Role for glutathione in the hyposensitivity of LPS-pretreated mice to LPS anorexia

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ABSTRACT. To study the role of the redox state regulator glutathione (GSH) in bacterial lipopolysaccharide (LPS)-induced anorexia we measured total reduced GSH (trGSH) in liver, serum and brain in response to intraperitoneal (ip) lipopolysaccharide (LPS, 4 µg/mouse) injection in LPS-naïve and LPS-pretreated (4 µg/mouse given 3 days earlier) mice. LPS reduced food intake in LPS-naïve mice and LPS pretreatment attenuated this effect. LPS decreased trGSH at 24 hours after injection in LPS-naïve mice but 4 days later trGSH levels were upregulated in brain and liver, and this was associated with a significant attenuation of LPS-induced anorexia. In addition, LPS increased mitochondrial GSH levels in brain and liver at 4 days after injection. Pharmacological GSH depletion with diethylmaleate and L-buthionine sulfoximine in LPS-pretreated mice ablated the hyposensitivity to the anorexic effect of LPS. Together, these findings suggest a prominent role for GSH and its intracellular repartition in LPS anorexia.

Keywords: reactive oxygen species, cytokine, food intake, liver, brain, mitochondria

Inflammation, infection and administration of bacterial lipopolysaccharide (LPS) cause oxidative stress, and this entails an increase in tissue concentrations of reactive oxygen species, which are believed to mediate tissue dysfunction and/or destruction. Consequently, antioxidants such as glutathione (GSH) [1] are major determinants of the degree of pathology in models of tissue inflammation [2]. GSH is mainly produced in the liver and controls the redox state in many tissues [3]. GSH has also been supposed to limit LPS-induced pathology [4], but so far it is unknown whether GSH also affects LPS anorexia. GSH can modify cytokine production by several mechanisms [5] and may therefore regulate anorexic cytokine production in response to LPS. Tissue GSH may, in fact, be one determinant of cytokine pathology. Some evidence indicates that, in addition to an increase in total GSH, an increase in mitochondrial GSH is important for its protective effect [6-9]. In this study, we investigated the role of GSH in the food intake response to LPS in LPS-naïve and LPS-pretreated mice. LPS pretreatment normally reduces the pathophysiological responses and the anorexia in response to subsequent LPS administrations, a state often referred to as LPS tolerance [10]. If GSH modulates LPS anorexia, its production and tissue levels in response to LPS may differ between LPS-naïve and LPS-pretreated animals. To examine further a possible causal relationship between GSH and LPS anorexia, we also determined whether pharmacological GSH depletion in LPS-pretreated animals changes the feeding response to LPS, and whether total or mitochondrial GSH levels in brain and liver are indicative of tolerance to LPS-induced anorexia.

METHODS

Mice, housing and diets

We used male, 8-16 week old, 25-30g (body weight) c57bl6 mice for all experiments. Mice were housed individually in stainless steel cages with a grid floor and kept on a 12:12 hour light:dark cycle. Drug solutions were freshly prepared and always injected 1-2 hours before the onset of the dark cycle. Mice had continuous *ad libitum* access to water and powdered laboratory chow (Nafag, Gossau, Switzerland). All procedures were approved by the Canton of Zurich Veterinary Office.

Experiment 1: Effects of LPS

Design (table 1) and feeding

LPS from *Eschericha coli* O111:B4 (L2630, Sigma, Buchs, Switzerland) was dissolved in pyrogen-free saline (Braun, Emmenbrucke, Switzerland) and injected intraperitoneally (ip) at a dose of 4µg/mouse twice, on days 1 and 4 of the experiment, when the animals' food intake had returned to pre-injection values. Control animals received

