Role for nerve growth factor in the *in vivo* regulation of glutathione in response to LPS in mice

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ABSTRACT. Since the redox state regulator glutathione (GSH) influences lipopolysaccharide (LPS) anorexia, we studied the roles of tumour necrosis factor-alpha (TNFα) and nerve growth factor (NGF) in the GSH response to intraperitoneal (ip) LPS injection in mice. Basal NGF and GSH levels were up-regulated in brain and liver of TNFα-knock-out (KO) mice, and this was associated with attenuated LPS anorexia. The increases in NGF and GSH presumably contributed to the attenuated anorexia in response to LPS because transgenic mice over-expressing NGF (NGF-tg) also had increased GSH levels and displayed attenuated anorexia compared to the corresponding wild type (WT) mice. Attenuated LPS anorexia in NGF-tg mice was accompanied by reduced serum TNFα and IFNγ levels compared to WT mice. In response to a second injection of LPS, NGF and GSH levels, but not TNFα levels changed. This suggests that *in vivo* tissue GSH changes following LPS in LPS-naïve or LPS-pretreated mice are regulated by NGF rather than TNFα. The finding that genetic TNFα deficiency did not inhibit the acute GSH response to LPS supports this interpretation. In sum, the results indicate i) that a decrease or increase in NGF is accompanied by a decrease or increase in GSH levels and ii) that elevated NGF and/or GSH levels attenuate some of the responses to LPS such as anorexia and cytokine production.

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

Pro-inflammatory cytokines are common mediators of oxidative stress [1], which is one important pathological mechanism of tissue dysfunction. Antioxidants have been shown to modify the inflammatory process by attenuating cytokine production and reducing oxidative stress [2]. In turn, cytokine signaling can be modified by antioxidants such as glutathione (GSH) [3, 4]. GSH is produced mainly in the liver and is taken up from the circulation by other tissues [5]. GSH has been implicated in limiting LPS-induced pathology [6]. One of the cytokines that supposedly enhances oxidative stress in response to LPS and other immune challenges is tumour necrosis factor-alpha (TNFα). Yet, although some studies implicate TNFα in reactive oxygen species toxicity [1], other findings suggested it has protective effects [7]. One possible explanation for these discrepancies is that there may be a tissue-specific, local cytokine and/or redox state regulation. Thus, we have seen tissue-specific redox regulation in a murine *Toxoplasma gondii* infection model, in which oxidative stress is reduced in the brain and markedly increased in the liver at the same time [10]. Circulating hormones may also play an important role in tissue redox regulation. It has been shown that growth hormone (GH) and leptin not only influence cytokine production, but also GSH levels [11, 12]. Furthermore, both hormones may regulate nerve growth factor (NGF), which is an important activator of antioxidant mechanisms ([13-16]. Based on results from *in vitro* studies [17], NGF may be particularly important in regulating GSH, but recently, TNFα has been implicated in the control of NGF [18], suggesting that there are complex interactions between these two cytokines and GSH. Therefore, the aim of the present study was to examine the roles of TNFα and NGF in the *in vivo* regulation of GSH in response to LPS in 1) acute LPS-naïve and 2) LPS-pretreated mice.

METHODS

Mice, housing and diets

We used male, 8-16 week-old 25-30 g (body weight) knockout (KO) or transgenic (tg) and corresponding wild type mice for all experiments. Tumour necrosis factor-alpha-knockout (TNFα-KO) mice on a c57/bl6 background [19], and corresponding wild-type (WT) mice were obtained from Dr. I. Garcia (University of Geneva, Switzerland). Nerve growth factor-over-expressing transgenic mice (NGF-tg) and corresponding WT mice on a b6 background were obtained from Dr. B. Onteniente

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(INSERM UMR 421, Creteil, France) [20]. 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 diet (Nafag, Gossau, Switzerland). Food intake after lipopolysaccharide (LPS) injections (see below) was measured (± 0.1 g) at various time intervals (6, 12 and 24 hours) by subtracting the weight of the container with the food and taking into account any spillage. All procedures were approved by the Canton of Zurich Veterinary Office.

Role of TNFα in LPS-induced changes in i) NGF and GSH levels in brain and liver and ii) serum levels of growth hormone and leptin

We used 16 TNFα-KO and 16 corresponding WT mice to examine whether the lack of TNFα changes NGF and total reduced GSH levels in brain and liver under baseline conditions or after intraperitoneal (ip) injection of LPS (4 µg/mouse). The LPS dose was chosen because it 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). In addition to NGF and GSH, TNFα was measured in brain and liver of WT mice. WT and TNFα-KO mice were injected with LPS (12 WT and 12 TNFα-KO) or saline (n = 4 of each genotype). Mice were sacrificed immediately after saline injection (time point 0, baseline) or at 15, 90 and 240 minutes (n = 4/genotype and time point) after LPS injection. Serum, brain and liver were collected and tissue levels of NGF, total reduced GSH, and TNFα were determined as described below. Growth hormone was determined using rat growth hormone immunoassay from Amersham (Otelfingen, Switzerland) and leptin was determined by mouse immunoassay kit from Crystal Chem (Chicago, USA).

