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Involvement of nitric oxide in lipopolysaccharide induced anorexia

Thomas Riediger *, Caroline Cordani, Catarina Soares Potes, Thomas A. Lutz

Institute of Veterinary Physiology and Centre of Integrative Human Physiology, University of Zurich, 8057 Zurich, Switzerland

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ABSTRACT

Treatment with the bacterial endotoxin lipopolysaccharide (LPS) is a commonly used model to induce disease-related anorexia. Following LPS treatment inducible nitric oxide synthase (iNOS) is expressed in the hypothalamic arcuate nucleus (ARC), where nitric oxide (NO) inhibits orexigenic neurons. Intracellular STAT signaling is triggered by inflammatory stimuli and has been linked to the transcriptional regulation of iNOS. We evaluated whether pharmacological blockade of iNOS by the specific inhibitor 1400W attenuates LPS-induced anorexia. Furthermore, we hypothesized that the tolerance to the anorectic effect occurring after repeated LPS treatment is paralleled by a blunted STAT3 phosphorylation in the ARC. Rats treated with a subcutaneous injection of 1400W (10 mg/kg) showed an attenuated anorectic LPS response relative to control rats receiving only LPS (100 µg/kg; i.p.). Similarly, iNOS blockade attenuated LPS-induced adipsia, hyperthermia, inactivity and the concomitant drop in energy expenditure. While single LPS treatment increased STAT3 phosphorylation in the ARC, rats treated repeatedly with LPS showed no anorectic response and also no STAT3 phosphorylation in the ARC after the second and third LPS injections, respectively. Hence, pSTAT3 signaling in the ARC might be part of the intracellular cascades translating pro-inflammatory stimuli into suppression of food intake. The current findings substantiate a role of iNOS dependent NO formation in disease-related anorexia.

1. Introduction

Disease-related anorexia is a phenomenon associated with a wide range of acute and chronic pathological conditions (Hart, 1988; Langhans, 2004; Plata-Salaman, 1996) characterized by an inflammatory state, such as bacterial and parasitic infection, cancer, acquired immune deficiency syndrome (AIDS), chronic obstructive pulmonary disease (COPD), chronic inflammatory bowel disease, chronic liver disease, chronic cardiovascular disease and rheumatoid arthritis. Pro-inflammatory cytokines are pivotal in the etiology of disease- and infection-related anorexia (Argiles and Lopez-Soriano, 1999; Kotler, 2000; Langhans, 2004, 2000; Matthys and Billiau, 1997; McNamara et al., 1992).

Treatment with the bacterial endotoxin lipopolysaccharide (LPS) is a commonly used model for acute bacterial infection and peripheral inflammation. LPS is a major constituent of the cell wall of gram-negative bacteria and represents a stimulatory molecule for the immune system (Rietschel et al., 1994, 1998) that initiates a generalized host defense reaction termed "acute phase response" (APR) (Baumann and Gauldie, 1994). The responses initiated during the APR involve leucocytosis, fever, hormonal and metabolic changes, anorexia and other behavioral responses (e.g. somnolescence and inactivity).

The brain represents a major target for pro-inflammatory stimuli that influence neuronal circuits controlling food intake and energy homeostasis. Although glial cells and neurons express pro-inflammatory cytokines within the brain itself (Gayle et al., 1997; Turrin et al., 2001; Vitkovic et al., 2000), peripherally produced cytokines can also reach central cytokine receptors via active or passive transport mechanisms through the blood–brain barrier or via circumventricular organs lacking this barrier (Banks, 2001; Banks and Kastin, 1996).

The pro-inflammatory neuromodulator nitric oxide (NO) seems to be part of the inflammatory signaling cascade triggered by LPS and might play an important role in transducing cytokine actions into a modulation of neuronal activity. NO is produced from the amino acid L-arginine by three different isoforms of nitric oxide synthase (NOS). Two constitutively expressed isoforms (endothelial NOS (eNOS) and neuronal NOS (nNOS) (Schmidt et al., 1993)) release NO in a calcium dependent manner in response to a cellular stimulation. In contrast, the inducible NOS (iNOS) is regulated at the transcriptional level and generates massive amounts of NO once it is expressed. In the brain iNOS is expressed in glial cells, endothelial cells and in neurons in response to LPS or pro-inflammatory cytokines (Moncada et al., 1991; Schmidt et al., 1993; Singh and Jiang, 2004). NO diffuses in a paracrine manner to activate the cytoplasmic soluble guanylate cyclase (sGC) that catalyzes the formation of the intracellular second messenger cyclic guanosine monophosphate (cGMP) (McDonald and Murad, 1996).

* Corresponding author. Institute of Veterinary Physiology, University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland. Tel.: +41 44 635 8815; fax: +41 44 635 8932.
E-mail address: triedig@vetphys.uzh.ch (T. Riediger).

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There is growing evidence that the induction of iNOS during inflammation and the subsequent release of NO in the hypothalamic arcuate (ARC) might be implicated in disease-related anorexia. The ARC is considered an important hypothalamic target for blood-borne hormonal and metabolic factors involved in the control of food intake and energy homeostasis.

The ARC shows a strikingly high induction of iNOS mRNA peaking 6 h after peripheral administration of LPS (Wong et al., 1996a). We recently demonstrated by electrophysiological and immunohistological in vitro studies that NO produced by an artificial NO-donor strongly inhibits ARC neurons that are activated by the orexigenic hormone ghrelin (Riediger et al., 2006). This effect was driven by an NO dependent formation of cGMP. Furthermore an anorectic dose of peripherally applied LPS attenuates the expression of c-Fos, a marker of neuronal activation, induced by food deprivation in the ARC (Becskei et al., 2008). These findings imply that orexigenic stimuli promoting energy intake via an activation of ARC neurons are antagonized by pro-inflammatory stimuli with NO being a possible mediator.