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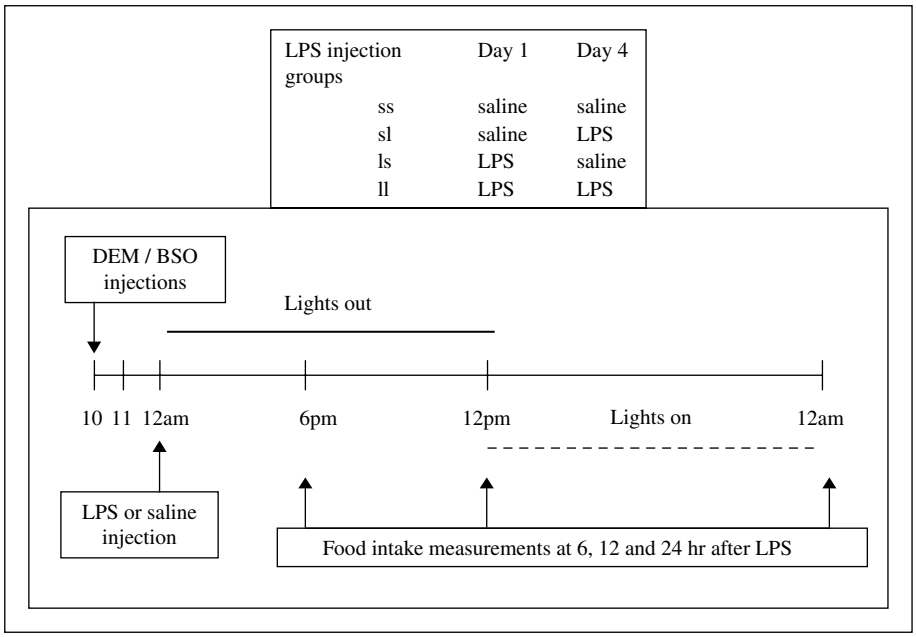
Table 1
Time lines for treatments and food intake measurements in mice



an equivalent volume of vehicle. The LPS dose chosen was the lowest dose which caused a reliable food intake reduction for 24 hours in a preliminary experiment in WT mice with 0, 0.4, 4 and 40 µg per 25 g body weight (n = 8/dose). Food intake was measured (± 0.1g) at 6, 12 and 24 hours after injection by subtracting the weight of the food container and taking into account any spillage. At the end of the experiment, i.e., on day 5, we collected blood, brain and liver to determine total reduced GSH (see below). Each treatment group shown in *table 1* consisted of 7-8 mice.

Glutathione (GSH) in serum, brain and liver

For determination of trGSH, mice were sacrificed with CO_2 24 hours after the second injection of LPS or saline. Blood was collected by heart puncture and centrifuged. Serum was kept at -80C° until analysis. Brains and livers were collected, snap frozen in liquid nitrogen, and stored at - 80C° until analysis. Throughout the extraction procedure for GSH, samples were kept on ice. Tissues were homogenized using a polytron PT1200 homogenizer (Kinematica, Lucern, Switzerland) with phosphate-buffered saline (600 µl/100 mg tissue). The solution underwent a precipitation reaction with phosphate solution (glacial metaphosphoric acid 1.67g, disodium EDTA 0.2 g, NaCl 30 g, in 100 ml distilled water). This solution was centrifuged, the supernatant was collected, and phosphate buffer (0.3M Na_2HPO_4) added. From this an aliquot was taken and the trGSH tissue levels were measured using a method based on the formation of a chromophoric product resulting from the reaction with 0.04% 5,5-dithiobis-2-nitrobenzoic acid (D8130, DTNB, Sigma Chemicals, St. Louis, MO, USA) and GSH (G4251, Sigma, Buchs, Switzerland) [11].

Mitochondrial GSH in brain and liver

In a separate group of mice, we also examined whether LPS resulted in altered mitochondrial GSH levels in brain and liver. On day, 5 mice (n=4, each) were perfused with ice-cold PBS, brains and livers were removed and mitochondria were isolated as follows [11]: tissues were homogenized in a glass tissue grinder in a solution corresponding to 600 µl (0.25 M sucrose – 1mM EDTA) per 100 mg of tissue. This suspension was overlayed onto 0.34 M sucrose – 1mM $MgCl_2$, and 20 mM phosphate buffer (pH 7.4) and centrifuged at 700 g (10 min, 4°C). The resulting supernatant was centrifuged at 5000 g for 15 min at 4°C. The pellet was re-suspended in 40 ml of 0.25 M sucrose – 1mM EDTA and centrifuged for 10 min at 24,000 g. The resulting pellet was then re-suspended in 3 ml of 0.25 M sucrose and 2 mM EDTA. Mitochondria numbers were estimated using the succinate dehydrogenase (SDH) activity assay from the mitochondrial preparation. Two µl were used to determine activity by the reduction of 200 µl of succinate/nitroblue tetrazolium solution. From the minimal activity of 0.14 optical density (OD)/min, all isolates were normalized. GSH was then determined as described above.

