

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), which influences lipopolysaccharide (LPS) anorexia, may be controlled by cytokines, we studied the roles of tumour necrosis factor-alpha (TNF α) and nerve growth factor (NGF) in the GSH response to intraperitoneal (ip) LPS injection in mice. Basal NGF and total reduced GSH (trGSH) levels were up-regulated in brain and liver of TNF α -knock-out (KO) mice, and this was associated with attenuated LPS anorexia. The increases in NGF and trGSH presumably contributed to the attenuated anorexia in response to LPS because transgenic mice over-expressing NGF (NGF-tg mice) also had increased trGSH 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 TNF α and IFN γ levels compared to WT mice. In response to a second injection of LPS, NGF and trGSH levels, but not TNF α levels changed. This suggests that *in vivo* tissue trGSH changes following LPS in LPS-naïve or LPS-pretreated mice are regulated by NGF rather than TNF α . The finding that genetic TNF α deficiency did not inhibit the acute trGSH 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 trGSH levels and ii) that elevated NGF and/or trGSH 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 signalling 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 (TNF α). Yet, although some studies implicate TNF α in reactive oxygen species toxicity [1], other findings suggested it has protective effects [7-9]. 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 was 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. Recently, TNF α 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 TNF α and NGF in the *in vivo* regulation of GSH in response to LPS in 1) 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 (TNF α -KO) mice on a c57/bl6 back-

ground [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 bl6 background were obtained from Dr. B. Onteniente (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 chow (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 TNF α 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 TNF α -KO and 16 corresponding WT mice to examine whether the lack of TNF α changes NGF and total reduced GSH (trGSH) levels in brain and liver under baseline conditions or after intraperitoneal (ip) injection of LPS (4 μ g/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 μ g per 25 g body weight ($n = 8$ /dose). In addition to NGF and trGSH, TNF α was measured in brain and liver of WT mice. WT and TNF α -KO mice were injected with LPS (12 WT and 12 TNF α -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, trGSH, and TNF α were determined as described below. Growth hormone was determined using rat growth hormone immunoassay from Amersham (Otefingen, 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 CO₂ 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 -80°C until analysis. Throughout the extraction procedure for GSH and cytokines, samples were kept on ice. Tissues were homogenized with phosphate-buffered saline (600 μ L/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 Na₂HPO₄) 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) [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 trGSH 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 TNF α -KO and corresponding WT mice (TNF α -KOuf and WTuf).

Role of TNF α and NGF in LPS tolerance

To investigate whether the lack of TNF α changes the evolution of NGF levels during induction of tolerance to LPS, we injected TNF α -KO and corresponding WT mice with 4 μ g 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 TNF α usually peaks at approximately 90 minutes after LPS injection, we compared plasma TNF α and NGF levels at this time point after LPS or saline injection in LPS-naïve and LPS-pretreated TNF α -KO and WT mice to see whether LPS pretreatment affected LPS-induced changes in plasma NGF and TNF α (in WT mice). Four mice for each subgroup of WT and TNF α -KO mice were used according to *table 1*. Blood was collected by heart puncture. Serum was frozen and stored at -80°C [24], and TNF α and NGF were measured using immunoassay kits from Amersham (Otefingen, Switzerland) and Catalys (Wallisellen, Switzerland), respectively.