In the current study we tested whether specific pharmacological blockade of iNOS attenuates LPS-induced anorexia and associated disease symptoms in rats. We used the specific and long-acting iNOS inhibitor N-(3-(aminomethyl)benzyl)acetamidine (1400W) (Garvey et al., 1997). 1400W shows a high specificity for iNOS because it is at least 5000-fold and 200-fold selective for human iNOS versus eNOS and nNOS, respectively, and 1000-fold more potent against rat iNOS than eNOS (Garvey et al., 1997). Further, 1400W is a long-acting compound that exerts an irreversible or at least slowly reversible iNOS inhibition (Garvey et al., 1997). Finally, 1400W seems to act centrally after peripheral application. Using metabolic cages we tested the effect of peripherally applied 1400W on LPS-induced effects on ingestive behavior, metabolism, body temperature and physical activity.

It was a further aim to evaluate whether LPS-anorexia is associated with the phosphorylation of the transcription factor STAT3 (signal transducers and activators of transcription 3) in the ARC. STAT signaling has been linked to the transcriptional regulation of iNOS expression and it is one of the intracellular pathways mediating neuroinflammatory processes following immune challenges (Kleinert et al., 2004) (Perez-Rodriguez et al., 2009). We hypothesized that LPS-sensitive animals showing LPS-induced anorexia also develop a STAT3 response in the ARC. In contrast, rats that were made unresponsive to the feeding inhibitory effects of LPS by repeated LPS treatment should not exhibit a LPS-induced STAT3 phosphorylation.

2. Materials and methods

2.1. Animals and housing conditions

Male Wistar rats (Elevage Janvier, Le Genest-St. Isle, France) were individually housed in metabolic cages on a layer of wood shavings or in stainless steel cages with wire mesh floor equipped with tubes and nesting material. The rats were adapted to a 12 h/12 h light–dark cycle and had free access to standard rodent chow (GLP 3433; Provimi Kliba AG, Kaiseraugst, Switzerland) and water. The animals were adapted to their housing conditions and handled for at least 10 days before the onset of the experiments. All experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland.

2.2. Effect of iNOS inhibition on LPS anorexia and associated disease symptoms

LPS (*Escherichia coli* O111:B4, L2630-25MG, Sigma Aldrich Chemie GmbH, Buchs, Switzerland) and 1400W (100050-5, VWR International AG, Dietikon, Switzerland) were diluted in sterile saline (Fresenius Kabi AG, Stans, Switzerland). The rats were divided into four groups consisting of four animals each, i.e. a saline control group, a LPS-treated group, a 1400W-treated control group and a group that was injected with LPS and 1400W. LPS (100 µg/kg; i.p.), 1400W (10 mg/kg; s.c.) or saline was injected before dark onset. The dose of 1400W was based on the in vivo effective dose range between 5 mg/kg and 20 mg/kg (Parmentier et al., 1999).

Food and water intake, body temperature, physical activity and gas exchange (to calculate energy expenditure and respiratory quotient; RQ) were measured during 23 h after injections as described below. The study was performed using a crossover design between the saline-treated and the LPS-injected animals, and between the 1400W and LPS/1400W-treated animals with 12 days recovery time between the two trials. Hence, each rat received only one LPS treatment to avoid the development of LPS tolerance (Langhans et al., 1993).

Sixteen metabolic cages in an open-circuit indirect calorimetry system were used (AccuScan Instruments Inc., Columbus, OH, USA). The plexiglas cages had a size of 42 cm × 42 cm × 30 cm and were closed with air-tight lids. Water bottles and food cups were placed on scales to measure water and food intake continuously. For these experiments powdered chow (GLP 3433-9.24; Provimi Kliba AG) was used to avoid food hoarding and to be able to detect small changes in food consumption. The measurements were saved on the computer in 5 min intervals and were used to calculate cumulative food and water intake.

DSI PhysioTel® TA-F40 small animal transmitters (Data Sciences International, St. Paul, MN, USA) were implanted into the peritoneal cavity of each animal for telemetric body temperature and activity measurements. Receiver plates were placed underneath the cage. From these plates, the readout was saved every 5 min and evaluated on a connected computer with the manufacturer's software (Acquisition Version 4.00, Analysis Version 4.00; Dataquest A.R.T.™). The activity data represent an arbitrary unit reflecting locomotor activity.

To measure energy expenditure and RQ, ambient air was pumped through the cage via a manually adjustable flow controller (flow rate set to 2 l/min). Air entering and leaving each cage was monitored for its O_2 and CO_2 concentration. The cages were connected to two analyzers measuring O_2/CO_2 concentration of each cage for a period of 30 s in 5 min intervals. This allowed to calculate energy expenditure and RQ using the manufacturer's software (PhysioPlot Version 1.80, Integra ME Version 2.21; AccuScan Instruments Inc.). This calculation was based on the following equation: total energy expenditure (kcal/kg/h) = $(3.9 \times V(O_2) + 1.1 \times V(CO_2))/1000$; $V(O_2)$ and $V(CO_2)$ were normalized for body weight.

2.3. Association between pSTAT3 formation and LPS-induced anorexia

First, the time course of LPS-induced pSTAT3-formation in the ARC was investigated. Animals weighting approximately 300 g were injected with either LPS (100 µg/kg; i.p.) or saline at dark onset. Two hours later half of the LPS and saline-treated rats were anesthetized and perfused transcardially; the remaining animals were perfused 4 h after treatment. In total 26 animals were divided into 4 groups ($n = 5$–10/group). Brains were frozen, sliced and stained according the protocol described below. The stained slices were evaluated using a fluorescent microscope by manually counting the number of pSTAT3 positive cells in every second slice of the ARC. The evaluating person was blind to the treatment of the animals.