Role of NGF in LPS-induced changes in i) GSH levels in brain, liver and plasma and ii) in LPS-induced anorexia

Four NGF-tg and four corresponding WT mice were used per group. Blood was collected in EDTA-tubes by heart puncture. Immediately thereafter, mice were sacrificed by CO_2 and brain and liver were removed, frozen in liquid nitrogen, and stored at –80°C. Furthermore, to determine whether NGF-tg mice had an altered anorexic response to LPS, 12 NGF-tg and 12 WT mice were injected (ip) with LPS (n = 6/genotype) or saline (n = 6/genotype) and food intake measured at 24h after injection. Serum, brain and liver were collected, snap frozen in liquid nitrogen, and stored at -80C° until analysis. Throughout the extraction procedure for GSH and cytokines, samples were kept on ice. Tissues were homogenized with phosphate-buffered saline (600 µl/100 mg tissue) using a polytron PT1200 homogenizer (Kinematica, Lucern, Switzerland). The solution underwent a precipitation reaction with phosphate solution (glacial metaphosphoric acid 1.67 g, 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 total reduced GSH 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) [21].

Effect of anorexia on brain and liver GSH levels

As the level of food intake can alter tissue GSH, we examined whether LPS-induced changes in total reduced GSH in brain and liver could be due to the decrease in food intake after LPS. Additional groups of NGF-tg and WT mice were underfed (NGF-tguf and WTuf, n = 4, each) to the same level as NGFtg and WT mice that received LPS. An identical experiment was performed in TNFα-KO and corresponding WT mice (TNFα-KOuf and WTuf).

Role of TNFα and NGF in LPS tolerance

To investigate whether the lack of TNFα changes the evolution of NGF levels during induction of tolerance to LPS, we injected TNFα-KO and corresponding WT mice with 4µg LPS twice, on day 1 of the experiment and on day 4 (see *table 1*), when the animals' food intake had returned to pre-injection values. As circulating TNFα usually peaks at approximately 90 minutes after LPS injection, we compared plasma TNFα and NGF levels at this time point after LPS or saline injection in LPS-naïve and LPS-pretreated TNFα-KO and WT mice to see whether LPS pretreatment affected LPS-induced changes in plasma NGF and TNFα (in WT mice). Four mice for each subgroup of WT and TNFα-KO mice were used according to *table 1*. Blood was collected by heart puncture. Serum was frozen and stored at -80°C [24],, and TNFα and NGF were measured using immunoassay kits from Amersham (Otelfingen, Switzerland) and Catalys (Wallisellen, Switzerland), respectively.

Statistics

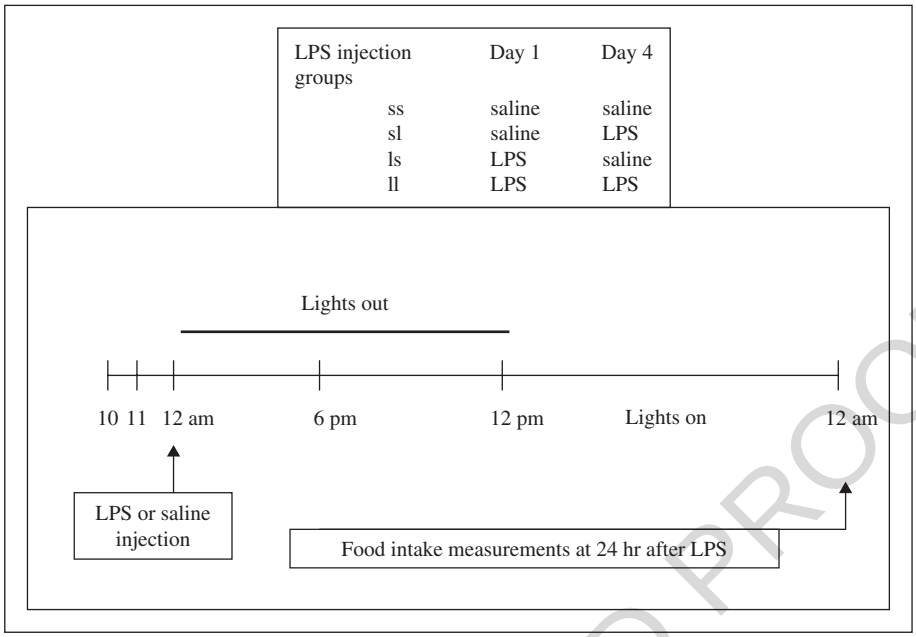
Differences between groups were determined by using Kruskal-Wallis test with a Dunn *post hoc* test. A value of $p<0.05$ was considered significant. Non-parametrical Spearman correlation coefficients were calculated using the Instat program (GraphPad Incorporation, San Diego, CA, USA).

