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ORIGINAL INVESTIGATION

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Endotoxin-induced reduction of social investigation by mice: interaction with amphetamine and anti-inflammatory drugs

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Abstract Previous studies indicate that some aspects of endotoxin-induced sickness behavior in rats may be mediated by interleukin-1 stimulated events and can be attenuated by corticosteroids, cyclooxygenase inhibitors and the interleukin-1-receptor antagonist. In the current studies, we replicate and extend these findings in adult male mice. A relatively low dose of lipopolysaccharide (LPS; 15 μg/kg, IP) was used to reliably induce a 50–60% reduction in the social investigation of a juvenile conspecific at 2–3 h after injection. Amphetamine (2.0–4.0 mg/kg, IP, 30 min pre-LPS) exacerbated LPS-induced decreases in investigation. Administration of methylprednisolone (10–30 mg/kg, IP), indomethacin (3–30 mg/kg, IP), and ibuprofen (1–100 mg/kg, IP) 1 h before LPS significantly reduced LPS-induced sickness behavior at several doses. Dexamethasone (0.1–10 mg/kg, IP) partially antagonized sickness. Representative flavonoids rohitukine (0.01–100.0 mg/kg, IP) and chrysin (0.01–10 mg/kg, IP) also antagonized LPS-induced deficits in social investigation. These studies replicate and extend previous studies in rat to demonstrate systematic effects of low doses of LPS, antagonism by anti-inflammatory drugs and enhancement of LPS effects by amphetamine. The latter findings are consistent with a modulatory role for adrenergic activation on interleukin-1 release stimulated by endotoxicity.

Key words Sickness behavior · Cyclooxygenase inhibitor · Corticosteroid · Rohitukine · Flavone

Introduction

Systemic administration of the cytokines interleukin-1β, interleukin-1α, and TNFα to rats initiates a complex pattern of physiological and behavioral changes manifested as hyperthermia, motor depression, piloerection, decreased feeding, and reduced social investigation (Dinarello 1988; Rothwell 1989; Bluthe et al. 1991, 1992b). Elements of this complex can be obtained after central or peripheral administration of cytokines suggesting that some aspects of the syndrome may be mediated by brain cytokine receptors. Indeed, cytokine-induced deficits in social investigation can be selectively reduced by central administration of the IL-1 receptor antagonist (IL1ra; Kent et al. 1992). In addition to the IL-1 receptor antagonist, drugs which interfere with other aspects of the inflammatory response such as corticosteroids and cyclooxygenase inhibitors also reduce cytokine-induced sickness in rats (Crestani et al. 1991; Plata Salaman 1991).

Lipopolysaccharide (LPS) is an endotoxic protein fragment from the cell wall of gram-negative bacteria (Kozak et al. 1994). In vitro, LPS stimulates cytokine release from macrophages (Perry et al. 1993) and cultured telencephalic cells (Romero et al. 1993). In vivo, both systemic and central administration of LPS increase levels of IL-1 or measures of IL-1 induced activity in several areas of the brain including the hippocampus, hypothalamus and diencephalic structures depending on dose and frequency of administration (Takao et al. 1993; Quan et al. 1994). Systemic administration of LPS is also associated with a behavioral syndrome comparable to cytokine-induced sickness in the rat (Bluthe et al. 1992b). Furthermore, pre-administration of the IL-1 receptor antagonist effectively attenuates LPS-induced sickness (Bluthe et al. 1992b), although only when both are administered peripherally. Taken together, these findings suggest that IL-1 and LPS may affect social investigation through a common mechanism.