Repeated LPS injections result in LPS-tolerance, leading to an attenuation of LPS-induced anorexia (Langhans et al., 1993). Based on these studies we hypothesized that the attenuation of LPS-anorexia after repeated LPS treatment might be associated with a blunted LPS-induced pSTAT3 formation in the brain. To test this hypothesis, 20 rats were used weighting approximately 200 g at the beginning of the experiment. The animals were subdivided into a saline ($n = 13$) and a LPS ($n = 7$) treated group. On day one of the experiment the rats were injected at dark onset either with saline or LPS. Food intake was

measured 4, 6 and 12 h after the treatment. After one day of recovery (day 2), the same procedure was performed on day 3 to induce LPS-tolerance. On day 5, the saline-treated rats were subdivided into one saline group (control; $n=6$), and one group of rats receiving a single LPS injection (1× LPS; $n=7$). The rats that had received LPS on the previous days received a third LPS treatment (3× LPS; $n=7$). Four hours after the last injections all animals were anesthetized and perfused transcardially; the animal had access to food after the treatments. The timepoint of perfusion was chosen according to the outcome of the previous experiments described above, showing a significant increase in the number of pSTAT3 positive cells 4 h after LPS injection. Brains were frozen, sliced and processed for pSTAT3 immunoreactivity.

For the immunohistochemical detection of pSTAT3, rats were deeply anesthetized by an IP-injection of pentobarbital (100 mg/kg Nembutal; Abbott Laboratories, Chicago, IL, USA). After reaching surgical tolerance, the thorax was opened and the animals were transcardially perfused with 0.9% NaCl (room temperature, 1.5 min) followed by ice-cold paraformaldehyde solution (2% PFA in phosphate buffer solution 0.1 M). The brains were postfixed in 2% PFA for 1 h at 4 °C and transferred to 0.02 M KPBS containing 20% sucrose for the following 48 h at 4 °C. After this cryoprotection, the brains were snap frozen using CO_2 gas (exposure time approx. 1 min). Coronal sections (20 µm) were cut in a cryosectioning system (Leica CM3050 S, Leica Microsystems, Nussloch, Germany), thaw-mounted on microscopic glass slides (Super Frost Plus slides; Faust, Schaffhausen, Switzerland) and stored at −20 °C until further processing.

To detect pSTAT3, the sections were air-dried at room temperature (1 h) and rehydrated in 0.02 M KPBS. Afterwards the sections were treated with 0.3% NaOH + 0.3% H_2O_2 (20 min), followed by 0.3% glycine (10 min) and 0.03% sodium dodecyl sulfate (SDS, 10 min; all in 0.02 M KPBS). Unspecific binding was blocked by 20 min incubation in blocking solution (KPBS containing 4% normal donkey serum, 0.4% Triton X-100, 1% bovine serum albumin). The primary antibody (rabbit anti-pSTAT3 1:500; Cell signaling; in blocking solution) was applied for 48 h at 4 °C, followed by the secondary antibody (donkey anti-rabbit Alexa-555 1:100; Jackson Immunoresearch) for 2 h at room temperature. Finally, the sections were coverslipped with citifluor (Citifluor Ltd).

2.4. Statistical analyses

For statistical evaluation of experiments conducted in metabolic cages, differences between the LPS-treated groups relative to their respective control groups were calculated at each time point. These values were then compared using Student's *t*-test. Daily food intake (23 h) relative to controls was evaluated by paired Student's *t*-test. In the feeding study of LPS tolerance, the effects of LPS were compared to saline injection by Student's *t*-test at the different time points. When data were not normally distributed a non-parametric Mann–Whitney rank sum test was used. The immunohistological data were analyzed using one-way ANOVA followed by Newman–Keuls multiple comparison test. In the immunohistological studies the mean value of the cell count/section of an individual animal was used for statistical analyses (n = number of animals per group). All data are presented as mean ± SEM. $p<0.05$ was considered significant.

3. Results

3.1. Effect of iNOS inhibition on LPS anorexia and associated disease symptoms

LPS induced a pronounced suppression of eating from about 2 h after injection over the entire 23 h measuring period ($t=23$ h; saline: 26.5 ± 4.8 g, LPS: 13.9 ± 2.6, paired *t*-test, $p<0.001$). 1400W partly reversed the LPS-induced reduction of food intake compared to the LPS group (Fig. 1A). 1400W alone slightly reduced food intake relative to the saline control group. However, this difference was not significant at the end of the measurement period ($t=23$ h; saline: 26.5 ± 4.8, 1400W: 23.8 ± 1.6, paired *t*-test, $p=0.22$). For statistical evaluation the differences in the LPS-treated groups relative to their respective control groups were compared for each time point of measurement (LPS vs. saline and LPS/1400W vs. 1400W; Fig. 1C). The LPS-induced reduction in eating of 1400W-treated animals was significantly smaller than in saline-treated animals between 8 and 23 h after injection.

Similar to the effects on food intake, the LPS-induced reduction of water intake was attenuated by treatment with 1400W. The LPS-induced reduction of water intake in 1400W-treated animals was significantly smaller between 5 and 23 h (Fig. 1B and D).

The LPS-induced hyperthermia was also partly prevented by 1400W treatment (Fig. 2A). LPS induced a stronger hyperthermia in the saline-treated group than in the 1400W-treated group at various time points between 2 and 22 h after injections (Fig. 2C).

1400W control animals exhibited a similar locomotor activity as saline-treated animals. LPS reduced locomotor activity in the dark phase; this was partly counteracted by 1400W because 1400W/LPS-treated animals showed a higher activity than the LPS-treated control group (Fig. 2B). The LPS-induced reduction of activity in 1400W-treated rats was significantly smaller at various time points between 6 and 17 h after injections (Fig. 2D).

LPS produced a pronounced decrease in energy expenditure, particularly during the dark phase (Fig. 3A). The LPS-induced reduction of energy expenditure was significantly attenuated by 1400W at various time points between 8 and 12 h after injections (Fig. 3C).

LPS-treated animals had a lower respiratory quotient relative to both saline and 1400W-treated rats; this indicates a shift towards increased lipid oxidation (Fig. 3A). 1400W did not significantly alter the LPS-mediated reduction in RQ (Fig. 3D).

3.2. Association between pSTAT3 formation and LPS-induced anorexia

LPS induced a clear pSTAT3 response 4 h but not 2 h after injection (Fig. 4). Based on this experiment, a follow-up study with repeated LPS treatments was performed to investigate a potential association between pSTAT3 formation and LPS-induced anorexia. On day one, rats were given a single i.p. injection of LPS (100 µg/kg; $n=7$) or saline ($n=13$) at dark onset and food intake was measured manually 4, 6 and 12 h after injections. Food intake of LPS-treated animals was significantly reduced 6 and 12 h after the injections (Fig. 5). On day three of the experiment, i.e. after the second daily LPS treatment, LPS did not reduce eating at any time point, indicating LPS desensitization to this effect of LPS.