RESULTS

Role of TNFα in LPS-induced changes in i) NGF and GSH levels in brain and liver and ii) serum levels of growth hormone and leptin

TNFα-KO mice had higher baseline, total reduced GSH levels than WT mice in brain ($p<0.05$) *(figure 1A)* and liver ($p < 0.001$) *(figure 1B)*. LPS reduced total reduced GSH in brain and liver at 15 minutes in TNFα-KO and WT mice and at 90 minutes in TNFα-KO mice. At 240 minutes after LPS injection, brain and liver GSH had returned to baseline levels in mice of both genotypes. LPS progressively increased brain TNFα in WT mice at 15, 90 and 240 minutes *(figure 1C)*. Also, LPS increased TNFα at

Table 1
Time lines for treatment and food intake measurements in WT and TNFαKO mice



15 and 90 minutes after injection, in livers of WT mice *(figure 1D)*. No TNFα was detected in brains and livers of TNFα-KO mice at any of the time points *(figure 1C and 1D)*. TNFα-KO mice had higher basal NGF levels than WT mice in brain ($p < 0.001$) *(figure 1E)* and liver ($p < 0.001$) *(figure 1F)*. LPS reduced brain and liver NGF levels at 15 and 90 minutes after injection in both TNFα-KO and WT mice. At 240 minutes after LPS injection, brain and liver NGF levels had increased again, but brain NGF was still lower than baseline in TNFα-KO mice *(figure 1E)*. Hepatic NGF and GSH levels after LPS treatment were correlated in TNFα-KO (Spearman correlation coefficient: 0.91) and WT mice (0.89). TNFα deficiency did not result in differences in basal growth hormone levels, and no differences where seen in the response to LPS throughout the measurement period *(figure 2A)*. Also, basal serum levels of leptin did not differ between WT and TNFα-KO mice, but serum leptin was decreased ($p < 0.001$) at 15 minutes after LPS injection in WT mice. This decrease did not reach significance in the TNFα-KO mice *(figure 2B)*. Thereafter, serum leptin returned to basal levels in WT mice and was increased in TNFα-KO mice at 240 minutes after LPS injection, compared to basal levels ($p < 0.01$) and compared to WT leptin levels at this time point ($p < 0.01$) *(figure 2B)*.

Role of NGF in LPS-induced changes in i) GSH levels in brain, liver and plasma and ii) in LPS-induced anorexia

Baseline total reduced GSH levels in brain, liver and plasma were higher (all $p < 0.001$) in NGF-tg than in WT mice *(figure 3A)*. Baseline food intake did not differ between WT and NGF-tg mice *(figure 3B)*. LPS decreased 24-hour food intake in both, WT and NGF-tg mice, but the LPS-induced anorexia was less pronounced, and of shorter duration in NGF-tg mice than in WT mice ($p < 0.001$) *(figure 3B)*. NGF-tg mice had higher serum levels of NGF, but lower serum levels of TNFα and IFNγ 240 minutes after LPS injection ($p < 0.001$ $p < 0.01$ and $p < 0.001$ for NGF, TNFα, and IFNγ, respectively) than WT mice.

Effect of anorexia on brain and liver GSH levels

Underfeeding (uf) did not cause significant changes in brain total reduced GSH compared to *ad libitum* feeding *(table 2)*. Underfeeding did, however, decrease liver GSH levels in both TNFα-KO and NGF-tg mice. Furthermore, GSH levels in TNFα-KO-uf and NGF-tg-uf mice remained higher than in their WT-uf counterparts *(table 2)*.

Role of TNFα and NGF in LPS tolerance

Baseline serum brain and liver GSH was generally higher in TNFα-KO than in WT mice, whether they received LPS or not (data not shown**).** As expected, 90 minutes after LPS injection TNFα was detected only in the serum of WT mice but not in TNFα-KO mice *(figure 4A)*. Compared to WT control mice (ss), serum TNFα was increased ($p < 0.01$) in WT mice receiving LPS for the first time (sl, 165 ± 11 pg/ml *versus* ss, 25 ± 5 pg/ml). In contrast, LPS did not increase serum TNFα in LPS-pretreated mice (ll). Serum NGF was significantly higher ($p < 0.001$) in TNFα-KO control mice (ss, 128 ± 9 pg/ml) than in WT control mice (ss, 62 ± 5 pg/ml) *(figure 4B)*. LPS decreased serum NGF in both WT (sl) and TNFα-KO (sl) mice to 23 ± 4 pg/ml and 43 ± 3 pg/ml, but NGF levels remained higher ($p < 0.01$) in TNFα-KO mice. LPS-pretreated mice (ls) of both genotypes had higher serum NGF levels than saline-pretreated control mice (ss). Also, in LPS-pretreated, saline-injected (ls) TNFα-KO mice, serum NGF levels (239 ± 15 pg/ml) were higher ($p < 0.001$) than in WT mice (117 ± 10 pg/ml) *(figure 4B)*. Serum NGF levels were still higher in twice LPS-injected mice of both genotypes than in twice saline-injected control mice.