Experiment 2: Effects of GSH depletion on the responses to LPS

Design and feeding response to LPS

To determine whether GSH depletion alters the animals' feeding response to 4µg LPS, we inhibited GSH production and, hence, reduced GSH levels by injecting saline or LPS-pretreated mice with diethylmaleate (DEM, D97703, Sigma, Buchs, Switzerland) and L-buthionine sulfoximine (BSO, B2640, Sigma, Buchs, Switzerland) (DEM/BSO = db) at 800µl/kg body weight sc and 200 mg/kg [12] body weight ip, 2 hours before the second LPS injection on day 4. Preliminary trials showed that at these doses, the combination of both inhibitors reduced trGSH in the liver 2 hours after administration. This resulted in the following six treatment groups (n=4, each; first injection/second injection with or without db treatment): 1) saline/saline no db (ss[-db]), 2) ss[+db], 3) LPS/LPS no db (ll[-db]), 4) ll[+db], 5) LPS/saline db treatment (ls[+db], and

6) saline/LPS db treatment (sl[+db]). Food intake was measured at 6, 12 and 24 hours after the second LPS injection.

Serum acute phase protein response to LPS

To determine whether GSH depletion altered the acute phase protein response, we determined serum proteins by cellulose gel electrophoresis (Paragon SLE gel – Beckman, Nyon, Switzerland). Mice groups (n = 4, each) were defined as ss[-db], ssdb[+db], ls[-db], sl[+db], ll[-db], lldb[+db] and lsdb[-db]. Mice were sacrificed with CO_2, 24 hours after the second injection of LPS or saline. Serum was collected by heart puncture and stored at -80°C prior to use. Two µl of serum were loaded on a tract and gels were run for 30 minutes in barbital buffer. Gels were then stained with paragon blue and scanned with the Scion Image program. We also determined whether GSH depletion by DEM/BSO altered serum proteins. Total serum proteins levels were determined by the Bradford protein assay (cat. No. 500-0006, Bio-Rad, Reinach, Switzerland).

Serum tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ) response to LPS

To determine whether GSH depletion in LPS-pretreated animals altered TNFα and IFNγ serum levels 24h after the second LPS injection, we used serum from the electrophoresis experiment (see above) and analyzed it for TNFα and IFNγ using IFNγ and TNFα murine ELISA kits from Amersham (Otelfingen, Switzerland) [13].

Statistics

Differences between groups were determined by using Kruskal-Wallis test with a Dunn *post hoc* test. A value of $p<0.05$ was considered significant. Non-parametrical Spearman correlation coefficients were calculated using the Instat program (GraphPad Incorporation, San Diego, CA, USA).

RESULTS

Experiment 1: Effects of LPS

Feeding

Administration of LPS (4 µg/mouse) on day 4 reduced food intake compared to twice saline injected (ss) mice at 6, 12, and 24 hours in LPS naïve mice (sl) *(figure 1)*. LPS-pretreated mice which received a second LPS injection (ll) showed a significant reduction in food intake at 6 and 12 hours compared to saline (ss) controls *(figure 1)*, but this difference did not persist over 24 hours. In addition, ll mice ate more than sl mice at 6, 12 and 24 hours after the second injection (*figure 1*, n = 8, $p < 0.01$).

Serum, brain and liver GSH response to LPS

LPS reduced serum and brain trGSH at 24 hours after the second injection in LPS-naïve mice (sl) compared to twice saline-injected mice (ss) ($p < 0.01$). LPS-pretreated mice, however, responded to a second LPS injection (ll) with a significant increase in serum and brain trGSH compared to their saline-injected controls ($p < 0.001$) *(figure 2)*. In addition, ls mice had higher trGSH levels in liver than saline controls (ss) ($p < 0.001$) *(figure 2)*.