Mice exhibit a robust repertoire of social investigatory behavior when presented with a conspecific (Winslow and Camacho 1995). Olfactory investigation is a species-typical, very reliable and highly motivated behavioral effort expressed by many species including rodents (Gheusi et al. 1994) and primates (Hennessy and et al. 1978; Boinski and Mitchell 1994). Consequently, social

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investigation may represent a useful behavioral background for interspecies comparisons of pharmacological manipulations. Previous studies of LPS and IL-1 administration in rats and mice have been limited to doses which produce near complete elimination of social behavior. Consequently, it has been difficult to assess the possible synergistic activities of drugs or non-linear dose effects of potential antagonists on reduced social interest. One objective of the current study was to assess the dose-effects of LPS on mouse social investigation and to identify dose and time points at which an approximately 50–60% reduction in investigation is reliably obtained. We were particularly interested in potential biphasic dose interactions between LPS and anti-inflammatory compounds. Ibuprofen, for example, has been reported to reduce or enhance neutrophil accumulation depending on dose and tissue substrate (Hellewell et al. 1995). Consequently, the comparative efficacy of several anti-inflammatory compounds against LPS-induced reduction in social investigation was examined across a broad dose range.

Several recent reports demonstrate that interleukins (particularly IL-1 and IL-6) stimulate significant central and peripheral noradrenergic neuronal activity (Dunn 1988; Ruhl et al. 1994) and that noradrenergic neurons in the ventral noradrenergic ascending bundle may be necessary for the expression of IL1β-induced ACTH release (Barbanel et al. 1993). Based on these findings, we postulated that amphetamine may modulate LPS-induced deficits in social behavior by increasing synaptic noradrenaline.

The following studies thus replicate and extend previous studies of the dose and time-course of LPS-induced sickness in mice. We also replicate interactions between a low dose of LPS, methylprednisolone, dexamethasone, and indomethacin and extend this examination of anti-inflammatory drugs to ibuprofen and a novel class of putative anti-inflammatory compounds (Middleton and Kandaswami 1992), the flavonoids. Finally, we examine the potential synergistic effects of amphetamine on LPS-induced deficits in social investigation.

Materials and methods

Subjects and housing

Adult male mice (CD-1, Charles River; 20–30 g) were housed under standard laboratory conditions as outlined in the "NIH guide for the Care and Use of Laboratory Animals" (revised 1985) in groups of 20 for 4–7 days after arriving in the vivarium. Food and water were freely available. Juvenile male mice (CD-1, Charles River, 21 days old) were used as social stimuli and were housed in groups of eight in clear plastic boxes (25 cm L×20 cm W×15 cm H) with sawdust bedding, wire tops and freely available food and water.

General procedure

Twenty-four hours prior to testing, adult mice were individually housed in clear plastic boxes with sawdust bedding and wire tops. Individual housing was provided to permit resident male mice an opportunity to scent mark and habituate to their home cage. Individual housing was limited to 24 h to reduce the likelihood of aggressive behavior associated with lengthier isolation (Valzelli 1969). Food and water remained freely available. On the day of testing, adult (n=32–40 per study) and juvenile mice (n=16–20 per study) were transported and allowed to acclimate to a test room illuminated by a red light (60 W) for at least 1 h prior to testing.

To establish each adult mouse's baseline social behavior, a pre-injection trial began each study when a juvenile mouse was placed into each adult's home-cage for a 2-min observation period. Mice express a complex repertoire of social behavior directed at intruders into their home cage. This is typically initiated as a vigorous bout of olfactory investigation and followed by predictable shifts to other behavioral strategies depending on the age, sex, and reproductive status of the intruder (Miczek and Winslow 1987; Winslow and Camacho 1995). In the current study, trained observers (>90% inter- and intra-observer reliability) measured the amount of time the resident mouse engaged in social contact with the juvenile. This time was primarily accounted for by ano-genital or body sniffing but also included bouts of allogrooming and close pursuit. Aggressive behaviors such as biting and mounting were rarely observed. At the end of the pre-injection trial, the juvenile mouse was removed and returned to its group home cage. Resident mice were then injected and at varying intervals after injection retested in an encounter with a second juvenile mouse. The injection-to-test interval depended on the treatment conditions detailed in the following sections.

Dose-effect and time-course of LPS

Based on previous reports in rats, initial studies examined the effects of several doses of LPS (1.25–100 μg/kg, n=8 per dose) administered IP 3 h prior to the second observation trial. A dose producing an approximately 50% reduction of social investigation was then selected to characterize time-course. For this study, groups of mice were treated with LPS (15 μg/kg) at varying time-intervals (30–300 min, n=8 per time point) prior to the second observation trial.