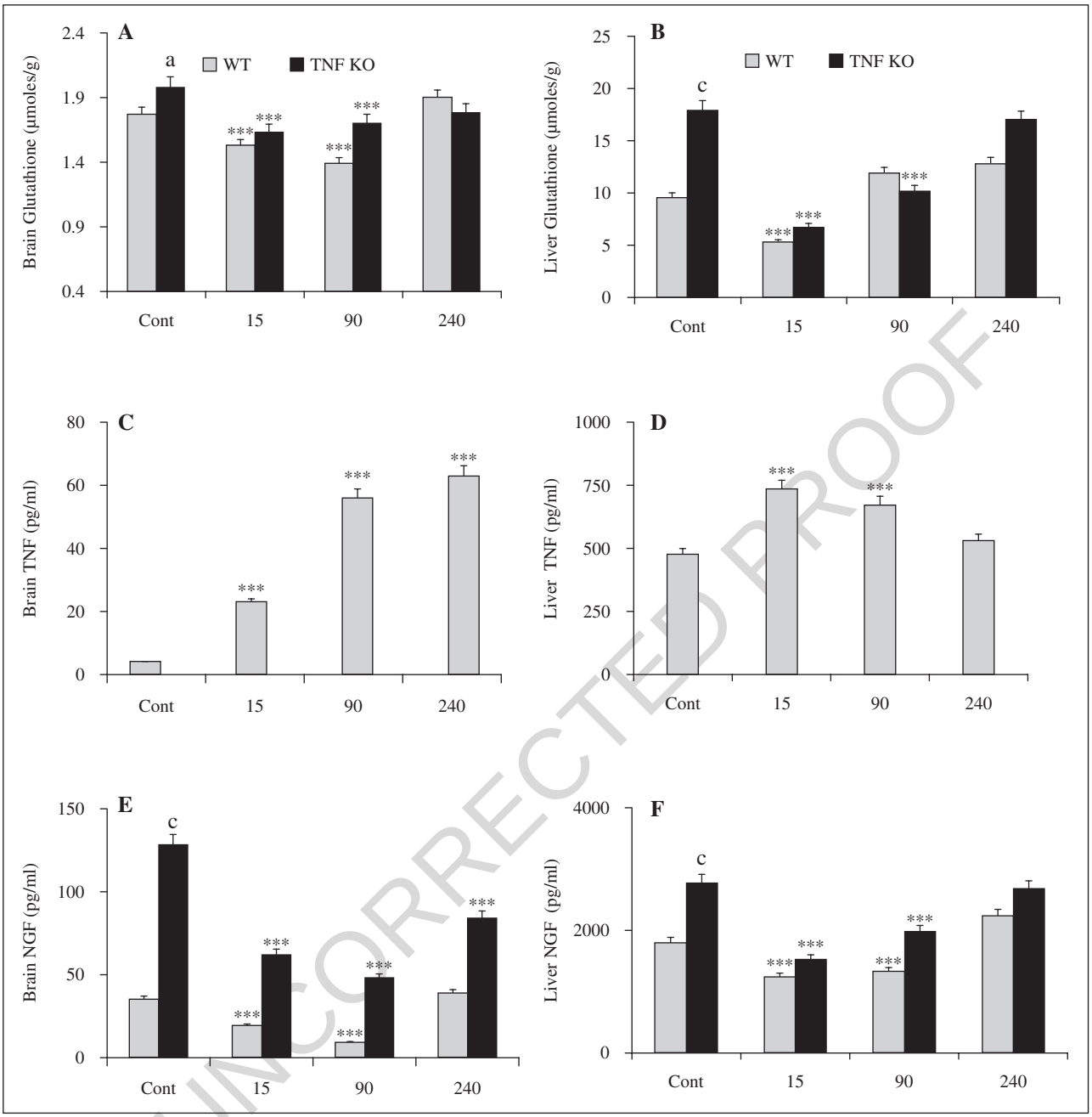


Figure 1

Acute changes in brain and liver total reduced GSH, TNFα and NGF levels in response to ip LPS (4 µg / 25 g BW). Fifteen, 90 and 240 minutes after injections mice were sacrificed, brain and liver tissues were collected and assayed for GSH (1A, 1B), TNFα (1C, 1D) and NGF (1E, 1F) as described in Methods and materials . Note that TNFα-KO mice showed significantly higher basal levels of GSH and NGF than WT mice. In both WT and TNFα-KO mice, a significant decrease in GSH and NGF in both brain and liver was observed at 15 and 90 minutes after LPS; n = 4 for all columns, a = $p < 0.01$; c and *** = $p < 0.001$; a, c = comparisons between the two genotypes at the same time point; *** = comparisons between LPS and control treatments within genotypes.

DISCUSSION

LPS has been shown to induce changes in GSH (1), and GSH may modify the severity of the LPS response [6]. Confirming and extending these findings, we have previously shown that resistance of LPS-pretreated mice to LPS anorexia is associated with an increase in GSH tissue levels [22], and here we again observed a negative correlation between liver GSH and the anorexic effect of LPS. Yet, while these data suggest that GSH plays a role in LPS anorexia, little is known about the control of GSH by cytokines, i.e., by the major endogenous mediators of LPS anorexia, and in particular about the mechanisms of tissue GSH regulation by cytokines *in vivo*. The results presented here begin to examine these mechanisms. The higher basal GSH and NGF levels observed in brains and livers of TNFα-KO compared to WT mice, and the fact that these differences more or less persisted after LPS administration, suggest that TNFα somehow decreases GSH and NGF. The latter finding is consistent with other studies showing that TNFα-KO mice had increased brain NGF (18). The decrease in GSH and NGF by TNFα may be due to the fact that TNFα is a major determinant of the level of oxidative stress in response to LPS, which then draws upon