Mitochondrial GSH in brain and liver

Four days after injection, mitochondrial GSH levels were significantly increased in brains and livers of LPS-treated mice compared to their corresponding controls *(figure 3)*.

Experiment 2: Effects of GSH depletion on the responses to LPS

Feeding response to LPS

In Experiment 2, we confirmed our previous results that LPS injection reduced food intake compared to saline injection in saline-pretreated control mice (sl[-db] *versus* ss[-db]) at 6, 12 and 24 hours after injection *(figure 4)*.

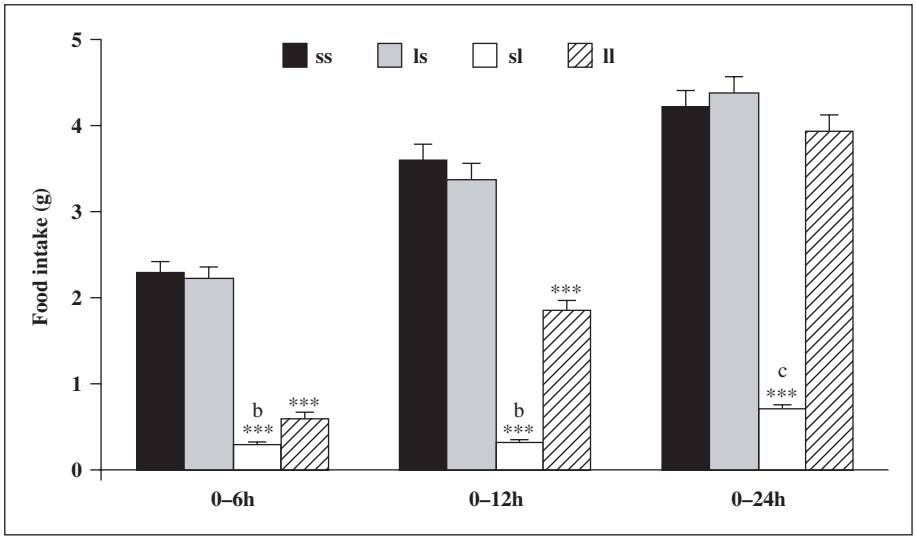


Figure 1

Cumulative food intake in LPS-naïve and LPS-pretreated mice at 6, 12 and 24 hours after LPS. Mice received a first saline (s) or LPS injection (4µg/mouse) (l) on day 1 and a second injection of saline or LPS on day 4. This resulted in four treatment groups: saline/saline (ss), LPS/saline (ls), saline/LPS (sl) or LPS/LPS (ll). Mice treated with only saline (ss, n = 8) and ls mice (n = 8) mice ate similar amounts of food after the second injection. Sl mice (n = 7) showed significant anorexia compared to ss mice at all time points ($p < 0.001$). LPS-pretreated mice (ll, n = 8) showed anorexia only at 6 and 12 hours after the second LPS injection, but this was less pronounced than in sl mice at all time points. ** = $p < 0.01$, *** = $p < 0.001$ compared to ss control mice; a = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$ compared to sl mice.