Antagonism of LPS-induced deficits in social interest

All compounds in these studies were compared for efficacy after a 1-h pretreatment against LPS-induced deficits. The various antagonists were administered IP to resident mice immediately following the baseline pre-injection trial. One hour later, LPS was also administered. Two hours after the LPS injection, juveniles were introduced to treated adult mice and social investigation quantified in a second observation trial. Consequently, the total injection-to-test interval was 3 h. The following drugs were examined: corticosteroids included methylprednisolone and dexamethasone, cyclooxygenase inhibitors included indomethacin and ibuprofen, and flavonoids including chrysin and rohitukine. In addition, the effects of *d*-amphetamine alone and as pre-treatment 30 min prior to LPS were examined.

Drug preparation

All drugs were administered in a volume of 1 ml/100 g body weight. LPS (1.25–100 μg/kg; Sigma) was sonicated and dissolved in distilled water. Dexamethasone (0.01–10.0 mg/kg), methylprednisolone (3.0–30.0 mg/kg), indomethacin (1.0–30.0 mg/kg), and *d*-amphetamine (2.0–4.0 mg/kg) were dissolved in distilled water. Ibuprofen (1.0–100.0 mg/kg) and chrysin (0.01–10 mg/kg) were dissolved in a solution of 50% distilled water, 40% ethyl alcohol and 10% propylene glycol. Rohitukine (0.01–100.0 mg/kg) was dissolved in 10 ml distilled water with 1 drop of 100% dimethyl sulfoxide (DMSO).

Statistical analysis

Treatment effects on mean duration of social investigation were analyzed with two-factor analysis of variance with repeated measures for pre- versus post-injection trial performance. Post-hoc Newman-Keuls test comparisons (q) were performed where indicated by significant interactions between treatment and repeated trials (Winer 1971).

Results

LPS dose-effect and time course

LPS (1.25–50 μg/kg, IP) produced significant dose-dependent changes in social investigation at 3 h after IP administration [$F(5,63)=18.71$, $P<0.05$; Fig. 1]. A 39% reduction in social investigation was detected at 5 μg/kg ($q=5.83$, $P<0.05$), 90% at 50 μg/kg ($q=14.11$, $P<0.05$) and complete suppression of behavior at 100 μg/kg (data not shown).

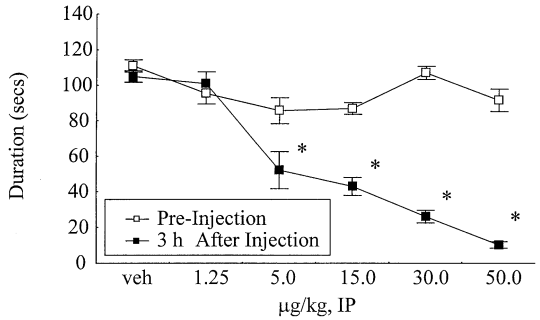

Fig. 1 LPS dose-effect. Doses of LPS were injected IP immediately after baseline and 180 min prior to efficacy testing. LPS (*closed symbols*) produced a dose-related decrease in mean duration of social investigation compared to pre-injection control levels (*open symbols*). Error bars depict ±1 SEM. *Asterisks* represent significant differences detected by Newman-Keuls test between LPS effect and corresponding pre-injection control, **P*<0.05

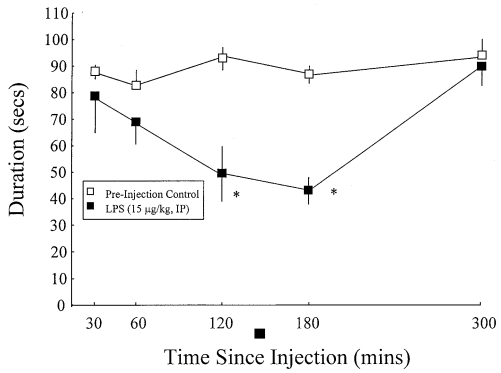

Fig. 2 LPS time-course. LPS 15 μg/kg (*closed symbols*) produced a significant reduction in mean duration of social investigation compared to pre-injection control levels (*open symbols*) measured at 120–180 min. Full recovery was detected at 300 min after injection. Error bars depict ±1 SEM. *Asterisks* represent significant differences detected by Newman-Keuls test between LPS effect and corresponding pre-injection control, **P*<0.05

A study of LPS time-course revealed that the reduction of social investigation measured after administration of 15 μg/kg [$F(4,56)=5.81$, $P<0.05$; Fig. 2] did not become statistically significant until 120 min after injection ($q=6.41$, $P<0.05$). Social investigation remained reduced at 180 min ($q=6.48$, $P<0.05$) and recovered to baseline levels by 300 min after administration.