Statistics

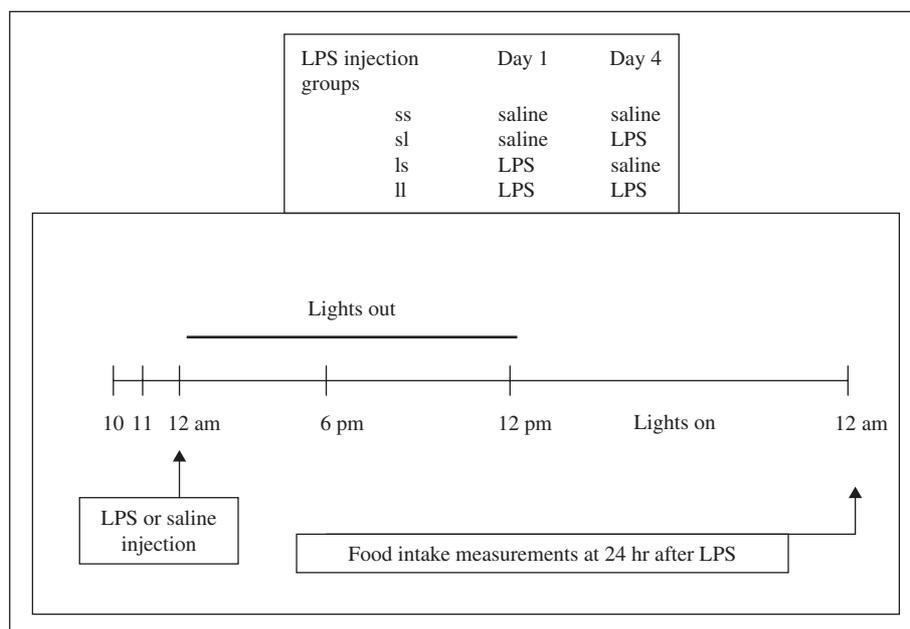
Differences between groups were determined by using Kruskal-Wallis test with a Dunn *post hoc* test. A value of $p < 0.05$ was considered significant. Non-parametrical Spearman correlation coefficients were calculated using the InStat program (GraphPad Incorporation, San Diego, CA, USA).

RESULTS

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

TNF α -KO mice had higher baseline trGSH levels than WT mice in brain ($p < 0.05$) (*figure 1A*) and liver ($p < 0.001$) (*figure 1B*). LPS reduced trGSH in brain and liver at 15 minutes in TNF α -KO and WT mice and at 90 minutes in

Table 1
Time lines for treatment and food intake measurements in WT and TNF α KO mice



TNF α -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 TNF α in WT mice at 15, 90 and 240 minutes (*figure 1C*). Also, LPS increased TNF α at 15 and 90 minutes after injection, in livers of WT mice (*figure 1D*). No TNF α was detected in brains and livers of TNF α -KO mice at any of the time points (*figure 1C and 1D*). TNF α -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 TNF α -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 TNF α -KO mice (*figure 1E*). Hepatic NGF and trGSH levels after LPS treatment were correlated in TNF α -KO (Spearman correlation coefficient: 0.91) and WT mice (0.89). TNF α deficiency did not result in differences in basal growth hormone levels, and no differences were seen in the response to LPS throughout the measurement period (*figure 2A*). Also, basal serum levels of leptin did not differ between WT and TNF α -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 TNF α -KO mice (*figure 2B*). Thereafter, serum leptin returned to basal levels in WT mice and was increased in TNF α -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 trGSH 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 TNF α and IFN γ 240 minutes after LPS injection ($p < 0.001$, $p < 0.01$ and $p < 0.001$ for NGF, TNF α , and IFN γ , respectively) than WT mice.

Effect of anorexia on brain and liver GSH levels

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

Role of TNF α and NGF in LPS tolerance

Baseline serum brain and liver trGSH was generally higher in TNF α -KO than in WT mice, whether they received LPS or not (data not shown). As expected, 90 minutes after LPS injection TNF α was detected only in the serum of WT mice but not in TNF α -KO mice (*figure 4A*). Compared to WT control mice (ss), serum TNF α 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 TNF α in LPS-pretreated mice (ll). Serum NGF was significantly higher ($p < 0.001$) in TNF α -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 TNF α -KO (sl) mice to 23 ± 4 pg/mL and 43 ± 3 pg/mL, but NGF levels remained higher ($p < 0.01$) in TNF α -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) TNF α -KO mice, serum NGF levels (239 ± 15 pg/mL) were higher

