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Biomarkers of Oxidative Stress Study VI. Endogenous Plasma Antioxidants Fail as Useful Biomarkers of Endotoxin-Induced Oxidative Stress

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Abstract

This is the newest report in a series of publications aiming to identify a blood-based antioxidant biomarker that could serve as an *in vivo* indicator of oxidative stress. The goal of the study was to test whether acutely exposing Göttingen mini pigs to the endotoxin lipopolysaccharide (LPS) results in a loss of antioxidants from plasma. We set as a criterion that a significant effect should be measured in plasma and seen at both doses and at more than one time point. Animals were injected with two doses of LPS at 2.5- and 5 µg/kg i.v. Control plasma was collected from each animal before the LPS injection. After the LPS injection, plasma samples were collected at 2 h, 16 h, 48 h and 72 h. Compared with the controls at the same time point, statistically significant losses were not found for either dose at multiple time points in any of the following potential markers: ascorbic acid, tocopherols (α , δ , γ), ratios of GSH/GSSG, cysteine/cystine (Cys/CySS), mixed disulfides and total antioxidant capacity. However, uric acid, total GSH, and total Cys were significantly increased, probably because LPS had harmful effect on liver. The leakage of substances from damaged cells into the plasma may have increased plasma antioxidants concentrations making changes difficult to detect. Although this study used a mini pig animal

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model of LPS-induced oxidative stress, it confirmed our previous findings in different rat models, that measurement of antioxidants in plasma is not useful for the assessment of oxidative damage *in vivo*.

Introduction

This is another report in the continuing series comparing, validating and identifying biomarkers that may be used for the measurement of oxidative stress in animal models and, ultimately, in humans. Exploration of oxidative stress biomarkers is a field of ever-increasing attention, both in science and in commerce. It is now generally recognized that the field is maturing, and there is a great effort to study and understand biomarkers at both a chemical and a molecular-biological level. Although a compelling body of evidence indicates that oxidative stress is involved in pathways leading to cell death and tissue damage, and the role of reactive oxygen species in the etiology of various diseases has been extensively reviewed [1], there is no agreement among scientists as to which is the best and most accurate measure of oxidative stress.

One common approach used in assessing oxidative stress is to measure the decreases of endogenous antioxidants. In two classical models of induction of oxidative stress, CCl₄ treatment and ozone inhalation exposure, we have previously shown that levels of endogenous plasma antioxidants were not reduced in a time- and dose-dependent pattern. Therefore, it was concluded that measurement of plasma antioxidants may not be used as biomarkers for oxidative stress. [2, 3]. In the present study, we used treatment with the endotoxin lipopolysaccharide (LPS) to investigate changes in endogenous plasma antioxidants in a porcine model of oxidative stress.

Intravenous infusion of endotoxin into the anaesthetized pig has been used to imitate the pathophysiological events during the early phase of severe gram-negative septic shock in man [4]. Endotoxemia promotes the production of arachidonic acid metabolites, which activate the complement and coagulation cascades and also play a significant role in pulmonary dysfunction during human sepsis. During this process, formation of free radicals accelerates in the body, which causes oxidative tissue damage and is followed by a high mortality rate [5]. LPS has frequently been used in experimental models of inflammation and oxidative stress. It is well known that LPS, an outer-membrane component of Gram-negative bacteria, interacts with CD14, which then presents LPS to the Toll-like receptor 4 [6, 7]; binding to this receptor activates inflammatory gene expression through nuclear factor κ B and mitogen-activated protein kinase signaling [8, 9]. The inflammatory response includes activation of free radical-generating enzymes in various types of cells that initiate host lipid peroxidation. In this study, LPS treatment of Göttingen mini pigs was chosen as a model of oxidative stress because the response of pig macrophages to LPS more closely resembles that of humans than mice in their set of macrophage-expressed systemic manifestations and LPS-inducible genes [10]. Therefore, the similarities between pigs and humans support the use of the pig as a more predictive model than the rodent in research studies [10].