Amphetamine exacerbates LPS

Amphetamine administered 30 min prior to LPS significantly changed the effects of LPS measured at 90 min after LPS administration [$F(3,28)=9.90$, $P<0.05$; Fig. 3]. Post-hoc analysis revealed significant exacerbation of LPS-induced deficits at 2.0 ($q=5.70$, $P<0.05$) and 4.0 mg/kg ($q=6.00$, $P<0.05$). Administration of amphetamine alone at 4.0 mg/kg did not affect social investigation in this paradigm.

Cyclooxygenase inhibitors antagonize LPS

Indomethacin significantly affected LPS-induced deficits [$F(5,66)=6.48$, $P<0.05$ Fig. 4A] measured as a reversal at 3.0 ($q=4.07$, $P<0.05$), 10.0 ($q=4.94$, $P<0.05$) and 30 mg/kg ($q=2.52$, $P<0.05$). A dose of 10.0 mg/kg indomethacin alone had no significant effect on social investigation at 3 h after injection compared to pre-treatment control.

Administration of ibuprofen 60 min prior to LPS also significantly affected LPS-induced reductions in social contact [$F(5,47)=10.61$, $P<0.05$; Fig. 4B]. Post-hoc anal-

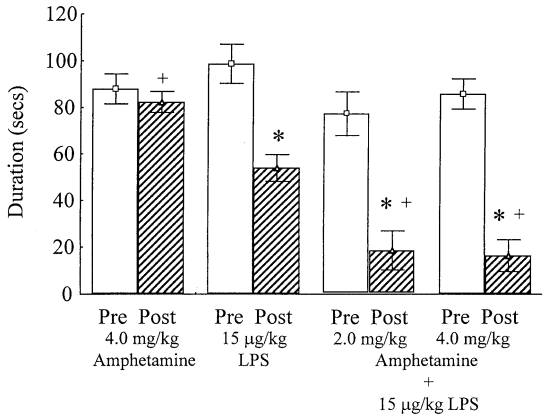

Fig. 3 Amphetamine enhances LPS-induced deficits. Doses of amphetamine were injected 30 min before LPS administration. Mice were re-tested 2 h after 15 μg/kg LPS injection. Amphetamine (4.0 mg/kg) had no measurable effect on social investigation when administered alone but significantly exacerbated LPS-induced reductions at both doses tested. Error bars depict ±1 SEM. *Asterisks* represent significant differences detected by Newman-Keuls test between treatment effect and corresponding pre-injection control, **P*<0.05. *Plus signs* represent significant differences detected between LPS alone and LPS combined with amphetamine