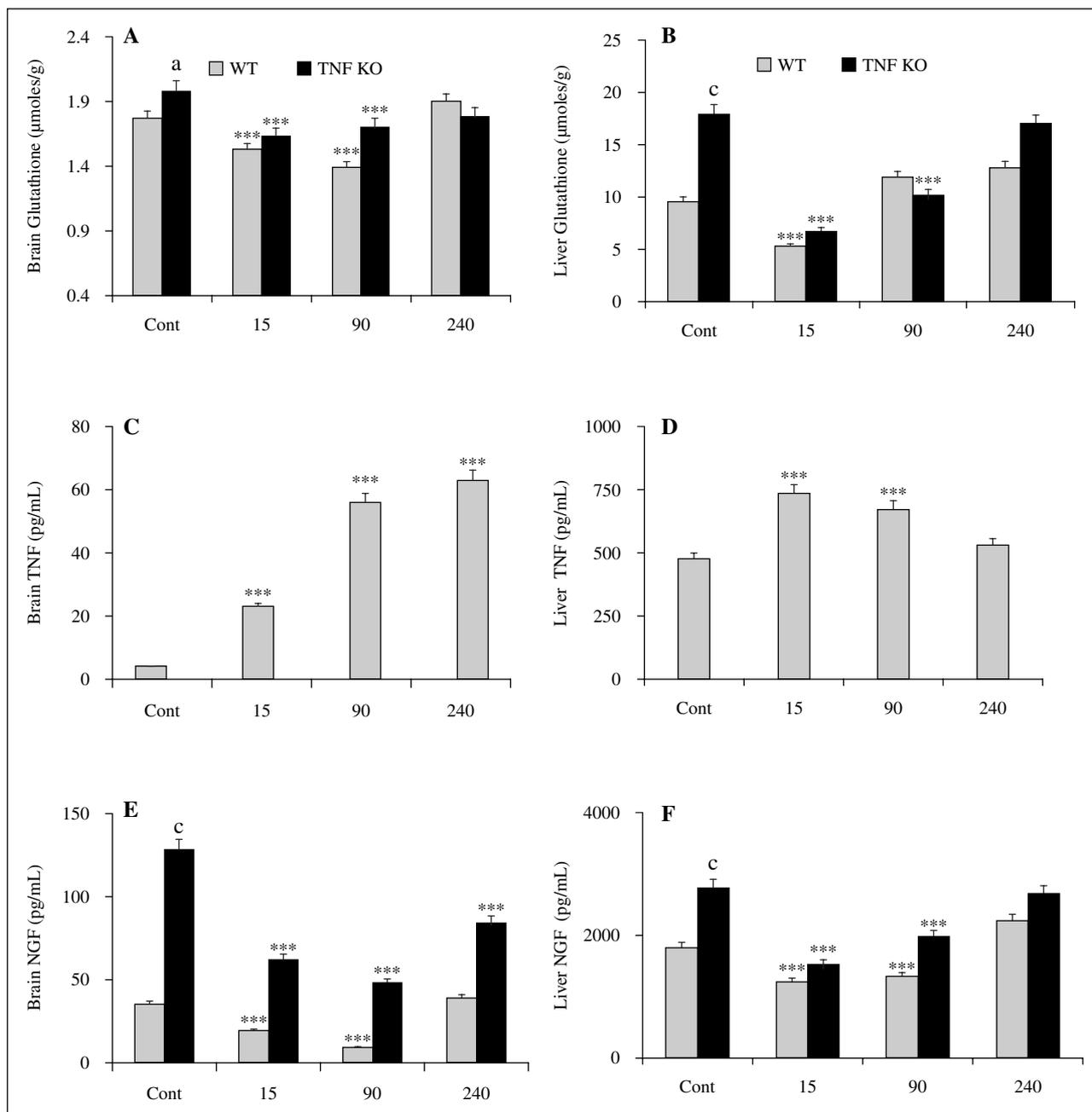


Figure 1

Acute changes in brain and liver total reduced GSH (trGSH), TNF α and NGF levels in response to ip LPS (4 μ g / 25 g BW). Fifteen, 90 and 240 minutes after injections mice were sacrificed, brain and liver tissues were collected and assayed for trGSH (A, B), TNF α (C, D) and NGF (E, F) as described in *Methods and materials*. Note that TNF α -KO mice showed significantly higher basal levels of trGSH and NGF than WT mice. In both WT and TNF α -KO mice, a significant decrease in trGSH 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.

(p < 0.001) than in WT mice (117 \pm 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.

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 hyposensitivity 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 trGSH and the anorexic effect of LPS. Yet, while these data suggest that trGSH 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 trGSH and NGF levels observed in brains and livers of TNF α -KO compared to WT mice, and the fact that these differences more or less persisted after LPS admin-