The objective of our new Biomarkers of Oxidative Stress Study (BOSS) was to assess the effect of LPS on different antioxidants in plasma in an animal model consisting of LPS administered as a single intravenous injection to Göttingen mini pigs. We assessed the time- (2, 16, 48 and 72 h) and dose- (2.5 µg/kg and 5 µg/kg i.v.) dependent effects of LPS on blood plasma levels of ascorbic acid, tocol, α-, δ-, and γ -tocopherols, glutathione (GSH and GSSG), cysteine (Cys), cystine (CySS), uric acid and total antioxidant capacity to determine whether they would be decreased and therefore identified as markers of oxidative stress by LPS.

Materials and Methods

Chemicals and Reagents

All chemicals and reagents used in the study were obtained from Sigma-Aldrich Corporation (St. Louis, MO).

Animals and Treatment Protocol

Göttingen SPF minipigs obtained from Ellegaard Göttingen Minipigs ApS, Sorø Landevej 302, DK-4261, Denmark Dalmose, were used in all experiments. All 12 male minipigs were housed individually in floor pens (1.2 m²) with sawdust ("Lignocel 3-4" from J. Rettenmaier & Söhne GmbH + Co, D-73494 Rosenberg, Germany) as bedding. The animals were 8-12 months old and the body weight was 20-25 kg. Animal rooms were maintained at 21°C-24°C with 55%-70% relative humidity with alternating 12-h light and dark cycles. The ventilation system was designed to give ten air changes per hour. Before animal arrival, the animal room was cleaned and disinfected according to established procedures. During the study, the animal room and the pens were washed regularly and rinsed with water. A pretreatment period of 3 weeks (including an acclimatization period of 5 days) was allowed during which the animals were observed daily.

The animals had free access to domestic quality drinking water and received an SDS minipig diet (SMP MOD) from Special Diets Services, Witham Essex, CM8 3AD, U.K. Food was offered twice daily in an amount of approximately 225g per animal per meal. The amount of food was constantly adjusted during the course of the study in order to allow reasonable growth of the animals.

In the metabolism cages the animals had free access to drinking water and feed. For welfare reasons and to avoid feces interfering with urine collection, a supply of autoclaved hay was offered daily during urine sampling. Treatment and care of animals met all appropriate regulations and guidelines of Ellegaard facility which is fully accredited by the "Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Study Design

LPS Treatment

Göttingen minipigs, six animals per group, were given a single intravenous injection of either 2.5µg/kg LPS or 5.0 µg/kg LPS. The vehicle for preparation of the dose formulation

was 0.9% NaCl. All 12 mini pigs were used as control animals and were pretreated with the vehicle 0.9% NaCl. Two additional animals served as non-treated controls for histopathological evaluation.

Specimen Collection

Animals were divided into two groups of 6 mini pigs each. One group received LPS at a dose of 2.5 µg/kg i.v and the second group received 5 µg/kg i.v. Control blood samples were collected from each animal 3 weeks before LPS injection. Experimental blood samples were collected at 2 h, 16 h, 48 h and 72 h post-LPS dosing. Each sample was marked with a code number so that the investigators conducting the assays were not aware of the treatment status of the animals. Animal treatment and sample preparation were performed by LAB Research (Scantox), Hestehavevej 36A, Ejby DK-4623 Lille Skensved, Denmark.

Plasma Preparation

Blood samples of approximately 35 ml were collected from the jugular vein/bijugular trunk into heparinized open vacutainer blood collection tubes. The tubes were gently inverted 2-3 times for mixing and immediately placed on ice. Blood samples were centrifuged (1270 × g, for 10 min at 4°C) within 30 minutes after collection. The plasma samples (0.5 ml) were placed into 25 Nunc cryotube [Nunc, Denmark]) immediately frozen at -70°C and stored for analysis.