On day 5, the animals were treated for the third time with LPS or saline for the detection of pSTAT3 immunoreactivity. In contrast to the positive controls (1× LPS), animals that had received repeated LPS injections (3× LPS) showed no LPS-induced pSTAT3 formation in the ARC 4 h after the last injection compared to saline-treated animals (Fig. 6).

4. Discussion

4.1. Effect of iNOS inhibition on LPS anorexia and associated disease symptoms

One aim of the present study was to test the possible involvement of NO in disease-related anorexia. In line with our hypothesis, peripherally applied 1400W partly reversed the reduction of food intake in LPS-treated animals. Hence, we demonstrated for the first time that iNOS-mediated NO formation appears to be part of the

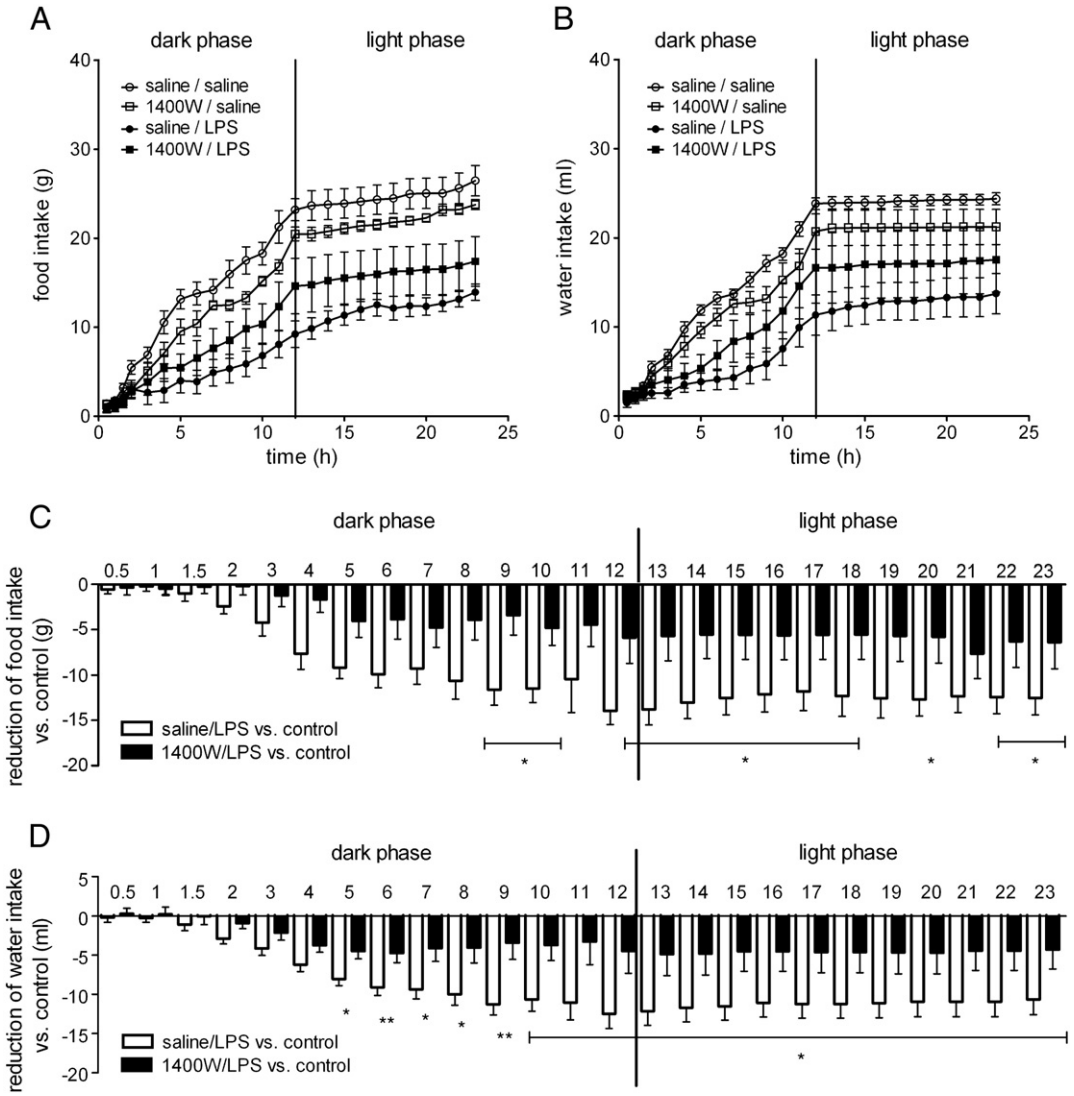

Fig. 1. Effect of the iNOS inhibitor 1400W on LPS-induced inhibition of ingestive behavior (A: food intake; B water intake). Comparisons of anorectic (C) and antidipsogenic (D) effect of LPS in 1400W-treated and saline-treated rats (*$p<0.05$, **$p<0.01$).

neuromechanism triggering disease-related anorexia, at least in the LPS inflammation model.

While there is evidence that NO might play a role in the control of food intake under normal, non-pathological conditions (Morley and Flood, 1992, 1991), studies investigating the relevance of NO in disease-related anorexia are scarce. In general support of our view that iNOS induction may be pathologically relevant in this context, iNOS expression is increased in the hypothalamic paraventricular nucleus and the lateral hypothalamic area of tumor bearing mice (Wang et al., 2005). These effects seemed to be specifically induced by tumor growth and not by anorexia per se, because healthy pair-fed animals receiving the same amount of food as the anorectic tumor bearing animals did not show this effect. Unfortunately, the ARC was not analyzed in these studies; however, a high iNOS expression has been described in another study in the ARC after LPS-induced inflammation (Wong et al., 1996b).