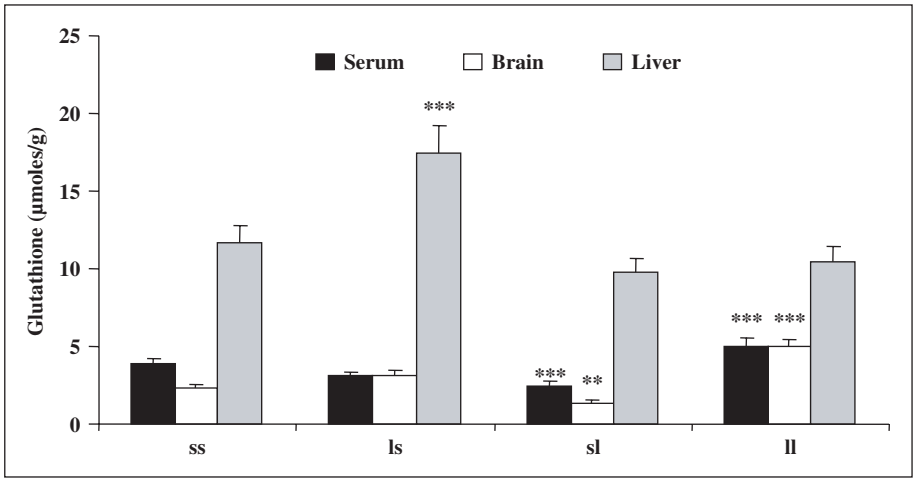


Figure 2
Total reduced GSH (trGSH) levels in plasma, brain and liver at 24 hours after LPS (4 µg/mouse) injection in LPS-naïve and LPS-pretreated mice. LPS-pretreated mice (ll, n = 8) had significantly higher plasma trGSH levels than LPS-naïve saline controls (ss, n = 8, $p < 0.001$). Ls mice had significantly higher liver trGSH levels than saline controls (ss, n = 8, $p<0.001$). ** = $p < 0.01$, *** = $p < 0.001$ compared to ss mice.

Again, LPS pretreatment attenuated the effect of LPS at all time points measured (sl[-db] *versus* ll[-db], $p < 0.01$) (*figure 4*, also see *figure 1*). GSH depletion with DEM/BSO (db) *per se* (ss[+db] and ls[+db] mice) did not affect food intake compared to saline pretreated mice (ss[-db]) or LPS-pretreated hyposensitive mice (ll[-db]). Yet, DEM/BSO treatment 2 hours prior to the second LPS injection antagonized the hyposensitivity to LPS-induced anorexia, *i.e.*, it re-established the anorexia, in ll[+db] mice *(figure 4)*. Ll[+db] mice showed significant anorexia compared to their controls (ll[-db], ss[+db]) at 6 hours and 12 hours (both time points $p < 0.01$), but the effect did not persist over 24 hours.

Serum acute phase protein response to LPS

GSH depletion *per se* resulted in no major change in serum protein levels (ss[+db] *versus* ss[-db]). Similar responses were observed in all groups, with the exception of the ll[+db] group, in which serum protein levels were significantly increased *(figure 5A)*. Serum electrophorograms indicate that this increase in ll[+db] mice was due to a general increase rather than an increase in a specific protein *(figure 5B)*.

Serum TNFα and IFNγ response to LPS

Circulating serum IFNγ levels were increased in mice that received LPS for the first time (sl[-db]) and in LPS-

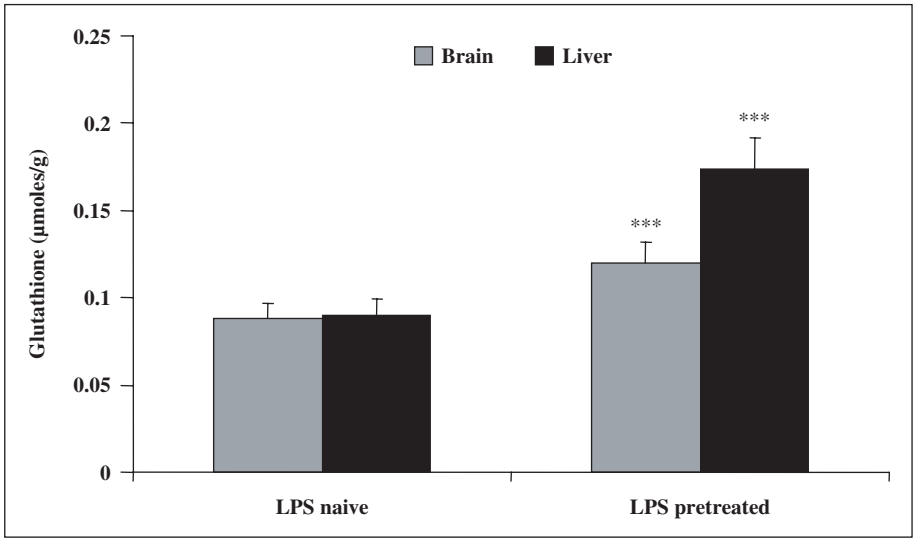


Figure 3
Mitochondrial GSH repartitioning in LPS-naïve and LPS-treated mice. Mice were injected either with saline or LPS (4 µg/mouse) and were sacrificed four days later to collect brain and liver. Tissues were assayed for mitochondrial GSH levels as described in Methods. LPS resulted in increased mitochondrial GSH in both brain and liver. n = 6, * = $p < 0.05$, *** = $p < 0.001$: * or *** = comparison between LPS and saline injected mice.