Tissue preparation for histopathology

Animals were anesthetized with an intramuscular injection (left hind leg) of 0.2 ml/kg of a mixture of Zoletil 50 Vet., Virbac, France (125 mg tiletamine and 125 mg zolaxepam), Rompun Vet., Bayer, Germany (20 mg xylazine/ml, 6.5 ml), Ketaminol Vet., Veterinaria AG, Switzerland (100 mg ketamine /ml, 1.5 ml) and Methadon DAK, Nycomed Danmark, Denmark (10 mg methadone/ml, 2.5 ml). The animals were sacrificed by exsanguination prior to necropsy during deep anesthesia. Tissue samples from the lungs, liver and spleen were collected and fixed in buffered 4% formaldehyde 72 h post exposure. Two additional animals were used as non-treated controls, three had received 2.5µg/kg of LPS, and three had received 5 µg/kg of LPS. Tissues were trimmed, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for histopathological evaluation. The histopathological evaluation was performed at NIEHS/NIH, Research Triangle Park, NC 27709, USA.

Analysis of Antioxidant Status

Tocopherols—α-, γ- δ-tocopherol; 5-nitro-γ-tocopherol and α-tocopherol-quinone were measured in plasma by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) [11] at OMRF, OK.

Ascorbic acid and uric acid: Plasma samples were assayed using amperometric detection [12] at USEPA, RTP, NC.

Glutathione—GSH and GSSG were analyzed by HPLC with fluorescence detection [13, 14] at Emory University, Atlanta, GA.

Cysteine/cystine oxidation of plasma was measured by HPLC [14,15] at Emory University, Atlanta, GA.

Plasma total antioxidant status was measured using a kit (Randox laboratories, Crumlin, U.K.) This assay is based on the absorbance of the colored 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation (ABTS^{•+}) which is decolorized by antioxidants according to their concentrations and antioxidant capacities. This change in color is measured spectrophotometrically as a change in absorbance at 660 nm. Results are expressed in mM units based on a calibration standard antioxidant (6-hydroxy-2,5,7,8-tetramethylchrooman-2-carboxylic acid) (16). All measurements were carried out at US EPA, RTP NC.

Statistical analysis

Since within each dose group repeated measurements were taken over time on the same animal, the time course data are intrinsically correlated. Consequently, we used mixed effects ANOVA to account for within-animal correlations. As is commonly done in such situations, we treated animals as the random effects. All analyses were performed using PROC MIXED in the statistical software SAS 9.1 (Copyright (c) 2002-2003 by SAS Institute Inc., Cary, NC, USA). Within each dose group, we compared each time point with the baseline and considered a point significant if the p-value was less than 0.05.

Results

Clinical signs by LPS

Clinical signs from LPS in both dosed groups included body tremors, redness of the skin, red eyes, and high temperature of 40.4°C for the first 2 h and 39.0°C thereafter. All animals treated with the low dose of 2.5 µg/kg LPS quivered and were passive up to 4 h post-dosing, whereas animals treated with the 5 µg/kg LPS dose quivered and were passive for a much longer period of time and also had fevers. In addition, loose stools and weight reduction occurred in most animals from the first day of treatment to the day of necropsy at 72 h post-treatment.

Histopathological review of liver, lung and spleen tissues

In general, a dose-related increase in the incidences and severities of cellular degeneration and cell necrosis were observed in the liver. Specifically, histopathological findings that appeared to be related to the administration of LPS consisted of multifocal centrilobular degeneration, observed in the livers of the higher dose (5µg/kg) animals, and minimal hemorrhagic coagulative hepatocyte necrosis. Microscopically, degeneration was observed in a few scattered centrilobular areas. In affected sites, there was variable loss of hepatocytes, disorganization and decrease in the size of the hepatic cords, and minimal to mild centrilobular sinusoidal dilation (Fig.1). Some of the affected centrilobular areas contained increased numbers of cells with plump nuclei, possibly Kupffer cells or reactive endothelial cells, along the luminal surface of the sinusoids. The liver of one animal also contained 3 discrete foci of coagulative hemorrhagic hepatocyte necrosis consisting of hypereosinophilic (necrotic) hepatocytes and cellular debris, mixed with varying amounts of

blood and surrounded by a mixed inflammatory cell infiltrate consisting mostly of macrophages with low numbers of lymphocytes and neutrophils (Fig. 2).