In the studies by Wang et al. (2005), unspecific NOS inhibitors were given via the drinking water to examine a possible role of NO formation in cancer anorexia. These inhibitors did not affect cancer anorexia. However, a specific role of iNOS signaling in disease-related anorexia should not be excluded based on these experiments. General blockade of NOS with unspecific antagonists is known to decrease food intake after peripheral and central administration in healthy animals (Morley and Flood, 1992, 1994, 1991; Squadrito et al., 1994, 1993). This well-known effect is unlikely to be mediated by iNOS that is hardly expressed under non-pathological conditions in the brain. The reduction of food intake seen after unspecific NOS blockade is thought to be mediated by an inhibition of neuronal NOS (nNOS) but the exact mechanisms, including the site of NO action, are not fully understood. Irrespective of the underlying mechanisms responsible for the decrease in food intake after unspecific NOS antagonisms at baseline, this effect may compensate or mask an increase in food intake resulting from concomitant iNOS inhibition when a similar approach is used under inflammatory experimental conditions.

In contrast to the suppression of feeding by non-selective NOS inhibitors (Morley and Flood, 1992, 1994, 1991; Squadrito et al., 1994, 1993), we observed only a weak and non-significant reduction in food intake after iNOS antagonism under baseline conditions. This in principle supports the idea that 1400W exerts a specific iNOS inhibition and that iNOS activity is low under unstimulated baseline conditions. These results further imply that 1400W treatment does not seem to induce strong side effects that might reduce food intake via aversive mechanisms, such as e.g. hemodynamic effects that occur after unspecific NOS inhibition (Torok, 2008).

Similar to the effect on food intake, the reduction of water intake after LPS treatment was also attenuated by 1400W. Irrespective of the

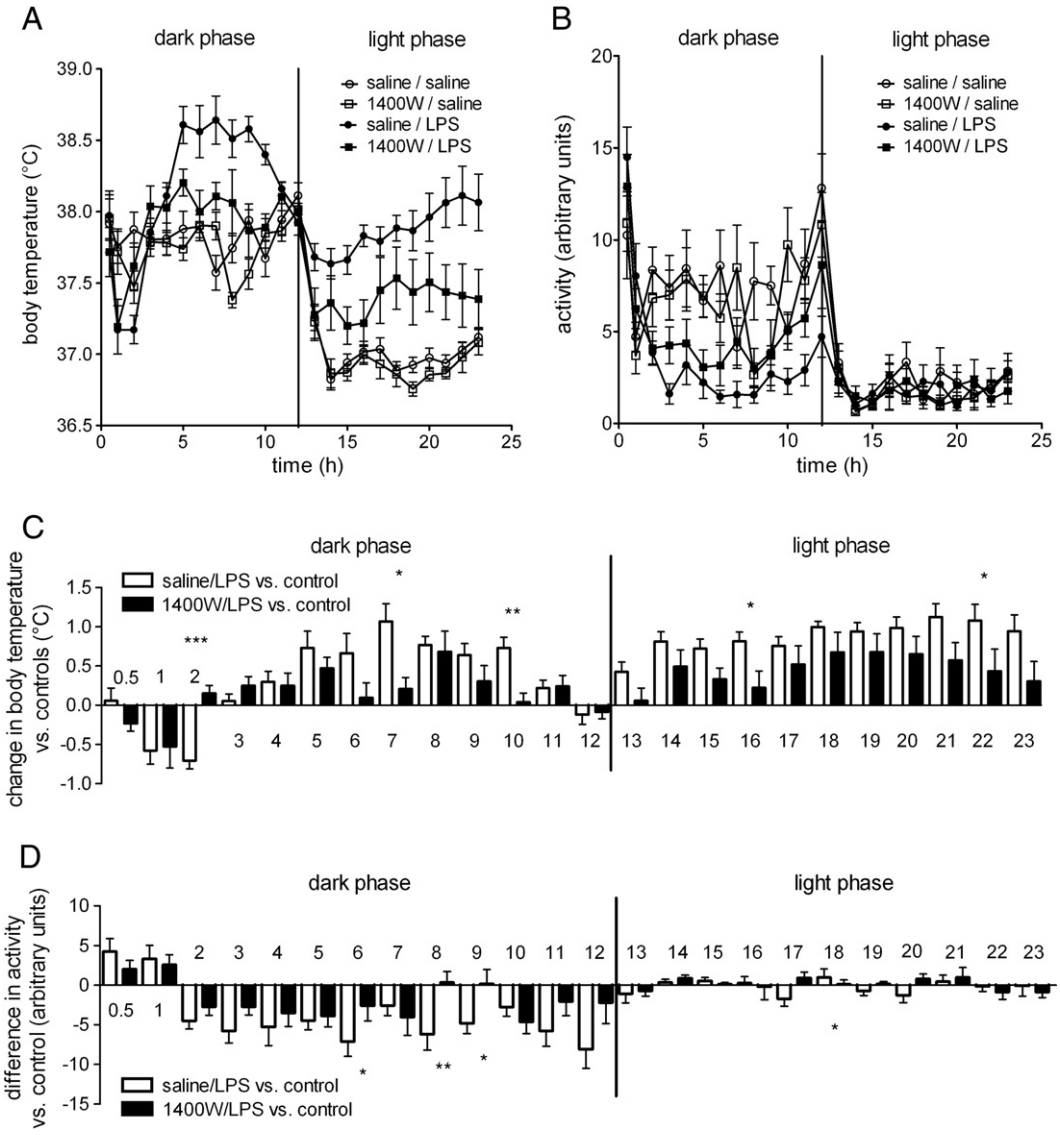

Fig. 2. Effect of the iNOS inhibitor 1400W on LPS-induced hyperthermia (A) and inhibition of physical activity (B). Comparisons of hyperthermic effect of LPS (C) and the LPS-mediated inhibition of physical activity in 1400W-treated and saline-treated rats (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

question whether this effect is directly mediated by the inhibition of NO formation or whether it is secondary to the attenuated anorexia, a reversal of adipsia is a beneficial effect contributing to the amelioration of disease-related symptoms. Adipsia and dehydration are common clinical problems during inflammation (Hart, 1988).