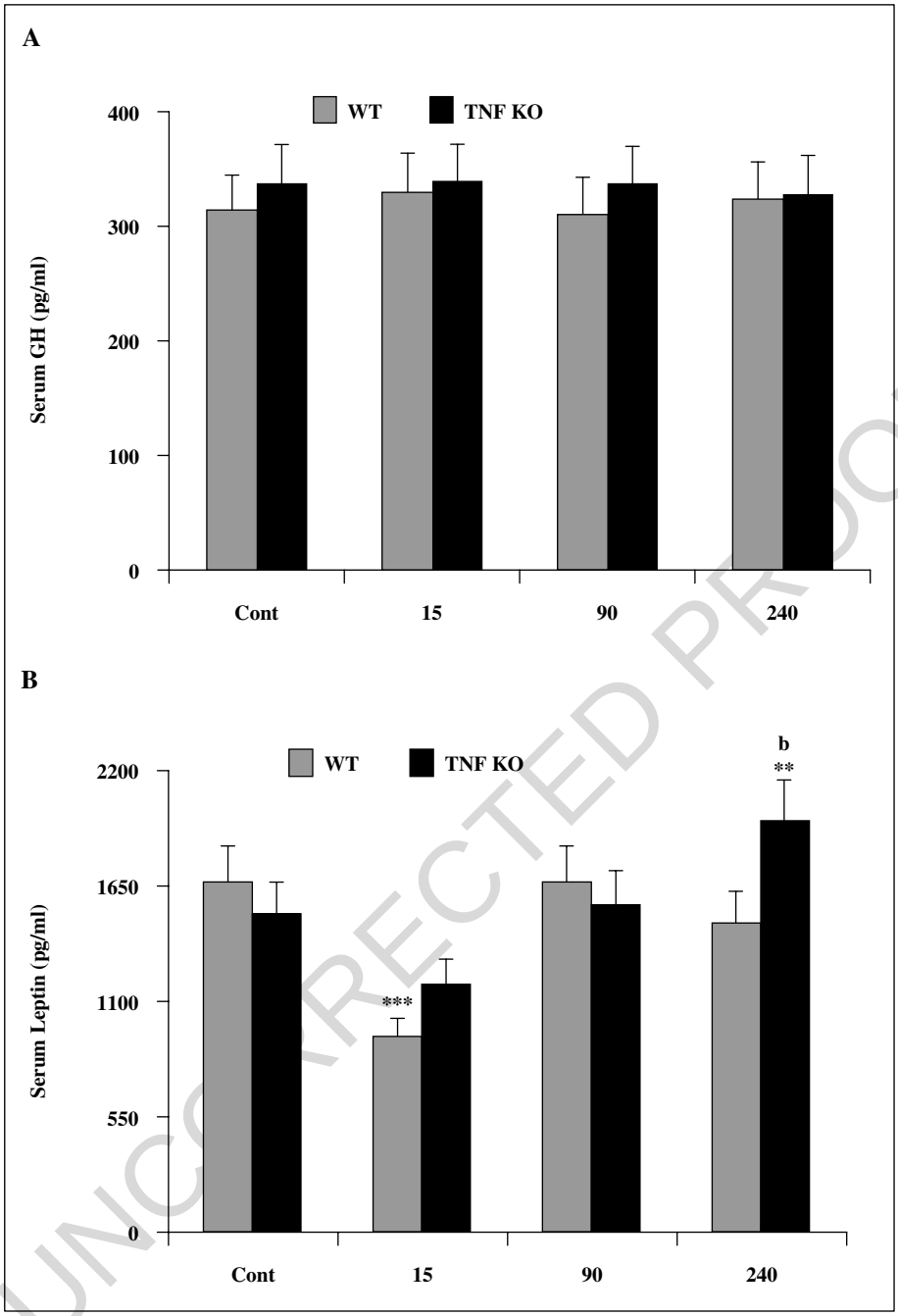


Figure 2

Acute changes in serum growth hormone (GH) and leptin levels in response to ip LPS (4 µg / 25 g BW). Fifteen, 90 and 240 minutes after injections mice were sacrificed, serum was collected and assayed for GH (2A) and leptin (2B) as described in Methods and materials. Note that TNFα-KO mice showed no significant differences in basal levels of GH and leptin compared to WT mice. Only in WT mice was leptin decreased at 90 minutes after LPS injection (n = 6, *** = $p < 0.01$). At 240 minutes after LPS injection, leptin was increased in only TNFα-KO mice; ** and b = $p < 0.01$; b = comparison between genotypes at the same time point; *** = comparisons between LPS and control treatments within genotypes.