Plasma level of ascorbic acid and uric acid

Using the same LPS treatment regimen of low (2.5 µg/kg iv) and high (5 µg/kg iv) doses, we examined the plasma levels of ascorbic acid and uric acid. Compared with the controls at the same time point, no statistically significant changes in levels of ascorbic acid were found in either dose group at 2h, 16 h, 48 h or 72 h (Table 1). However, plasma levels of uric acid had a tendency to be higher in the treatment group at early times, with the difference being significant at 2 h (almost 2-fold) for both doses. No significant effect on uric acid was found for either dose at the 16 h, 48 h or 72 h time points studied (Table 1). Both dose groups had similar time course patterns for ascorbic acid as well.

Tocopherols: α-, γ- δ-tocopherol; 5-nitro-γ-tocopherol

Except for low-dose LPS at 48 hrs, none of the other time points and dose groups showed a statistically significant change in α-tocopherol plasma concentration. Mean levels of the other tocopherols did not change significantly for any of the doses or at any of the time points (Table 1).

GSH and GSSG

Administration of both doses of LPS (2.5 µg/kg i.v. and 5 µg/kg i.v.) resulted in a statistically significant increase in plasma GSH level at the 2 h time point. An increase was also measured at 48 and 72 h after LPS treatment. However, statistically significant differences were found only for the lower LPS dose of 2.5 µg/kg and not for the higher dose (Table 1). GSSG measurements also showed an increase which happened to be marginally significant for the lower dose of LPS 2 h after treatment and for the higher dose 16 h after treatment (Table 1). The ratio of GSH to GSSG showed a decreasing pattern at all time points for the low dose of LPS, but none of the changes, except at 16 hours, were statistically significant using a one-sided test. For the high dose, we saw no significant decrease at any of the time points (one-sided p-values).

Cysteine and Cystine

Assessments of cysteine (Cys) and its disulfide cystine (CySS) were also conducted because they constitute an abundant low-molecular-weight thiol/disulfide redox couple in the plasma, and Cys homeostasis has been shown to be adversely affected during acute lung injury [14]. Plasma levels of CySS at the low LPS dose showed a significant increase at 2 hours and no significant changes at subsequent time points. The high LPS dose, however, showed a non-significant increase at 2 hours but significant increases at 48 and 72 hours with a significant decrease at 16 hours relative to the untreated control. An identical pattern of response was seen for the total CyS plasma concentrations for the two dose groups. However, for CyS we saw a non-significant increase at 2 hours in both dose groups but a significant decrease at 16 hours in both dose groups. At 48 hours, although we saw a decrease in CyS for both dose groups, only the high-dose group was significant. At 72 hours, while there was a significant decrease in the low dose, the response did not change at

the high dose (Table 1). In addition, no adverse effects of LPS were observed in the measurement of the mixed disulfides of Cys and glutathione with the exception of the effect for the low dose of LPS at 2 h (Table 1).

Total antioxidant capacity of plasma

The total antioxidant capacity of the plasma was unaffected by either dose of LPS at any of the 4 time points studied (Table 1).

Discussion

This study was designed to measure a series of antioxidants to identify oxidative stress biomarkers in blood plasma collected from similarly LPS-exposed Göttingen mini pigs. As in our previous CCl₄ and ozone exposure studies in rats, the experimental exposures and sample collections were all done in one laboratory under identical conditions. Göttingen mini pigs were chosen as a clinically relevant species, resembling humans in various functions as assessed by cardiovascular, respiratory, and biochemical parameters [10, 17, 18]. In addition, the use of larger animals as an LPS model of oxidative stress allowed their use as an internal control prior to treatment. Thus, each animal was used as a control before treatment for its own LPS injection. The sample collection design allowed direct comparison of different methods using the same samples. Samples were sent to recognized laboratories with known experience in the corresponding analytical procedures. Investigators were not aware of the treatment status. Thus, the design excludes possible errors due to lack of experience in complicated analytic techniques and also avoids any possible bias in sample analysis and reporting of data.