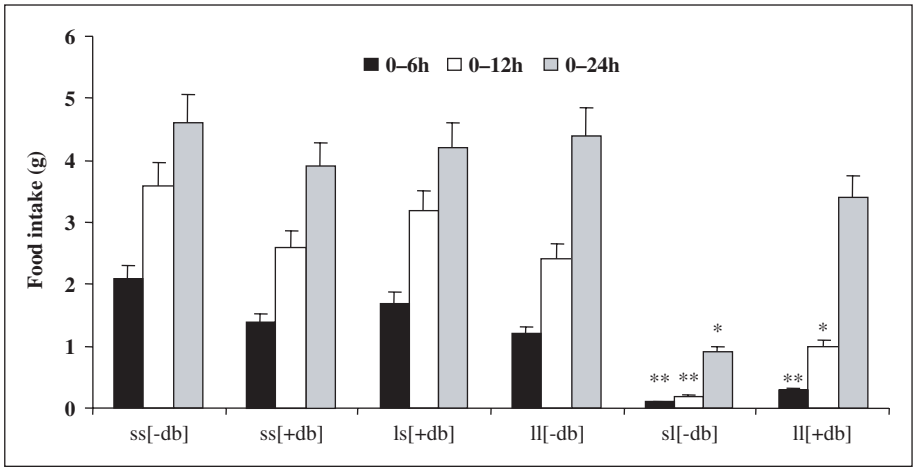


Figure 4

Effect on LPS anorexia of GSH depletion by DEM/BSO (db) in mice. LPS-naïve or LPS-pretreated mice received either saline or db and were then given either saline or LPS as described in Methods. After the final injection, food intake was measured at 6, 12 and 24h. Mice receiving LPS for the first time sl[-db] ate less than their controls (ss[-db] or ll[-db] mice) at all time points measured (all $p < 0.01$). DEM/BSO attenuated the LPS hyposensitivity, i.e., re-established the feeding inhibitory effect of LPS at 6 and 12 h in mice (ll[+db] *versus* ls[+db] or ss[+db] mice, $p<0.01$). n=4 animals in all groups. * = $p < 0.05$, ** = $p < 0.01$ compared to ss[-db] mice; b = $p < 0.01$ compared to ll[-db] mice.

