.

Metabolic activity, oxygen consumption, ATP: ADP ratio and lactate production

Investigation of the functional capacity of mitochondria after 7d septic insult was achieved by measuring the metabolic activity as determined by the rate of reduction of Alamar blueTM by mitochondrial respiratory complex activity, total oxygen consumption, ATP:ADP ratios and lactic acid production (Table 2). The metabolic activity in cells treated with auranofin plus LPS/PepG was significantly higher than all other cell treatments (p<0.001). Cells treated with

LPS/PepG had significantly reduced oxygen consumption rates (p=0.028) and ATP: ADP ratios (p<0.001) and increased lactate formation (p<0.001). However, cells treated with LPS/PepG plus auranofin had a considerably lower oxygen consumption rate (p=0.02) and ATP: ADP ratio (p<001) and a large increase in lactate formation (p<0.001).

DISCUSSION

We have shown that inhibition of the mitochondrial thioredoxin-2 or glutathione systems in our endothelial cell model of sepsis resulted in higher total intracellular radical production and loss of mitochondrial membrane potential, with altered protein expression of various components related to these systems. Inhibition of the TRX-2 system resulted in higher mitochondrial metabolic activity, lower ATP/ADP ratios, lower oxygen consumption, increased lactate formation and evidence of caspase 3/7 activation. Inhibition of the mGSH system had little effect on mitochondrial function. This suggests that the TRX-2 system may be more important preserving mitochondrial function in endothelial cells under conditions of sepsis.

Sepsis is associated with increased pro-inflammatory cytokine production, oxidative stress, consumption of endogenous antioxidants and mitochondrial damage [3-8]. Tight control of cellular redox homeostasis is essential for protection against oxidative damage and for maintenance of normal metabolism. Therefore, mitochondrial generation of ROS is strictly regulated by mitochondrial antioxidant enzymes including MnSOD, mGSH/GPX-1 and the TRX-2/PRX-3 systems [19,30]. Although both systems remove mitochondrial hydrogen peroxide, redox potentials differ substantially between the two, to facilitate the complex multiple signalling roles of ROS [31]. Auranofin is a co-ordinated gold(I) compound that reacts with selenol-containing residues and is a potent inhibitor of purified thioredoxin reductase protein. At the concentrations used in our *in vitro* study, we showed inhibition of mitochondrial TRX-R only, which resulted in no loss in cell viability in unstimulated cells (higher concentrations were required to inhibit the cytosolic TRX-R isoform and caused 50% reduction in cell viability). This concentration effect could be due to auranofin's characteristics as it belongs to the phosphine class of compounds that can be selectively concentrated within mitochondria at low concentrations'. In addition, the concentrations required to inhibit the isoforms of THX-R is cell type specific indicating the complex nature of the thioredoxin system, extensively reviewed in [32,33]. Thiomalic acid is a glutathione mimetic that can bind to active site –SH groups. We inhibited mGPX-1 exclusively at the concentrations used here. It been shown previously that mitochondrial GPx is much more sensitive to thiomalic acid than the cytosolic isoform [34].

During sepsis, the basal rate of ROS production increases, specifically mitochondrial superoxide formation [4,13,14]. This is presumed to deplete antioxidants, increase free radical formation, and is associated with pro-inflammatory cytokine production and ultimately damage to mitochondria [3-11,16]. Antioxidant therapies directed at mitochondria are now being studied, and we and others have demonstrated beneficial effects on mitochondrial function both *in vitro* and in animal models of sepsis [16,17]. In endothelial cells treated with LPS/PepG plus inhibitors, the rate of total radical formation were enhanced above that of cells treated with LPS/PepG alone. However, this increase did not augment IL-6 or IL-8 production when compared to LPS-/PepG stimulated cells.

Mitochondrial oxidative stress was assessed by measuring intra-mitochondrial TRX-2 expression and intra-mitochondrial GSH:GSSG ratio compared to total GSH:GSSG ratio. After 24h (data not shown) and at 7d, we found that LPS/PepG treatments increased TRX-2

expression. A higher level of expression was seen in LPS/PepG treated cells treated with thiomalic acid and is likely to be compensatory for mGPx-1 inhibition. In addition, LPS/PepG treatment resulted in smaller decrease in both intra-mitochondrial and total GSH:GSSG ratios. However, auranofin caused an even lower intra-mitochondrial GSH:GSSG ratio without affecting total GSH:GSSG ratios, suggesting mitochondrial oxidative stress. We performed redox western blotting by covalent modification of any remaining reduced (after experimental treatment) TRX-2 in the mitochondrial lysates to assess the redox form of TRX-2. However, we found only one strong band indicating no oxidised TRX-2 in any treatment group. This result is consistent with a previous study using bovine aortic endothelial cells where TRX-2 was present only in the reduced form even when these cells were exposed to extreme oxidative stress (12mM hydrogen peroxide) [35].