In this investigation, we used LPS to simulate a systemic oxidative inflammatory environment in Göttingen mini pigs as the third testing model for biomarkers of oxidative stress study (BOSS) after CCl₄ and ozone exposure [2, 3]. As already mentioned, LPS has frequently been used in experimental models of inflammation since it interacts with the Toll-like receptor 4 and then activates inflammatory gene expression through nuclear factor κ B and mitogen-activated protein kinase signaling [6-9]. The endotoxin also stimulates the production of arachidonic acid metabolites, complement factors, the cytokine network, and coagulation cascades [19, 20]. We anticipated that LPS treatment, with its induction of the proinflammatory factors, consequent free radical generation, and the resultant enhanced lipid peroxidation, would be accompanied by a decrease of antioxidants in the plasma. Therefore, our aim was to evaluate major antioxidant substances in blood plasma and to determine if the loss of antioxidants could be used to assess the anticipated oxidative effects by LPS.

To understand the degree of systemic tissue damage caused by the LPS in our experimental porcine model, sections of tissues from the liver, lungs and spleen were studied before and 72 h after treatment. No abnormalities were seen in the control animals. Histopathological results indicated that 72 h after administration of both doses of LPS, changes occurred in the liver but not in the lungs or spleen. It has been reported that LPS induces time-dependent liver tissue reactions with edema, sinusoidal dilation, packing of red cells and leukocyte infiltration, progressing to endothelial cell and hepatocyte damage, formation of thrombi,

and necrosis during porcine endotoxemia for 6 h [21]. In the same study, liver tissue changes after 72 h consisted mainly of multifocal centrilobular degeneration, coagulative hepatocyte necrosis, and accumulation of neutrophils and macrophages within the affected centrilobular areas. Changes in the groups with the higher dose of 5 µg/kg LPS were relatively more prominent than in groups with the lower dose of 2.5 µg/kg. Other studies found similar LPS-induced liver tissue reactions with sinusoidal dilation, packing of red cells and leukocyte infiltration, progressing to endothelial cells and hepatocyte damage [21-23]. All these findings suggest that antioxidant concentrations in plasma might be altered in response to systemic tissue damage caused by LPS, especially in the high-dose animals.

Ascorbic acid is a well-studied antioxidant in plasma and tissues, where it efficiently scavenges superoxide, hydrogen peroxide, hypochlorite, and the hydroxyl and peroxy radicals and recycles other important antioxidant molecules [24-28]. Studies in different animal models have considered the potential for different antioxidants to treat and even prevent the development of diseases [29-31]. We expected to see a decline in plasma ascorbic acid concentrations in the treated groups. However, results from the present study of endotoxemic Göttingen mini pigs demonstrate that the ascorbic acid concentration did not show a dose response and was not significantly changed at any of the four time points. This large animal model allowed us to compare ascorbic acid concentrations in the plasma of the same animal before and after LPS treatment, which differs from the rat model used in similar studies [2, 3]. Using a rat model of ozone exposure, we have previously reported that the ascorbic acid concentration was significantly decreased in the BALF and in plasma, but was not changed in another rat model of oxidative stress by CCl₄ [2, 3]. Although free radical-mediated lipid peroxidation, induction of pro-inflammatory factors and COX activation have been shown to be early phenomena in porcine endotoxemia [32], in this study we could not demonstrate any effect of LPS on plasma ascorbic acid concentration. Therefore, because there was no decrease in ascorbic acid, regardless of the endotoxin dose, measurement of the ascorbic acid in plasma should not be used to assess the anticipated systemic oxidative effects by LPS.