It was beyond the scope of our investigation to dissociate indirect effects on drinking behavior from a possible direct influence of NO on brain structures involved in the control of water balance. Nevertheless it should be noted that the subfornical organ (SFO), which belongs to the circumventricular organs, has been implicated in NO-dependent effects on drinking behavior. Injection of the artificial NO donor sodium nitroprusside (SNP) into the SFO exerts an antidipsogenic effect on angiotensin II-induced drinking (Nicolaidis and Fitzsimons, 1975). In line with this observation, SNP inhibits angiotensin II sensitive SFO neurons via cGMP formation (Rauch et al., 1997). Since LPS has been shown to induce a marked iNOS gene expression in the SFO (Wong et al., 1996b) it appears plausible that an iNOS-mediated NO formation in the SFO might contribute to LPS-induced adipsia. Hence, in addition to an indirect effect of iNOS antagonism on water via increased food intake, dipsogenic neurons might be directly affected.

1400W also partly reversed the LPS-induced hyperthermia. NO plays a crucial role in the induction of hyperthermia (Parrott et al., 1998; Roth et al., 1998). Our data are in line with studies showing that the NOS inhibitor L-NAME significantly attenuated LPS-induced hyperthermia (Soszynski, 2001). Furthermore Roth et al. (1999) demonstrated a dose-dependent antipyretic effect of the selective iNOS inhibitors aminoguanidine and S-methylisothiourea. Hence, since the attenuated hyperthermia response after 1400W was expected, this observation might be considered as an internal positive control confirming the effectiveness of 1400W to reverse centrally mediated iNOS actions under our experimental conditions.

Lethargy and reduced physical activity belong to the most commonly recognized behavioral patterns of sick animals (Hart, 1988). The LPS-induced reduction in locomotor activity was also partly reversed by 1400W treatment. Hence, the higher activity of LPS/1400W-injected rats might be interpreted as improved general health status and is in line with the increased food intake and attenuated hyperthermia after 1400W. The neuronal mechanisms involved in LPS-induced lethargy and inactivity are poorly understood. Interestingly, in our previous studies (Becskei et al., 2008), peripherally applied LPS reduced c-Fos expression in orexin positive neurons of the lateral hypothalamic area

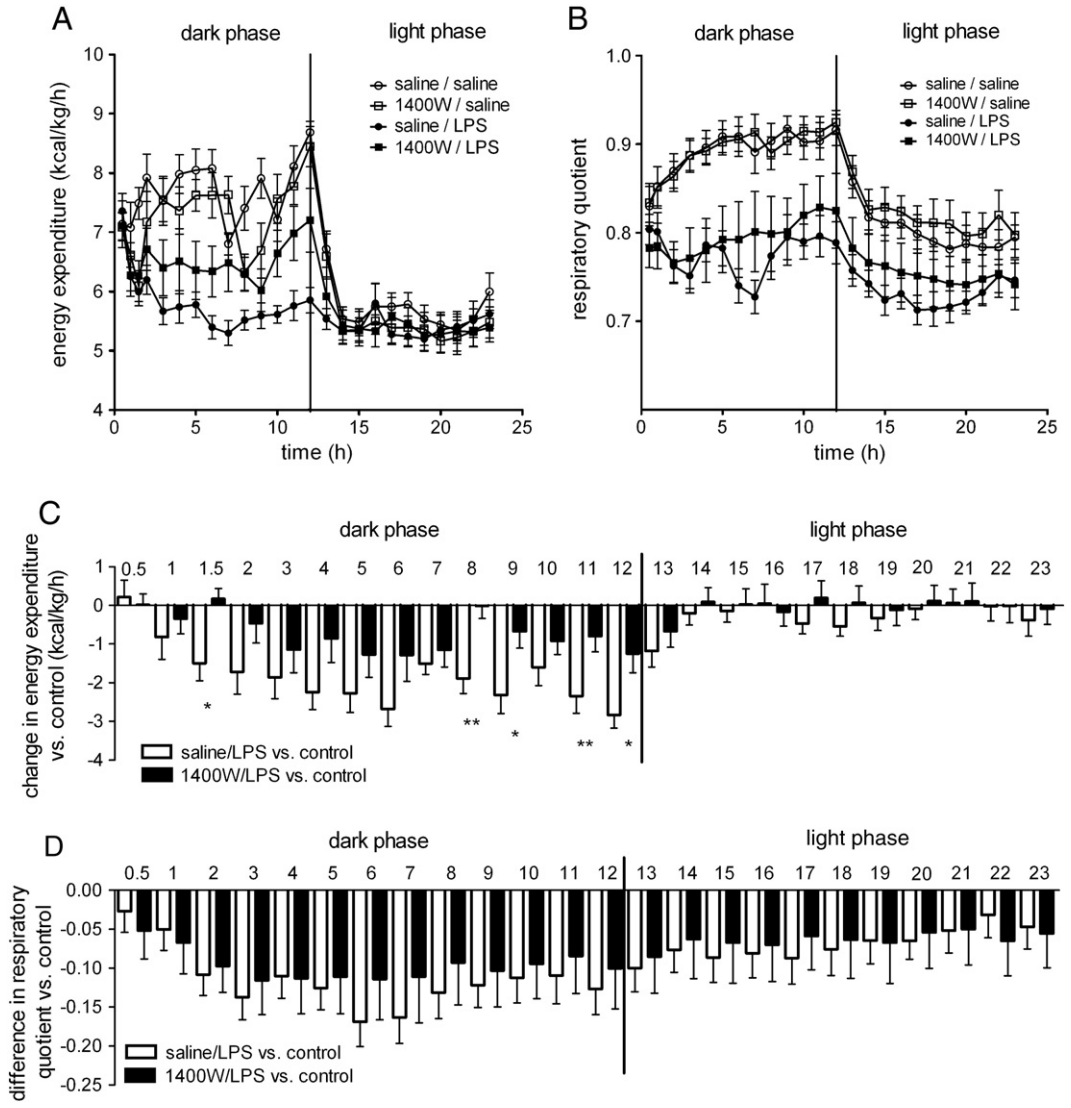

Fig. 3. Effect of the iNOS inhibitor 1400W on LPS-induced reduction of energy expenditure (A) and respiratory quotient (B). Comparisons of the inhibitory effects of LPS on energy expenditure (C) and the LPS-mediated reduction of the respiratory quotient in 1400W-treated and saline-treated rats ($^{*}p<0.05$, $^{**}p<0.01$).