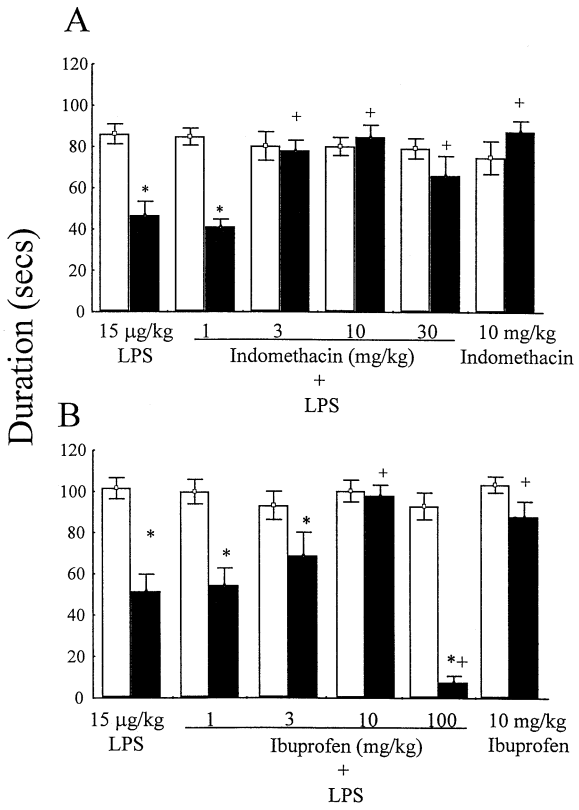

Fig. 4A, B Cyclooxygenase inhibitors antagonize LPS-induced sickness. Doses of ibuprofen and indomethacin were injected 1 h before LPS administration. Mice were re-tested 2 h after 15 µg/kg LPS injection. At comparable doses both inhibitors produced full recovery of social investigation compared to LPS and pre-treatment levels. Error bars depict ±1 SEM. *Asterisks* represent significant differences detected by Newman-Keuls test between treatment effect and corresponding pre-injection control, **P*<0.05. *Plus signs* represent significant differences detected between LPS alone and LPS combined with cyclooxygenase inhibitor

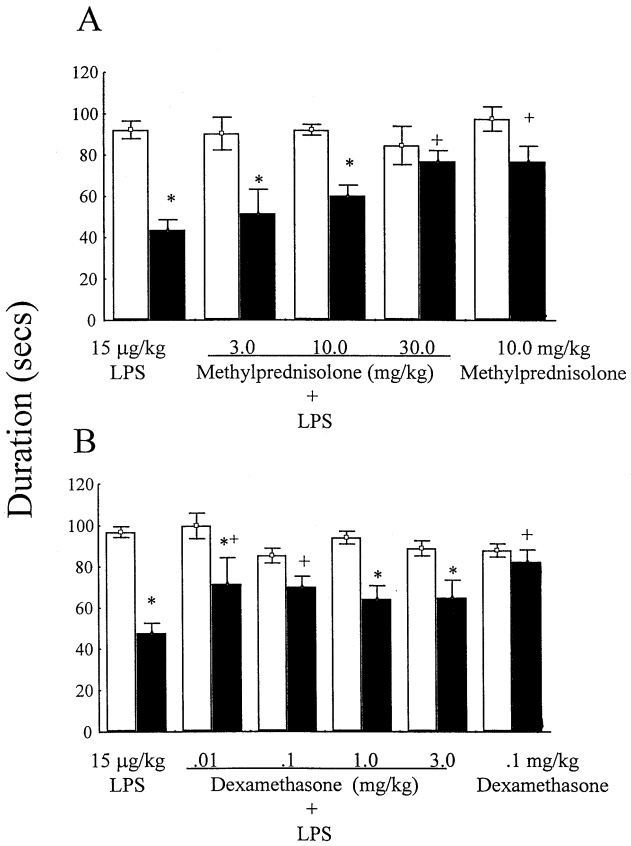

Fig. 5A, B Adrenocortical steroids antagonize LPS-induced sickness. Doses of methyprednisolone and dexamethasone were injected 1 h before LPS administration. Mice were re-tested 2 h after 15 mg/kg LPS injection. Both adrenocortical steroids produced significant recovery of social investigation compared to LPS and pre-treatment levels. Error bars depict ±1 SEM. *Asterisks* represent significant differences detected by Newman-Keuls test between treatment and corresponding pre-injection control, **P*<0.05. *Plus signs* represent significant differences detected between LPS alone and LPS combined with adrenocortical steroid

ysis revealed that ibuprofen prevented LPS-induced behavioral deficits at 10 mg/kg ; q=6.57, P<0.05) and enhanced LPS effects at a high dose (100 mg/kg; q=5.19, P<0.05). A dose of 10 mg/kg ibuprofen alone had no significant effects on social investigation at 3 h after injection.