Uric acid is a byproduct of purine metabolism, and hyperuricemia has been shown to be an independent predictor of poor outcome in the general population and in patients with stroke and heart failure [33-38]. A number of studies have linked the inflammatory response to uric acid levels because uric acid is a potent hydrophilic antioxidant that scavenges certain oxygen radicals [33]. It has also been shown to stabilize ascorbic acid in human plasma at physiological concentrations [34]. Others have concluded that the reducing and acidic properties of urate are important in the effective scavenging of peroxynitrite and that Cys and ascorbic acid enhance urate's antioxidant effect by reducing urate-derived radicals [35]. The findings in this study of increases in uric acid in plasma by both doses of LPS in the early post injection period 2 h after the treatment might be attributed to increased xanthine oxidase activity. Moreover, literature data demonstrate an association between the uric acid level and increased xanthine oxidase activity during endotoxemia and heart failure [36-38]. It is also possible that the elevated level of uric acid at the early time point is due to enhanced purine nucleotide catabolism in the liver with increased degradation of xanthine. Our histopathology findings and other studies have demonstrated that LPS induces apoptosis and necrosis in tissues.

α -Tocopherol (vitamin E), an endogenous, well-studied, lipid-soluble, chain-breaking antioxidant, is known to have an effect on radicals in membranes and lipoprotein particles and is crucial in preventing lipid peroxidation [39], although under some circumstances it may show a prooxidant activity [40]. The tocopherols (α , γ , and δ) have a chromanol ring and a phytyl tail, and differ in the number and position of the methyl groups in the ring [41]. The physiological role of these antioxidants is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals. Therefore, we expected to see a decline in plasma tocopherol concentrations after LPS treatments. However, our time course measurements of α -tocopherol and its isomers in minipig plasma revealed no significant changes after injection at 2 h or 16 h for either the low or high doses of LPS. LPS increased α -tocopherol levels 48 h and 72 h after toxic challenge only for the low dose. These findings differ from previous studies that had shown a reduction of plasma levels of α -tocopherol during experimental porcine endotoxemia, with the reduction being more pronounced in non-surviving than surviving animals [42]. Under various experimental conditions, other studies have reported evidence of elevation of α - and γ -tocopherol in the plasma after LPS exposure [43]. In addition, the results from our model of porcine endotoxemic challenge failed to show variations in plasma levels of other tocopherols (δ , γ). These findings are consistent with our previous reports of lack of changes in these lipid-soluble plasma antioxidants in rodent models of oxidative stress by CCl_4 and ozone exposure [2, 3].

Another potential target in the endotoxemic pig could be GSH since GSH/GSSG, conjugates, disulfides, and other GSH-derived products have been studied as biomarkers implicating the depletion and/or oxidation of GSH in a wide variety of human and experimental toxicities. Glutathione is a ubiquitous thiol tripeptide that acts alone or in concert with enzymes within cells to reduce superoxide radicals, hydroxyl radicals, and peroxynitrites. It has been reported that GSH in plasma provides an alternative indicator of systemic oxidative stress because of the continuous interaction of plasma with tissues and organ systems [44]. Despite the extensive evidence, critical examination of such studies frequently reveal that injury is not related in a simple way to glutathione status [44]. Indeed, our studies show that LPS caused neither a significant decrease in plasma concentrations of GSH nor a significant increase in GSSG. The marked GSH increase that occurred in plasma for the low LPS dose 2h, 48 h and 72 h after treatment and for the high dose only at 2 h is most likely attributed to its release from damaged hepatic cells, since intracellular GSH is normally found at a much higher concentration in tissues than in plasma.

The ratio of the reduced-to-oxidized forms of glutathione has been used as an important in vitro and in vivo indicator of the redox balance in the cell and, consequently, of cellular oxidative stress [14, 45]. Our data showed no significant change in plasma levels of GSH, GSSG or their ratio in either dose group; thus, it cannot be identified as a marker of oxidative stress in vivo in our experimental porcine model. It has been reported that the reduction potentials (E_h) for the redox couples GSH/GSSG and Cys/CySS in plasma are useful indicators of systemic oxidative stress and other medically relevant physiological states [45]. In contrast, plasma E_h (GSH) and E_h (Cys) in this study showed no statistically significant differences as compared to control values [-94.9 ± 2 mV for E_h (GSH) and -56.1 ± 4.4 mV for E_h (Cys)]. Cysteine and its disulfide cystine constitute the most abundant low-