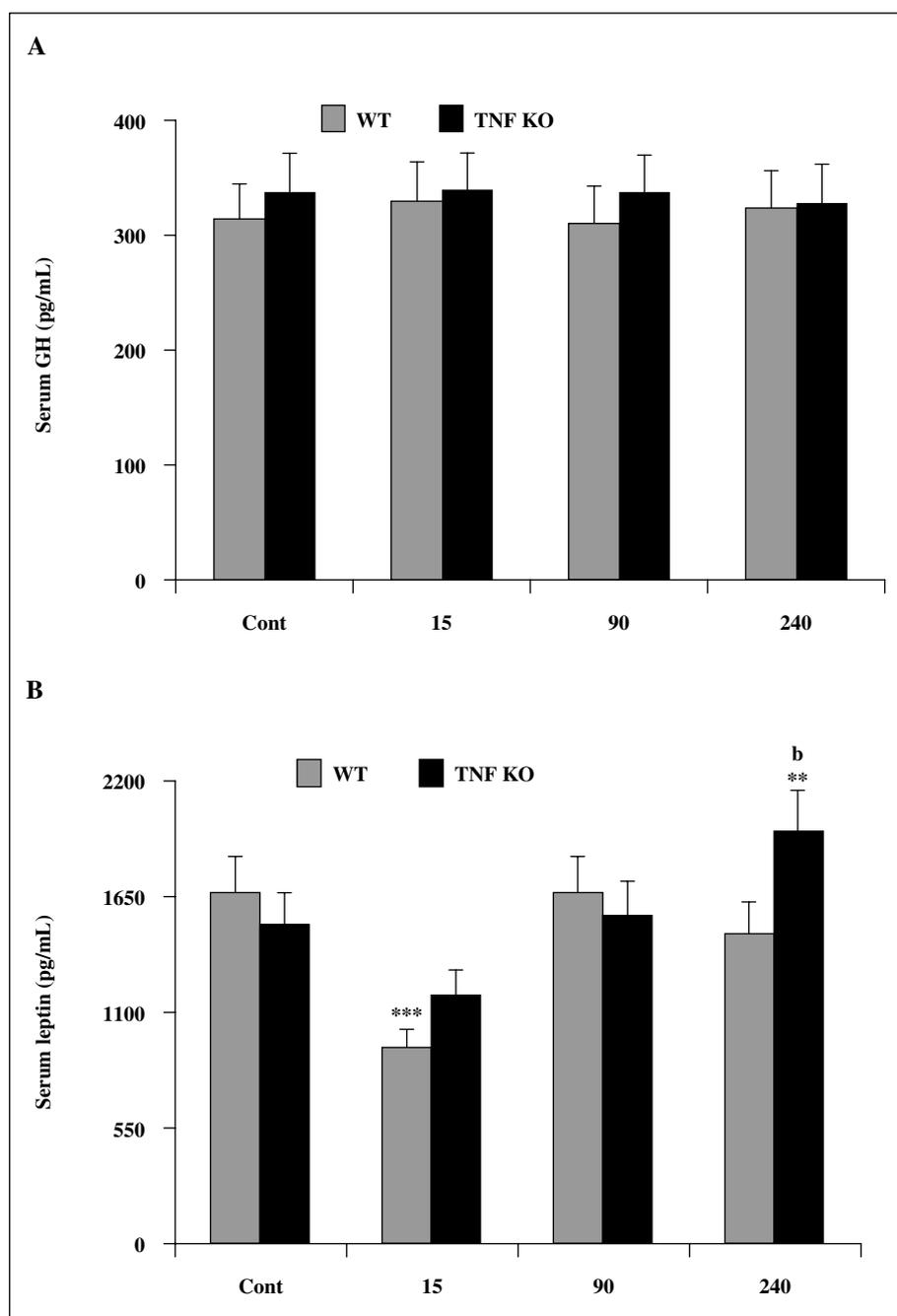


Figure 2

Acute changes in serum growth hormone (GH) (A) and leptin (B) 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 and leptin as described in Methods and materials. Note that TNF α -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 TNF α -KO mice; ** and b = p < 0.01; b = comparison between genotypes at the same time point; *** = comparisons between LPS and control treatments within genotypes.

istration, suggest that TNF α somehow decreases trGSH and NGF. The latter finding is consistent with other studies showing that TNF α -KO mice had increased brain NGF (18). The decrease in GSH and NGF by TNF α may be due to the fact that TNF α is a major determinant of the level of oxidative stress in response to LPS, which then draws upon the existing pool of antioxidants including GSH and NGF. Other cytokines may also affect GSH however, because TNF α -KO mice have been shown to have lower serum IFN γ levels than LPS-naïve WT mice in response to LPS (23). The results show that TNF α 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 TNF α because TNF α -KO mice had elevated circulating leptin levels at 240 minutes after LPS injection. In contrast, TNF α 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 TNF α -KO mice than in WT mice. [24]. Previous studies have suggested a