Peroxiredoxins may be damaged by excessive ROS production such that a condition termed hyper-oxidation and inactivation occurs [20]. Previous work has shown that endothelial mitochondrial PRX3 is much less sensitive to oxidation and inactivation than cytosolic PRX isoforms [26]. We found only reduced PRX-3 in all cell treatments using redox western blotting. This may be due to the effects of the enzyme sulfiredoxin, which is able to reactivate peroxiredoxins following oxidation. This enzyme is up-regulated during oxidative stress and sepsis and can migrate into mitochondria, reactivating oxidised PRX-3 [36]. We propose that sulfiredoxin expression may be higher in endothelial cells than other cell types, explaining the increased resistance of PRX-3 to oxidation.

ROS removal in oxidative stress and sepsis is controlled in part by the level of endogenous antioxidant defences. The rate of removal of hydrogen peroxide *in vivo* by GPX-1 has been presumed to be independent of GSH concentration. However, kinetic studies have now revealed that this is not the case [37]. It has been shown *in vitro* using purified enzyme that the total upper limit of hydrogen peroxide removal by GPX-1 is approximately 700nM, although under conditions of oxidative stress and sepsis, levels of intracellular hydrogen peroxide may reach 5μ M [38]. This would cause the pool of mGSH to fall rapidly, which is replaced very slowly by diffusion of cytosolic GSH into the mitochondrion, reviewed in [39]. The relative reactivity of PRX-3 with hydrogen peroxide is approximately the same as mGPX-1. However, peroxiredoxins have been shown to be expressed at higher levels than glutathione peroxidases/glutaredoxins [31,36] and TRX-2 contains a mitochondrial targeting sequence and is actively transported into the mitochondrion as our results suggest.

Mitochondrial dysfunction is thought to be linked to the development of sepsis-induced organ dysfunction [3-11,17,40]. Many studies have shown that derangements in cellular oxygen utilization result from damage to the electron transport chain and damage to the inner mitochondrial membrane that can result in impairment of ATP generation, increased lactic acid production and mitochondrial swelling. We assessed the relative roles of the TRX-2 and mGSH systems on mitochondrial dysfunction in LPS/PepG treated cells. Collapse of the mitochondrial membrane potential, which may further impact on the oxidative phosphorylation pathway for ATP generation, may trigger apoptotic cell death pathways. The loss of mitochondrial membrane potential with a low ATP:ADP ratio, reduced oxygen consumption and increased lactate production, but no change in metabolic activity was seen in LPS/PepG treated cells and was similar to cells where mGPX was inhibited. However, inhibiting TRX-2R caused further loss of membrane potential, substantially reduced oxygen consumption, a greater increase in lactate production, and a large reduction in the ATP:ADP ratio, accompanied by a subsequent 5 fold

increase in metabolic activity. The increase in metabolic activity is most likely due to the low ATP concentration acting as a switch to produce more ATP. AlamarBlueTM is an accurate and sensitive indicator of mitochondrial function as it is an exceptional detector of reduction of all the elements of the electron transport chain that are not affected by oxidants [41].

Changes to intracellular redox state (oxidative stress) have important implications for cell survival. Oxidation of TRX-2 and/or mGSH increases the susceptibility of the cell to oxidants and promotes apoptosis through the intrinsic pathway [42,43]. The low ATP: ADP ratio and mitochondrial dysfunction seen upon inhibition of TRX-R2 in LPS/PepG treated cells was accompanied by increased caspase 3/7 activation and a 30% reduction in cell viability. This shows that the TRX-2 system in endothelial cells is important to prevent cell death via adequate LPS/PepG-induced hydrogen peroxide removal.

In conclusion we have shown that in our *in vitro* endothelial cell model of sepsis, the proteins of the TRX-2 system are better resistant to the effects of oxidative stress and appear to have a larger protective role against mitochondrial dysfunction induced by LPS/PepG exposure than the mGSH system. Endothelial cells have an important role in host defence and inflammation during sepsis. However, further studies will be necessary to evaluate the relative importance of the mitochondrial hydrogen peroxide removal systems of other cell types during LPS/PepG insult.

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LEGENDS TO FIGURES

Fig. 1.

Inhibition of TRX-R and GPx in mitochondrial (panels A and C) and cytosolic (panels B and D) fractions from endothelial cells treated with solvent control (untreated), auranofin or thiomalic acid. Box and whisker plots show median, interquartile and full range (n=6). P value shown is Kruskal Wallis. * = significantly different from untreated cells (Mann Whitney p<0.05).

Fig.2

Interleukin (IL)-6 and IL-8 concentrations in cell culture supernatants from endothelial cells treated with solvent control (untreated), $2\mu g/ml$ LPS plus $20\mu g/ml$ PepG with $2\mu M$ auranofin or 4mM thiomalic acid for 24h. Box and whisker plots show median, interquartile and full range (n=6). P value shown is Kruskal Wallis. * = significantly different from untreated cells and (Mann Whitney p<0.05).

Fig. 3.