(LHA). Orexins are implicated in the control of sleep and arousal (Chemelli et al., 1999). nNOS expressing neurons are co-distributed with orexin neurons (Cutler et al., 2001). Furthermore, iNOS expression is upregulated in the LHA of tumor bearing rats (Wang et al., 2005). Hence, it is in principle plausible that a NO-dependent reduction of neuronal activity in orexinergic LHA neurons might be involved in both LPS-induced anorexia and inhibition of locomotor activity. To our knowledge this has not yet been investigated.

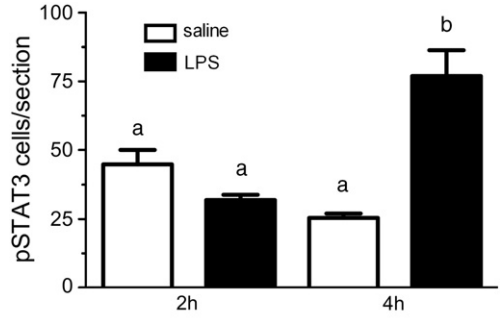

Fig. 4. Effect of LPS treatment on STAT3 phosphorylation in the hypothalamic arcuate nucleus 2 and 4 h after injection. Different letters indicate significant differences between the experimental groups ($p<0.05$).

LPS treatment reduced energy expenditure, and 1400W partly reversed this effect. We assume that the LPS-mediated reduction in energy expenditure is at least in part a consequence of reduced physical activity and food intake. To our knowledge, there are no published studies directly investigating the involvement of NO in energy expenditure. A specific effect on energy expenditure could for example be investigated in pair-feeding experiments. Such experiments were beyond the scope of our present study. It is important to note that it may be difficult to control for all confounding factors because pair feeding of healthy controls to LPS-treated animals is likely to increase physical activity in the control animals due to increased food seeking behavior.

Besides energy expenditure, we also determined the effect of LPS on the RQ. Possibly due to the strong reduction of food intake, the RQ of LPS-treated animals was between 0.7 and 0.8, whereas the RQ of the control animals was about 0.9. This indicates a shift towards lipid oxidation in LPS-treated rats. Despite a partial reversal of LPS-induced anorexia, 1400W had no obvious influence on the LPS-induced reduction of the RQ. A possible explanation is that the partial reversal of LPS anorexia did not compensate sufficiently for

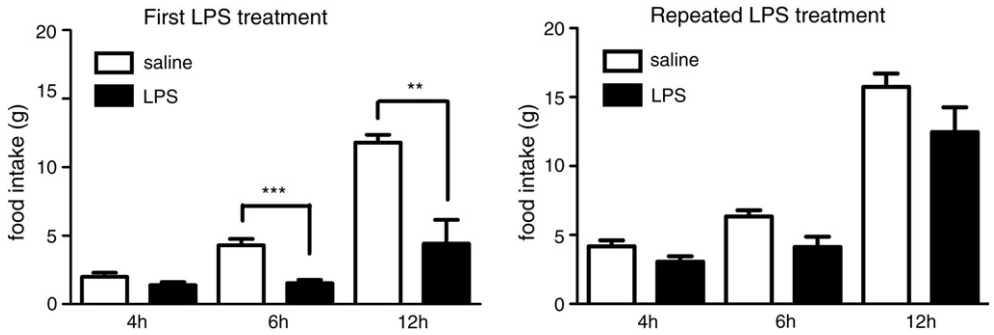

Fig. 5. Induction of LPS tolerance by repeated LPS treatment. Left: anorectic effect of LPS after the first treatment. Right: absence of anorectic LPS action after the second treatment (**$p<0.01$; ***$p<0.001$).

the negative energy balance to shift back from fat towards carbohydrate oxidation.

4.2. Association between pSTAT3 formation and LPS-induced anorexia

Previous studies had demonstrated that LPS triggers a time-dependent nuclear STAT3 translocation in the ARC and other brain structures of guinea pigs (Rummel et al., 2005) and rats (Gautron et al., 2002; Rummel et al., 2005). However, to our knowledge there are no immunohistochemical studies quantifying the LPS-induced pSTAT3 response in the ARC of rats after different exposure times. LPS-treated rats showed a markedly increased number of pSTAT3 positive cells in the ARC 4 h but not 2 h after treatment relative to saline-injected controls. Our data are therefore consistent with previous findings demonstrating that peripheral LPS administration induced nuclear translocation of STAT3 in the hypothalamus peaking between 2 and 4 h after injection (Gautron et al., 2002).

pSTAT3 formation in the brain is stimulated by different cytokines which are the putative endogenous mediators of LPS-induced STAT signaling (Imada and Leonard, 2000). IL-1β (Samavati et al., 2009) and IL-6 (Zhong et al., 1994) are potent stimulators of the STAT3 pathway after peripheral administration (Harre et al., 2002). It is therefore well accepted that these and probably other cytokines may contribute to LPS-induced STAT3 activation in the brain.