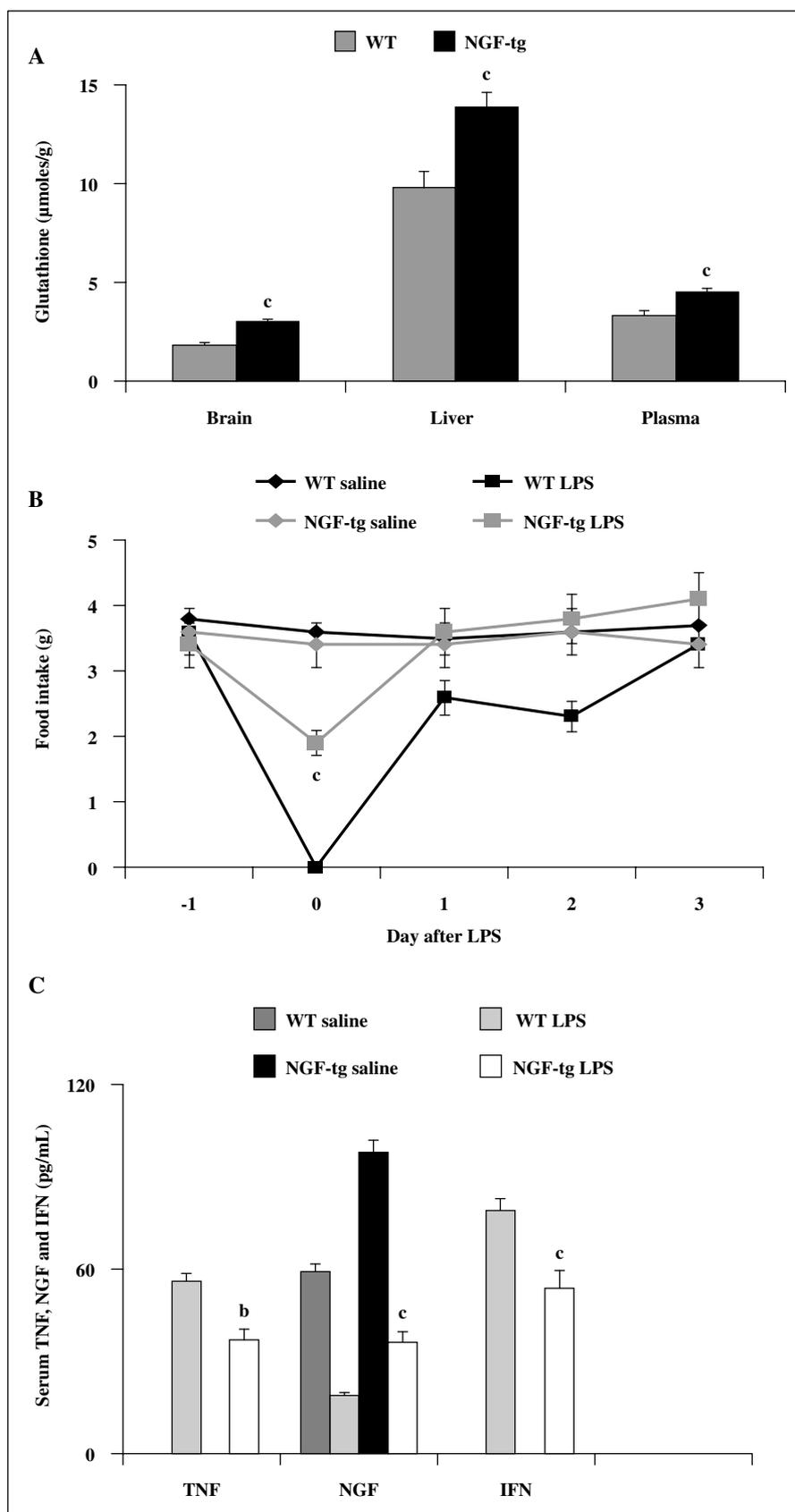


Figure 3

Basal total reduced GSH (trGSH) levels (A), anorexic response to ip LPS (B), and serum TNF α , NGF and IFN γ responses to ip LPS in WT and NGF-tg mice. Mice were sacrificed as described in the *Methods and materials* section to determine basal trGSH levels in brain, liver and serum (A). All tissues of NGF-tg mice showed significantly higher trGSH levels than WT mice ($n = 6$, $c = p < 0.001$). WT and NGF-tg mice were injected ip with LPS ($4 \mu\text{g}/25 \text{ g BW}$) 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 (B). Serum TNF α and IFN γ were reduced in NGF-tg mice at 240 minutes after LPS injection compared to WT mice (C). $n = 6$, $b = p < 0.01$, $c = p < 0.001$; $b, c =$ comparisons between genotypes at the same time point.