molecular-weight thiol/disulfide redox couple in the plasma, and Cys homeostasis is adversely affected during the inflammatory response to infection and injury [46]. Although higher plasma levels of total Cys were found in the plasma of endotoxemic pigs for the high dose of LPS, and some changes in both CySS and Cys were obtained for both LPS doses at different time points, the calculated redox states of the Cys/CySS couple showed no statistically significant differences between the experimental and the control groups. A previous study in mice also found decreased Cys apparently associated with anorexia following LPS treatment [14]. These results strongly suggest that perturbations in the extracellular thiol/disulfide redox environment are not associated in a simple manner with the progression and severity of acute systemic oxidative injury. In contrast, studies of plasma E_h as a biomarker of oxidative stress show that Cys/CySS and/or GSH/GSSG are oxidized in association with established risk factors for a number of diseases including age [47].

Mixed disulfide (CySSG) is the intermediate product formed from this reaction between GSH and CySS and has been shown to assess overall body oxidative stress because it is a product of a reaction between the predominant extracellular disulfide (CySS) and the predominant intracellular thiol (GSH) [48, 49]. Since the high dose of LPS did not cause statistically significant changes in plasma mixed disulfide at any time point and the low dose caused an increase only at 2 h and 48 h after treatment, we conclude that this biomarker does not assess overall oxidative stress under our experimental conditions.

There has been growing interest in the role of antioxidant function in controlling inflammatory disease states [50, 51]. Overall plasma antioxidant status has been considered to provide more relevant biological information compared to that obtained by the measurement of individual components, as it reflects the cumulative effect of all antioxidants present in plasma and body fluids [52]. We also checked whether LPS treatment alters the total antioxidant status in the present porcine model of systemic oxidative stress. We found no evidence for significant differences in plasma for either the low or high dose at all time points after treatment. Therefore, the present results do not support the measurement of total antioxidant status in plasma as a marker of oxidative stress.

In conclusion, our results indicate that measurements of plasma antioxidants failed to identify oxidative damage by LPS in the mini pig model. The criterion used to recognize an antioxidant marker was that a significant effect could be identified and measured in plasma seen at both doses at more than one time point. We previously found in rodent models that measurements of plasma antioxidants were also unsuccessful biomarkers of oxidative damage for CCl_4 and ozone [2, 3]. This new experimental porcine model is certainly a better model of oxidative stress than rodent models since the porcine physiology and organs are very similar to humans and have been successfully used in earlier studies to evaluate oxidative stress (20, 53). Together with previous Biomarkers of Oxidative Stress Study results, this investigation with an LPS porcine model of oxidative stress supports the overall conclusion that measurement of antioxidants in plasma is not a useful choice for the assessment of oxidative damage in vivo.

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- Antioxidants were measured to test whether they identify oxidative damage by LPS
- Ascorbic acid and tocopherols (α , δ , γ) in plasma resulted in no significant change
- GSH/GSSG, cysteine/cystine, disulfides and total antioxidant capacity did not change
- None of the antioxidants detected oxidative damage at 5 time points and two LPS doses
- Plasma antioxidants may not be used as biomarkers of LPS-induced oxidative damage

Figure 1. Centrilobular degeneration (arrows) in the liver of a Götting minipig 72 h after i.v. injection of 5 μg/kg LPS. There is a minimal loss of hepatocytes, disorganization and decrease in the size hepatic cords, and minimal centrilobular sinusoidal dilation.

Figure 2.

Focal coagulative necrosis in the liver of Göttingen mini pig 72 h after i.v. injection of 5 μg/kg LPS. The necrotic focus (arrows) consists of a mixture of hypereosinophilic (necrotic) hepatocytes and cellular debris, red blood cells surrounded by a mixed inflammatory cell infiltrate of mostly macrophages and low numbers of lymphocytes and neutrophils.