We observed pSTAT3 positive cells in the ARC under basal conditions. Leptin is known to induce STAT3 phosphorylation in ARC neurons (Hakansson and Meister, 1998), but it remains to be determined whether the basal pSTAT3 formation in general is driven by leptin. We did not identify the phenotype of LPS responsive cells. There is evidence that the STAT3 response induced by LPS occurs in endothelial cells which are positive for von Willebrand factor and in astrocytes which are positive for glial fibrillary acidic protein (GFAP) (Rummel et al., 2005) in the ARC of guinea pigs. Whether these cell types are the only target cells and which proportion of these subsets of cells shows a LPS-induced pSTAT response has not yet been determined in

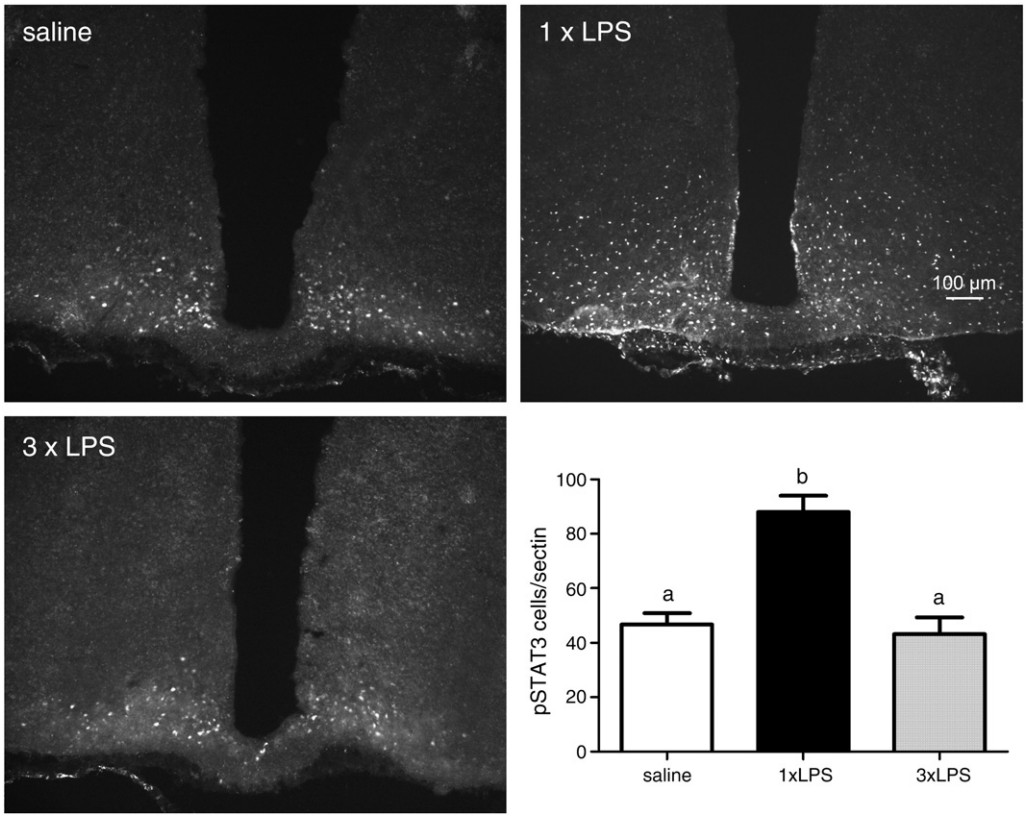

Fig. 6. Effect of LPS treatment on STAT3 phosphorylation in LPS sensitive rats (1× LPS) vs. LPS tolerant rats that received repeated LPS injection (3× LPS). Different letters indicate significant differences between the experimental groups ($p<0.05$).

detail. In addition to endothelial cells and possibly astrocytes, microglia cells might also respond to inflammatory stimuli by a pSTAT3 response (Arimoto and Bing, 2003; Beutler, 2000).

We further analyzed pSTAT3 formation in the ARC after single or repeated injections of LPS. We demonstrated a higher number of pSTAT3 positive cells after a single LPS treatment compared to controls, but the number of pSTAT3 positive cells was similar to control conditions after repeated LPS injection. Similar to the blunted pSTAT3 formation in the brain, there was no hypophagic effect of LPS after repeated injections because LPS did no longer reduce food intake compared to control animals after two LPS injections given over three days. This was expected based on previous studies under similar experimental conditions (Langhans et al., 1991, 1993). Our study indicates that the LPS-induced pSTAT3 formation in the ARC might contribute to the transcriptional processes that may ultimately mediate the anorectic effect to LPS. It has to be noted, however, that the blunted pSTAT3 response after repeated LPS treatment is most likely a more generalized effect also occurring in other brain areas and affecting other disease-related symptoms. While there is evidence that the ARC is involved in anorexia caused by inflammatory stimuli (Reyes and Sawchenko, 2002) the importance of STAT signaling in this context has not yet been assessed. Intracerebroventricular (icv) injection of the JAK inhibitor AG-490 has been shown to block the anorectic action of icv administered leptin (Morrison et al., 2007), which acts via ARC neurons to reduce feeding. Hence, this compound could also be useful to investigate whether pSTAT3 formation is necessary for LPS anorexia.

In this context it is important to consider that pSTAT3 (transcriptional level) and iNOS (functional level) do not represent the only inflammatory mediators of LPS anorexia. There are well-established roles of NF-κB/cyclooxigenase/PGE_2 and other determinants in LPS anorexia (Langhans, 2007). Our immunohistological data indicate that pSTAT3 is not induced within the first 2 h after LPS injection. The reported onset of LPS anorexia varies between different studies but it usually occurs between 2 and 6 h when a dose of 100 μg/kg is injected intraperitoneally (Langhans et al., 1991, 1993). When correlating the time course of the induction of transcription factors with behavioral parameters such as a feeding effects, a certain delay in the onset of the behavioral response seems plausible. We therefore believe that the time course of pSTAT3 formation in the ARC in principle matches the time course of LPS anorexia.

In summary our data indicate that NO seems to be involved in LPS-induced symptoms such as disease-related anorexia and some other disease-related symptoms (e.g. adipsia, inactivity and hyperthermia). The ARC has been suggested as a possible central site where NO might act to reduce food intake. LPS anorexia is associated with pSTAT3 formation in the ARC, which could represent one of the contributing pro-inflammatory intracellular pathways leading to iNOS expression and the release of the paracrine neuromodulator NO, which inhibits orexigenic ARC neurons. Further studies should address whether iNOS inhibition might be a therapeutic approach to treat illness anorexia under chronic conditions.

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