Table 2
Effect of underfeeding on brain and liver glutathione

	Brain GSH $\mu\text{mol/g}$ of tissue	Liver GSH $\mu\text{mol/g}$ of tissue
WT	1.6 \pm 0.03	9.3 \pm 0.2
WTuf	1.4 \pm 0.02	5.8 \pm 0.1**
TNF α KO	1.9 \pm 0.03	17.1 \pm 0.6
TNF α KOuf	2.1 \pm 0.02	14.8 \pm 0.3**, ^a
WT	1.1 \pm 0.02	7.8 \pm 0.2
WTuf	1.2 \pm 0.02	4.3 \pm 0.2***
NGF-tg	3.0 \pm 0.03	15.8 \pm 0.2
NGF-tguf	3.2 \pm 0.02	13.8 \pm 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 *** - $p < 0.001$.

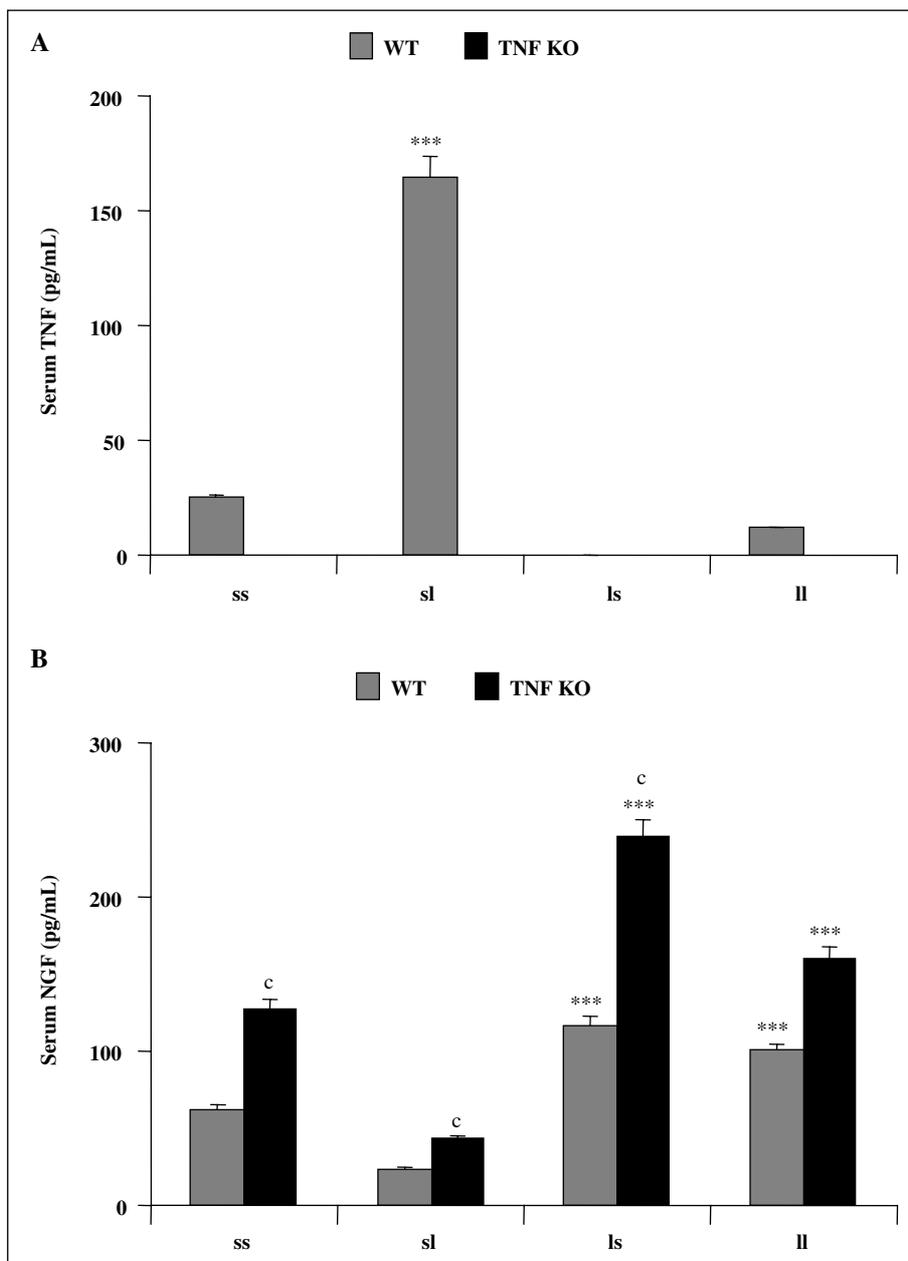


Figure 4

Serum TNF α and NGF response to ip LPS in LPS naïve and LPS pretreated TNF α -KO and WT mice. Serum TNF α was increased after LPS in naïve WT mice (sl) but not in LPS pretreated WT mice (ls or ll), and no TNF α was detected in TNF α -KO animals at 90 minutes after LPS injection (4A). Basal serum NGF was significantly higher in TNF α -KO mice than in WT mice ($p < 0.001$). Note that LPS-pretreated TNF α -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.

role for leptin in GSH regulation [25]. We report here a similar pattern of changes in serum leptin and liver trGSH levels in response to LPS. Serum leptin and liver trGSH 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 trGSH. Whether this similar reaction pattern in response to LPS reflects causality, and if so, whether leptin influences hepatic trGSH or vice versa, remains to be determined. LPS administration resulted in decreases of trGSH, which occurred in brains and livers of LPS naïve TNF α -KO and WT mice. Although TNF α -KO mice had increased basal NGF and trGSH levels, LPS still decreased trGSH in these animals, suggesting that factors other than TNF α mediated this effect. It is presently unknown whether the LPS-induced changes in tissue trGSH were mediated by cytokines other than TNF α or by other, "non-cytokine" factors. Although trGSH first decreased in response to LPS and then returned to near basal levels at 240 minutes after LPS injection, trGSH 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 trGSH [26], 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 trGSH 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 trGSH levels, although a reduction in liver trGSH did occur in food-restricted mice as after LPS injection. In addition, the reduction in trGSH at 15 and 90 minutes after LPS injection cannot be explained by