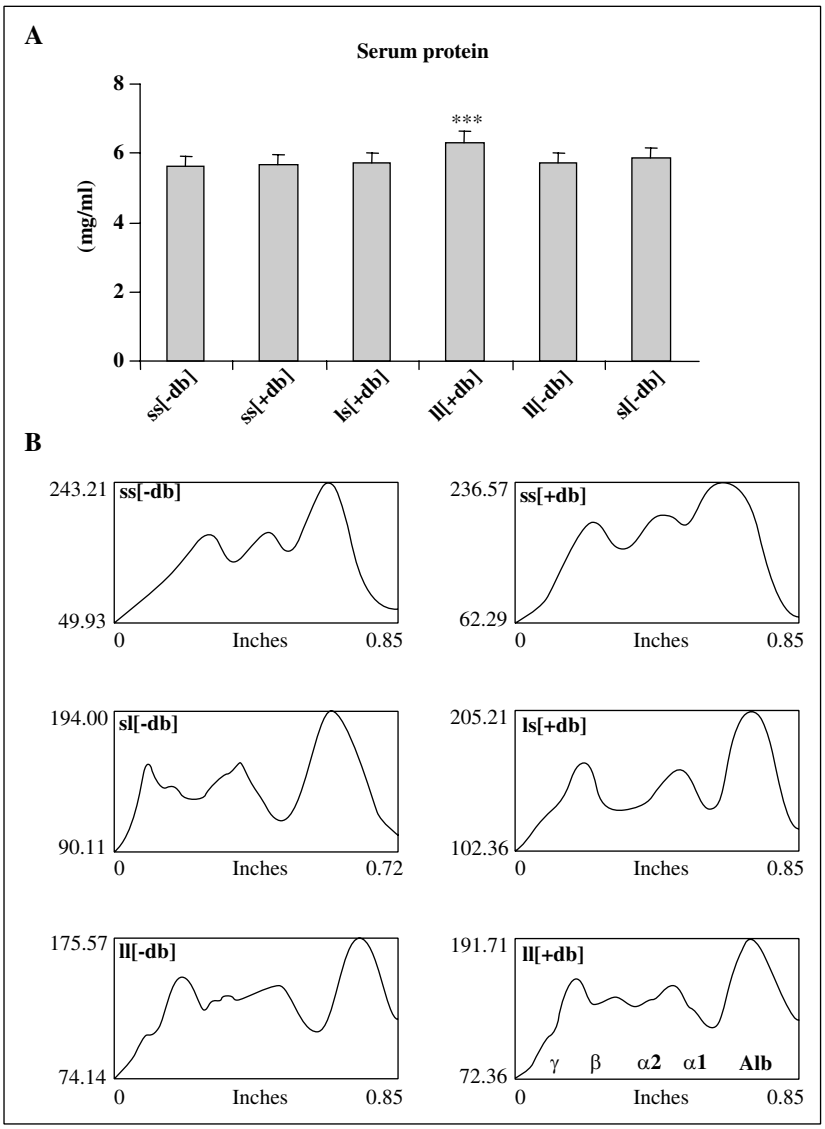


Figure 5

Serum protein levels were similar in all treatment groups at 24h after the second injection, except for ll[+db] mice which had higher serum protein levels than ll[-db] or ss[+db] mice ($n = 4$, $p < 0.001$) (5A). In contrast, qualitative changes due to the different treatments were revealed by the electrophoretogram of serum protein (5B), with albumin being the least affected (large band on the left of each electrophoretogram). The location of the gammaglobulin (γ), beta-globulin (β), alpha-1-globulin (α1), alpha-2-globulin (α2) and albumin (Alb) bands are shown in 5B.

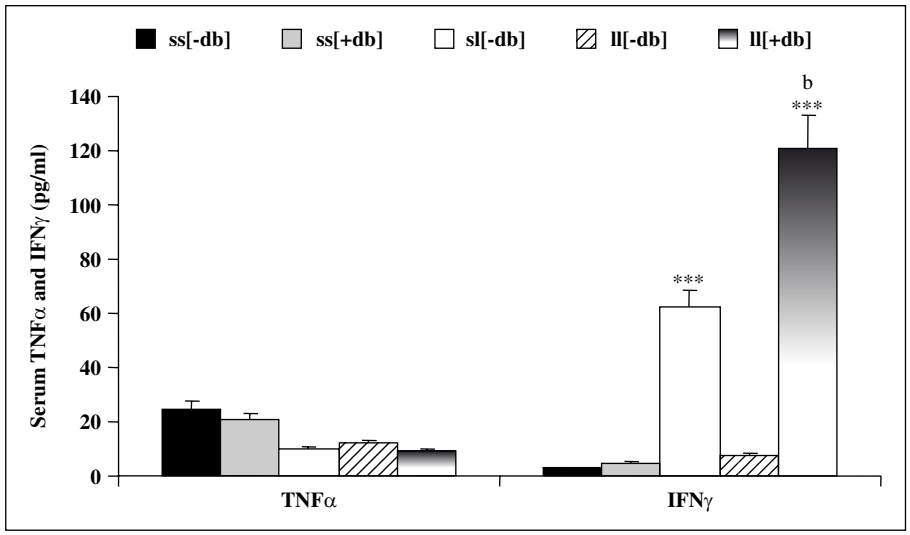


Figure 6

Serum TNFα did not increase 24 hours after the second LPS injection, contrastingly, IFNγ was increased in naïve mice (sl), DEM/BSO increased IFNγ in ll[+db] mice compared to ll[-db] 24 hours after the second LPS injection. n = 4 animals in all groups. *** = $p < 0.001$, compared to ss[-db] mice; b = $p < 0.01$ compared to sl[-db] mice.

pretreated animals treated with DEM/BSO and receiving a second LPS injection (ll[+db]), whereas LPS-pretreated animals without db treatment (ll[-db]) showed no increase in either cytokine one day after the second LPS treatment *(figure 6)*.