the existing pool of antioxidants including GSH and NGF. Other cytokines may also affect GSH however, because TNFα-KO mice have been shown to have lower serum IFNγ levels than LPS-naïve WT mice in response to LPS (23). The results show that TNFα deficiency does not alter basal serum growth hormone or leptin levels. This suggests that growth hormone does not mediate acute changes in the GSH in response to LPS, but it may still play a role later [15]. Leptin's response to LPS appeared to be largely independent of TNFα because TNFα-KO mice had elevated circulating leptin levels at 240 minutes after LPS injection. In contrast, TNFα appears to be a major stimulus of leptin secretion in mice with high fat diet-induced obesity because in this model the increase in circulating leptin was shown to be much smaller in TNFα-KO mice than in WT mice. [24]. Previous studies have suggested a role for leptin in GSH regulation [25]. We report here a similar pattern of changes in serum leptin and liver GSH levels in response to LPS. Serum leptin and liver GSH levels were decreased at 15 minutes after LPS in WT mice. Likewise, at 240 minutes after LPS injection there was an increase in serum leptin and liver GSH. Whether this

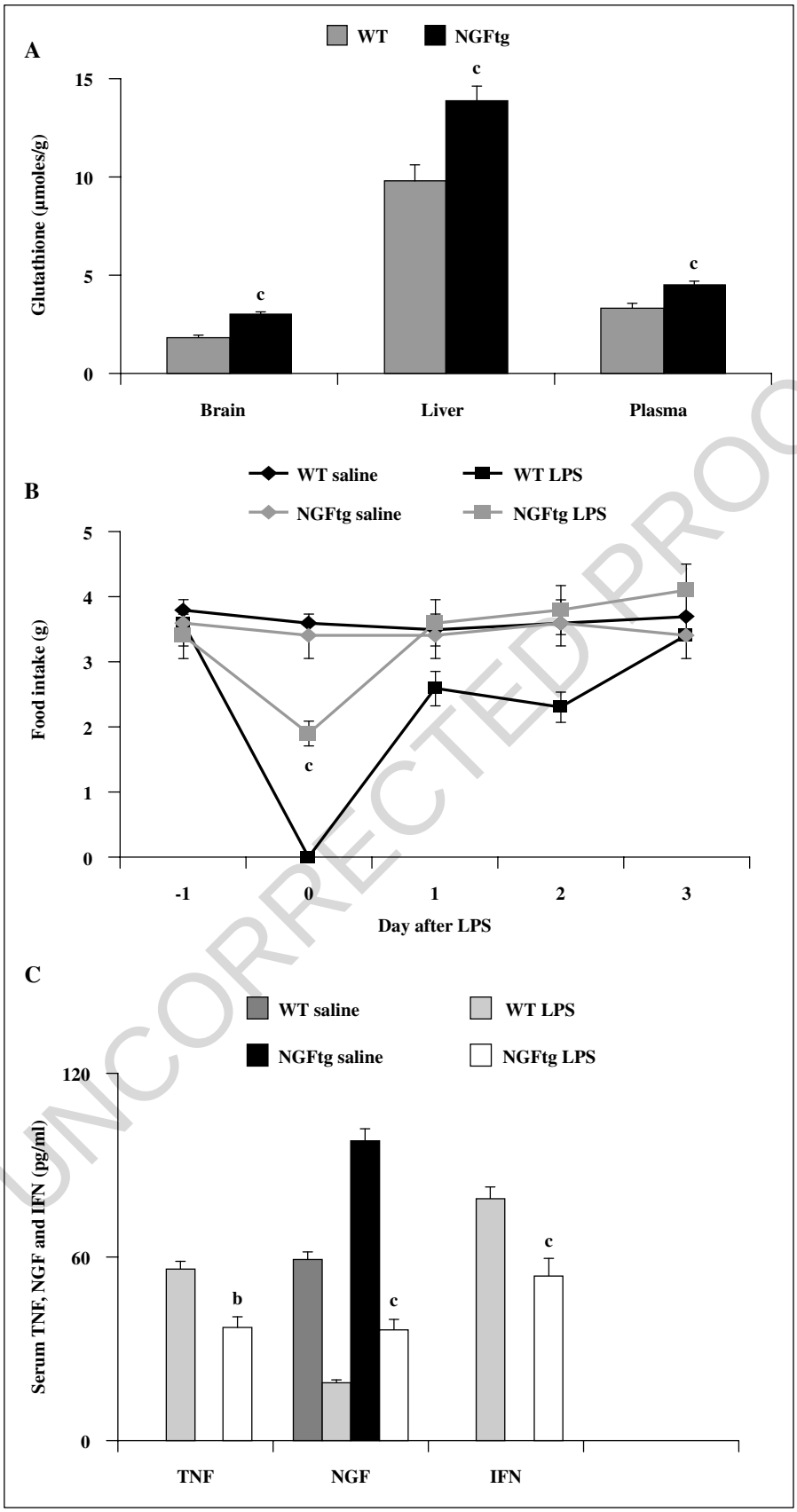


Figure 3

Basal total reduced GSH levels (3A), anorexic response to ip LPS (3B), and serum TNFα, NGF and IFNγ responses to ip LPS in WT and NGFtg mice. Mice were sacrificed as described in the Methods and materials section to determine basal GSH levels in brain, liver and serum (3A). All tissues of NGF-tg mice showed significantly higher GSH levels than WT mice ($n = 6$, $c = p < 0.001$). WT and NGF-tg mice were injected ip with LPS (4 µg/25 g BW) and food intake was measured every 24 h for three days. LPS reduced food intake in mice of both genotypes, but NGF-tg mice showed less anorexia than their WT counterparts (3B). Serum TNFα and IFNγ were reduced in NGF-tg mice at 240 minutes after LPS injection compared to WT mice (3C). $n = 6$, $b = p < 0.01$, $c = p < 0.001$; b, c = comparisons between genotypes at the same time point.