Table 1

Effect of LPS on Plasma Antioxidants of Göttingen Mini-pigs

Assays	Control		LPS							
	0h		2h		16h		48h		72h	
	For 2.5 µg/kg group	For 5 µg/kg group	2.5 µg/kg	5 µg/kg	2.5 µg/kg	5 µg/kg	2.5 µg/kg	5 µg/kg	2.5 µg/kg	5 µg/kg
Ascorbic Acid (µM)	23 ± 1	22 ± 6	29 ± 6	26 ± 6	18 ± 2	15 ± 2	18 ± 2	17 ± 3	30 ± 6	26 ± 5
Uric Acid (µM)	0.8 ± 0.2	0.6 ± 0.3	1.5 ± 0.1*	1.8 ± 0.1*	1.0 ± 0.1	1.2 ± 0.5	1.0 ± 0.2	1.0 ± 0.3	0.5 ± 0.2	0.9 ± 0.2
α-Tocopherol (µM)	2.6 ± 0.6	2.7 ± 0.6	2.5 ± 0.7	2.1 ± 0.1	1.8 ± 0.5	1.4 ± 0.4	4.5 ± 0.7*	2.6 ± 0.1	4.1 ± 0.5	2.4 ± 0.2
γ-Tocopherol (µM)	0.012 ± 0.001	0.02 ± 0.008	0.006 ± 0.004	0.003 ± 0.001	0.008 ± 0.003	0.007 ± 0.002	0.011 ± 0.005	0.010 ± 0.001	0.006 ± 0.001	0.008 ± 0.001
γ-Tocopherol (µM)	0.102 ± 0.025	0.112 ± 0.028	0.141 ± 0.063	0.105 ± 0.021	0.148 ± 0.014	0.108 ± 0.041	0.097 ± 0.041	0.172 ± 0.042	0.181 ± 0.002	0.104 ± 0.060
Total GSH (µM)	0.56 ± 0.05	0.55 ± 0.04	0.95 ± 0.05*	0.78 ± 0.05*	0.55 ± 0.05	0.57 ± 0.07	0.69 ± 0.05*	0.63 ± 0.05	0.71 ± 0.03*	0.69 ± 0.07
GSH (µM)	0.19 ± 0.01	0.22 ± 0.02	0.41 ± 0.04*	0.45 ± 0.06*	0.18 ± 0.01	0.19 ± 0.02	0.26 ± 0.03*	0.24 ± 0.03	0.26 ± 0.03*	0.25 ± 0.03
GSSG (µM)	0.019 ± 0.001	0.018 ± 0.001	0.030 ± 0.004*	0.018 ± 0.006	0.019 ± 0.004	0.028 ± 0.004*	0.026 ± 0.005	0.025 ± 0.003	0.025 ± 0.006	0.020 ± 0.003
Total Cys (µM)	96 ± 10	78 ± 8	123 ± 11*	99 ± 13	74 ± 10*	52 ± 8*	103 ± 8	103 ± 10*	93 ± 7	101 ± 10*
CysSS (µM)	46 ± 5	37 ± 4	59 ± 5*	47 ± 7	56 ± 5	25 ± 4*	50 ± 4	50 ± 5*	50 ± 4	48 ± 5*
Cys (µM)	4.1 ± 0.8	3.9 ± 0.6	5.0 ± 0.8	4.8 ± 1.0	2.2 ± 0.2*	2.4 ± 0.5*	2.6 ± 0.4	2.6 ± 0.5*	2.3 ± 0.4*	3.9 ± 0.9
Mixed disulfide Cys+GSH (µM)	0.33 ± 0.06	0.30 ± 0.02	0.47 ± 0.02*	0.29 ± 0.03	0.33 ± 0.04	0.32 ± 0.04	0.37 ± 0.02	0.33 ± 0.05	0.39 ± 0.03	0.39 ± 0.02
Total Antioxidant Capacity (mmol/l)	1.5 ± 0.1	1.4 ± 0.1	1.6 ± 0.3	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.7 ± 0.2	1.5 ± 0.1

Values are means ± SE for six Göttingen Mini-pigs in a group.