changes in food intake. TNF α -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 TNF α is neither necessary for the development of hyposensitivity to LPS anorexia nor for the LPS-induced changes in tissue trGSH [data not shown, 22]. On the other hand, development of hyposensitivity to LPS anorexia was accompanied by a blunted TNF α response to LPS, suggesting that TNF α 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 trGSH is handled in different ways in TNF α -KO and WT mice, in both the acute responses to LPS in LPS-naïve mice and during hyposensitivity to LPS. We also found a high correlation between liver trGSH and NGF. This correlation appears to reflect a causal relationship because NGF-tg mice had higher brain and liver trGSH levels. NGF apparently regulates trGSH 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 trGSH because liver, plasma and brain trGSH levels were higher in NGF-tg than in WT mice. As NGF-tg mice also had lower serum levels of TNF α and IFN γ 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 IFN γ apparently have antagonistic effects [27]. IFN γ has previously been shown to be an important mediator of LPS-induced anorexia, probably more important than TNF α [23, 28]. Also, IFN γ is a major contributor to LPS-induced oxidative stress [10, 29], which may be re-

sponsible for some of the pathological changes induced by LPS. Given the role of IFN γ in LPS-induced oxidative stress, the decrease in liver and brain trGSH levels associated with the increase in serum IFN γ presumably reflects the enhanced demand on antioxidant mechanisms after LPS injection. Nevertheless, IFN γ does not appear to be involved in basal trGSH regulation because it was not detected in the plasma of LPS naïve mice. It is interesting that LPS increased trGSH in brain and liver more, and circulating TNF α and IFN γ 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 TNF α -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 TNF α regulates trGSH *via* NGF in a tissue-specific manner and that liver trGSH 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 TNF α , 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 IFN γ show changes in trGSH levels and whether these changes control the mice's sensitivity to LPS-induced anorexia remains to be examined.

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