Table 2
Effect of underfeeding on brain and liver glutathione

	Brain GSH µmol/g of tissue	Liver GSH µmol/g of tissue
WT	1.6 ± 0.03	9.3 ± 0.2
WTuf	1.4 ± 0.02	5.8 ± 0.1**
TNFα KO	1.9 ± 0.03	17.1 ± 0.6
TNFα KOuf	2.1 ± 0.02	14.8 ± 0.3**,[a]
WT	1.1 ± 0.02	7.8 ± 0.2
WTuf	1.2 ± 0.02	4.3 ± 0.2***
NGF-tg	3.0 ± 0.03	15.8 ± 0.2
NGF-tguf	3.2 ± 0.02	13.8 ± 0.1***,[a]

** or *** are comparison of the underfed group to its respective ad lib control; [a] is the comparison between the underfed genetically modified group to its underfed WT group. ** - $p < 0.01$; a and *** - $p < 0.001$.

similar reaction pattern in response to LPS reflects causality, and if so, whether leptin influences hepatic GSH or vice versa, remains to be determined. LPS administration resulted in decreases of GSH, which occurred in brains and livers of LPS naïve TNFα-KO and WT mice. Although TNFα-KO mice had increased basal NGF and GSH levels, LPS still decreased GSH in these animals, suggesting that factors other than TNFα mediated this effect. It is presently unknown whether the LPS-induced changes in tissue GSH were mediated by cytokines other than TNFα or by other, "non-cytokine" factors. Although GSH first decreased in response to LPS and then returned to near basal levels at 240 minutes after LPS injection, GSH was reduced in the brains of WT mice at 24 hours after a single LPS injection. This temporal difference could be due to the cyclic fluctuations in GSH [27], to the anorexia itself, or to the time course of cytokine changes after LPS. According to our findings, the LPS-induced anorexia *per se* cannot explain the GSH changes at 24 hours after LPS in our experiments because food restriction to the level of LPS-injected mice did not lead to the same reduction in brain GSH levels, although a reduction in liver GSH did occur in food-restricted mice as after LPS injection. In addition, the reduction in GSH at 15 and 90 minutes after LPS injection cannot be explained by changes in food intake. TNFα-KO mice developed hyposensitivity to LPS anorexia in response to a single LPS pretreatment, as did their corresponding WT mice [data not shown, 22]. This indicates that TNFα is neither necessary for the development of hyposensitivity to LPS anorexia nor for the LPS-induced changes in tissue GSH [data not shown, 22]. On the other hand, development of hyposensitivity to LPS anorexia was accompanied by a blunted TNFα response to LPS, suggesting that TNFα contributes to LPS anorexia [8]. Altering antioxidant levels in various animal models has been shown to alter the release of cytokines. Our data show that the antioxidant GSH is handled in different ways in TNFα-KO and WT mice, in both the acute responses in naïve mice and during hyposensitivity. We also found a high correlation between liver GSH and NGF. This correlation appears to reflect a causal relationship because NGF-tg mice had higher brain and liver GSH levels. NGF apparently regulates GSH levels [17]. Furthermore, NGF overexpressing NGF-tg mice naïve to LPS showed less anorexia than their WT counterparts in response to LPS, i.e., high levels of NGF protected against LPS anorexia. This effect may, at least in part, be due to GSH because liver, plasma and brain GSH levels were higher in NGF-tg than in WT mice. As NGF-tg mice also had lower serum levels of TNFα and IFNγ than WT mice in response to LPS administration, the different sensitivity to LPS anorexia may also be due to this different cytokine response. It is of interest to note that NGF and IFNγ apparently have antagonistic effects [28]. IFNγ has previously been shown to be an important mediator of LPS-induced anorexia, probably more important than TNFα [23, 29]. Also, IFNγ is a major contributor to LPS-induced oxidative stress [10, 30], which may be responsible for some of the pathological changes induced by LPS. Given the role of IFNγ in LPS-induced oxidative stress, the decrease in liver and brain GSH levels associated with the increase in serum IFNγ presumably reflects the enhanced demand on antioxidant mechanisms after LPS injection. Nevertheless, IFNγ does not appear to be involved in basal GSH regulation because it was not detected in the plasma of LPS naïve mice. It is interesting that LPS increased GSH in brain and liver more, and circulating TNFα and IFNγ levels less in NGF-tg mice than in corresponding WT mice, suggesting that NGF is involved in the response of all these parameters to LPS. Consistent with the higher NGF levels in liver and brain, serum NGF was also always higher in TNFα-KO mice than in WT mice, under baseline conditions and after LPS injection. Serum NGF was particularly high at 24 h after LPS injection, when the animals had almost completely lost their sensitivity to the anorexic effect of LPS. This finding is again consistent with a role of NGF in reducing the anorexic effect of LPS. In summary, our study suggests that TNFα regulates GSH *via* NGF in a tissue-specific manner and that liver GSH levels are associated with the degree of LPS-induced anorexia in LPS-naïve mice. Interestingly, we also show that increases in serum NGF levels occur during the development of LPS tolerance, indicating that NGF, unlike TNFα, still responds to LPS injection in LPS-pretreated mice. The generality of this phenomenon, i.e., whether for instance NGF- or NGF receptor-deficient mice or mice deficient in IFNγ show changes in GSH levels and whether these changes control mice sensitivity to LPS-induced anorexia remains to be examined.