DISCUSSION

Consistent with previous findings [14, 15], we report here that ip LPS reduced food intake in LPS-naïve mice, whereas LPS-pretreated mice showed an attenuated anorexia in response to the second LPS injection. Interestingly, this phenomenon was associated with increased glutathione (GSH) levels in brain and liver, raising the possibility that the observed hyposensitivity to the second LPS injection was due to high tissue GSH levels. Altering antioxidant levels in various animal models has been shown to change the release of cytokines in response to immune challenges. Thus, LPS-induced cytokine production was modified by decreasing or increasing the GSH content with DEM/BSO [18, 19] or N-acetylcysteine [20, 21], respectively. Serum IFNγ levels appeared to be sensitive to GSH changes in LPS-pretreated animals as they were increased 24 h after the second LPS treatment when GSH was decreased. Our data suggest that LPS has a biphasic effect on tissue GSH levels. Whereas the first LPS injection led to an acute decrease in serum and brain GSH, presumably because the organism used GSH to cope with the LPS-induced oxidative stress, four days after the first LPS injection, mice had increased liver GSH, perhaps as a result of stimulated hepatic GSH production in response to the initial demand. This additional GSH appeared to be rapidly available when the mice were confronted with the second LPS injection because in this situation they showed higher serum and brain GSH levels when compared to the twice saline-injected control mice. Thus, the stimulated production and rapid distribution of GSH rather than elevated tissue levels of GSH alone may contribute to LPS hyposensitivity. In line with this hypothesis, depletion of GSH rendered LPS-pretreated mice susceptible to LPS anorexia, suggesting that GSH can curb LPS-induced anorexia. Of course, we can also not exclude that a difference in GSH turnover rates between LPS hypo-responsive and LPS-naive mice contributed to the observed differences in GSH levels because we did not measure GSH turnover. Finally, we also demonstrate that altered intracellular GSH repartitioning occurs in LPS hyposensitivity in both brain and liver. Thus, we observed an increase in mitochondrial GSH in LPS-pretreated mice compared to LPS-naïve mice. In other oxidative stress models, increases in mitochondrial GSH are associated with an increased resistance to the degree of pathology [6-9], and yet other studies have shown that modification of mitochondrial activity can result in resistance to LPS-induced anorexia [21-23]. Further studies using mice with genetically altered mitochondrial GSH levels could be used to critically examine the role of mitochondrial GSH in LPS anorexia by using 1) increased mitochondrial GSH transport [24] in LPS-naïve mice and 2) decreased mitochondrial GSH [25, 26] in LPS tolerance studies. A change in the redox state can alter the immune response due to changes in the production of cytokines and/or acute phase proteins. It has been shown that acute phase proteins, in particular an increase in lipoproteins (found in the beta-globulin faction), can attenuate some of the responses to LPS and their severity [16, 17]. Our electrophoretograms revealed that depletion of GSH *per se* did not result in a marked, qualitative decrease in the beta-globulins. The lack of major changes between ss[-db] and ss[+db] serum protein levels and the bands at 24 hours after treatment suggests that the difference between ll[-db] and ll[+db] mice was due to specific effects of GSH depletion on the response to LPS. In addition, we can exclude a general liver toxicity effect of db treatment at this time point because albumin levels were not reduced in db treated mice. This suggests that the reduction of GSH by db was not due to general liver toxicity/failure. In summary, these results suggest that LPS affects GSH levels in a tissue-specific manner and that stimulation of GSH production and its rapid distribution in the organism rather

than absolute GSH levels confer hyposensitivity to LPS anorexia. The intracellular repartition of GSH may also be important because hypo-responsive animals had increased mitochondrial GSH and did not display anorexia 24 hours after the second LPS treatment. Further support for a role of GSH in the feeding response to LPS is derived from the fact that mice that are hypo-responsive to the anorexic effect of LPS because of LPS pretreatment can be rendered sensitive again by pharmacological GSH depletion. The generality of this phenomenon, i.e., whether for instance IFNγ-KO mice or other proinflammatory cytokine-deficient mice can also be made more responsive to LPS-induced anorexia by pharmacological GSH depletion remains to be examined.

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