Oxidative stress (total radical production), measured as the rate of change in fluorescence in endothelial cells treated with solvent control (untreated), $2\mu g/ml$ LPS plus $20\mu g/ml$ PepG with and without $2\mu M$ auranofin or 4mM thiomalic acid or $50\mu M$ hydrogen peroxide. Box and whisker plots show median, interquartile and full range (n=6). P value shown is Kruskal Wallis. * = significantly different from untreated cells and + = significantly different from LPS-PepG alone (Mann Whitney p<0.05).

Fig. 4.

Expression of key proteins in mitochondria. Mitochondria were extracted from endothelial cells treated with solvent control (untreated), $2\mu g/ml$ LPS plus $20\mu g/ml$ PepG with and without $2\mu M$ auranofin or 4mM thiomalic acid, or $50\mu M$ hydrogen peroxide. HPRT was used a loading control. Densitometry analysis and representative western blot of protein expression of A. TRX-2. B. mGPx-1. C. PRX-3. Box and whisker plots show median, interquartile and full range from six separate cultures (n=6). P value shown is Kruskal Wallis. * = significantly different from untreated cells and + = significantly different from LPS-PepG alone (Mann Whitney p<0.05).

Fig. 5.

Caspase 3/7 activation and mitochondrial membrane potential. Endothelial cells were treated with solvent control (untreated), $2\mu g/ml$ LPS plus $20\mu g/ml$ PepG with and without $2\mu M$ auranofin or 4mM thiomalic acid, $50\mu M$ hydrogen peroxide (or 1mM rotenone as control, JC-1 only). A. Caspase 3/7 activation measured by luminescence. C. Mitochondrial membrane potential measured using JC-1. Box and whisker plots show median, interquartile and full range (n=6). P value shown is Kruskal Wallis. * = significantly different from untreated cells and + = significantly different from LPS-PepG alone (Mann Whitney p<0.05).

Table 1	Ratio of reduced/oxidised glutathione (GSH/GSSG)
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Treatment	Total GSH/GSSH	Mitochondrial GSH/GSSH
Laterated	42 7 [4] 1 51 0]	40 5 [46 9 5] 0]
Untreated	43./ [41.1 - 31.9]	49. 5 [40.8 - 51.9]
LPS/PepG	35.8 [34.6 - 36.0]*	14.3 [13.9 - 15.6]*
Auronofin	36.0 [34.6 - 39.4]*	30.6 [29.8 - 32.3]*+
Auronofin + LPS/PepG	34.4 [33.2 - 35.4]*	6.5 [5.6 - 7.5]*+
Thiomalic acid	37.3 [32.5 - 39.6]*	36.2 [34.9 - 39.0]*
Thiomalic acid + LPS/PepG	35.9 [35.6 - 36.5]*	45.7 [42.0 - 52.3]
Hydrogen peroxide	41.1 [40.5 - 41.7]*	16.2 [16.0 - 17.1]*

Median [interquartile range], n=6. P=0.0089 for total GSH/GSSG and p=<0.0001 for mitochondrial GSH/GSSH (Kruskal Wallis).

LPS= lipopolysaccharide, PepG = peptidoglycan G

*= significantly different from untreated cells (p<0.05)

+=significantly different from LPS/PepG alone (p<0.05)

B

Treatment	Metabolic activity Units/min/10 ⁶ cells	Oxygen consumption nMol/min/10 ⁶ cells	ADP/ATP ratio 10 ⁶ cells	Lactate nmol/10 ⁶ cells
Untreated	365 [272 - 450]	6.5 [6.1-7.8]	6.3 [6.0 - 6.6]	2.3 [2.0 - 2.5]
LPS/PepG	340 [238 - 352]	4.9 [4.7 - 5.3]*	4.7 [4.0 - 5.0]*	3.0 [2.9 - 3.6]*
Auronofin	343 [323 - 468]	5.3 [4.7 - 6.0]*	5.3 [4.7 - 5.4]	2.3 [2.0 - 3.1]
Auronofin + LPS/PepG	1250 [1050 - 1416]*	2.9 [2.7 - 3.3]*+	2.3 [2.0 - 2.7]*	4.1 [3.8 - 5.4]*+
Thiomalic acid	346 [227 - 467]	5.0 [4.7 - 5.4]*	5.6 [5.1 - 6.0]	2.0 [1.8 - 2.2]
Thiomalic acid + LPS/PepG	341 [252 - 668]	5.3 [4.6 - 5.9]*	4.4 [4.2 - 4.4]*+	2.6 [2.4 - 2.9]*
Hydrogen peroxide	378 [221 - 418]	4.5 [4.2 - 4.6]*	6.1 [5.3 - 6.6]	2.2 [1.8 - 3.3]

Table 2 Mitochondrial metabolic activity, oxygen consumption, ADP/ATP and lactate

Median [interquartile range], n=6. For Kruskal Wallis analysis of variance p values, see text.

LPS= lipopolysaccharide, PepG = peptidoglycan G

* = significantly different from untreated cells (p<0.05)

+ = significantly different from LPS/PepG alone (p < 0.05)



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