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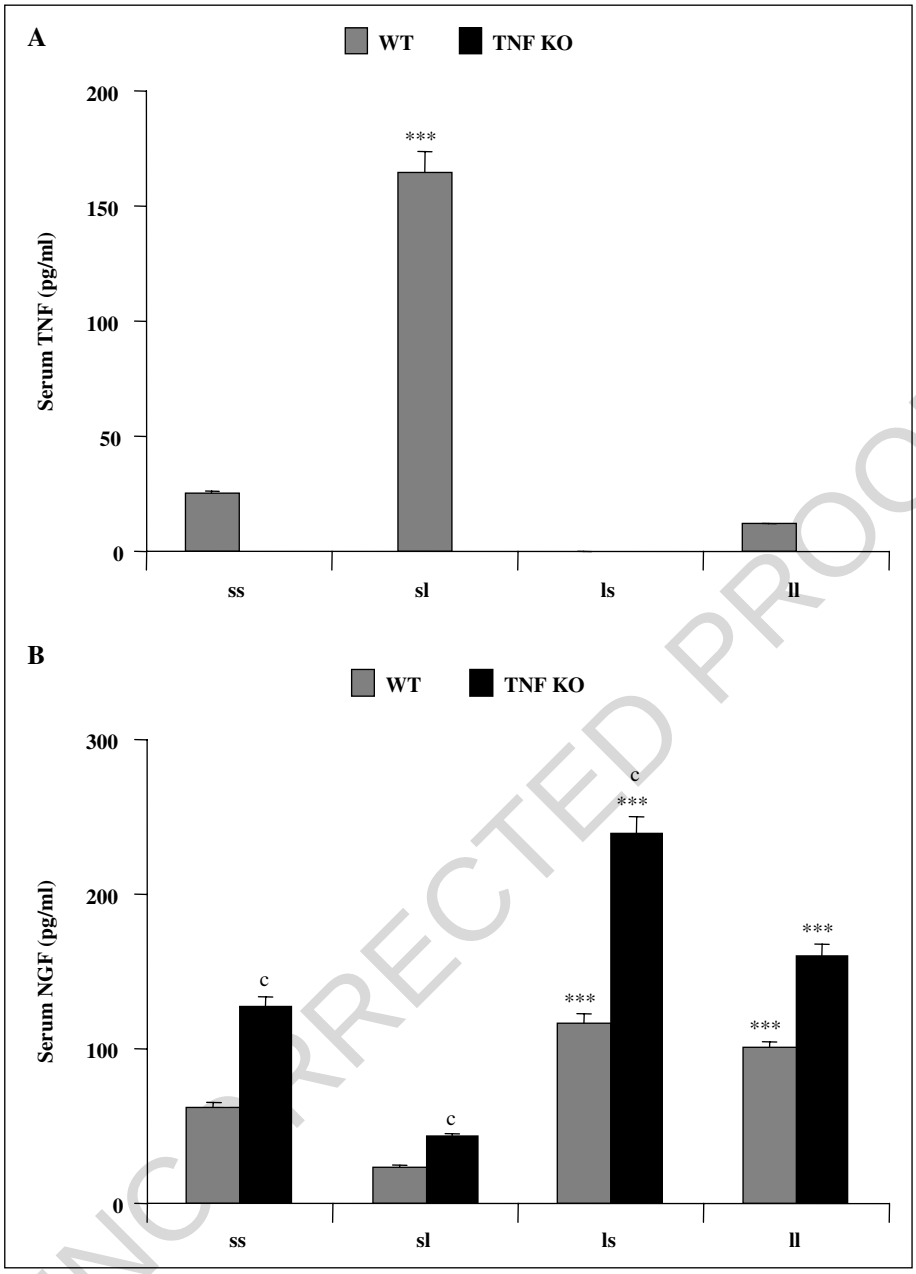

Figure 4

Serum TNFα and NGF response to ip LPS in LPS naïve and LPS pretreated TNFα-KO and WT mice. Serum TNFα was increased after LPS in naïve WT mice (sl) but not in LPS pretreated WT mice (ls or ll), and no TNFα was detected in TNFα-KO animals at 90 minutes after LPS injection (4A). Basal serum NGF was significantly higher in TNFα-KO mice than in WT mice ($p < 0.001$). Note that LPS-pretreated TNFα-KO mice (ls) had higher NGF levels than LPS-pretreated WT mice (ls) and that the second LPS injection decreased NGF in the former but not the latter (4B). c and *** = $p < 0.001$; *** = comparison to ss within genotype; c = comparison between genotypes.

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