Adrenocortical steroids

Administration of methylprednisolone 60 min prior to LPS significantly affected LPS-induced reductions in social contact [F(5,102)=2.97, P<0.05; Fig. 5A]. Post-hoc analysis revealed that methylprednisolone completely blocked LPS-induced behavioral deficits at 30 mg/kg (q=4.13, P<0.05). A dose of 10 mg/kg methylprednisolone alone had no significant effects on social investigation at 3 h after injection.

Administration of dexamethasone 60 min prior to LPS also significantly affected LPS-induced reductions in social contact [F(5,102)=5.19, P<0.05; Fig. 5B]. Post-hoc analysis revealed that dexamethasone reduced LPS-induced behavioral deficits at 0.01 (q=3.31, P<0.05) and 0.1 mg/kg (q=3.10, P<0.05). A dose of 0.1 mg/kg dexamethasone alone had no significant effects on social investigation at 3 h after injection.

Flavonoid compounds

Administration of chrysin produced a dose-dependent change in LPS-induced deficits [F(6,65)=7.53, P<0.05; Fig. 6A]. Post-hoc analysis revealed that 1.0 (q=3.58, P<0.05) and 10.0 mg/kg (q=5.55, P<0.05) significantly blocked LPS effects, but had no effect on social investigation when administered alone.

LPS-induced deficits were significantly affected by administration of rohitukine [F(7,88)=5.12, P<0.05; Fig. 6B]. Post-hoc analysis revealed that rohitukine reversed LPS effects at 0.25 mg/kg (q=3.89, P<0.05). Rohitukine had no

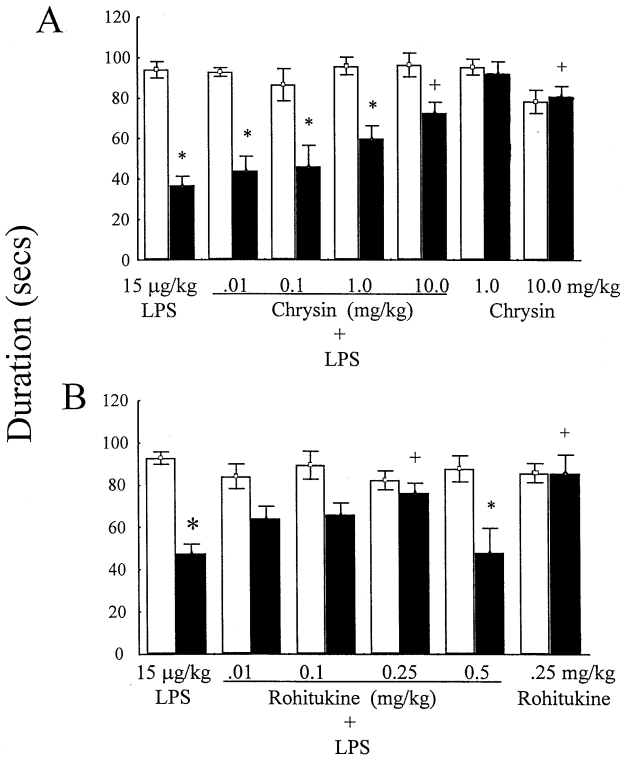

Fig. 6A, B Flavonoid drugs antagonize LPS-induced sickness. Doses of chrysin and rohitukine were injected 1 h before LPS administration. Mice were re-tested 2 h after 15 mg/kg LPS injection. Both flavonoids produced significant recovery of social investigation compared to LPS and pre-treatment levels. Error bars depict ±1 SEM. *Asterisks* represent significant differences detected by Newman-Keuls test between treatment effect and corresponding pre-injection control, **P*<0.05. *Plus signs* represent significant differences detected between LPS alone and LPS combined with adrenocortical steroid

measurable effect on social investigation when administered alone at 0.25 mg/kg.

Discussion

Adult male mice vigorously investigate novel conspecifics introduced into their home cage. Olfactory investigation can occupy 50–80% of a mouse's behavioral activity during brief (2–5 min) encounters depending on the age, sex, and prior experience with the conspecific (Winslow and Camacho 1995). Intraperitoneal injections of endotoxin produced a dose-dependent reduction in social investigation of a juvenile by an adult male mouse with reductions evident after as little as 5 µg/kg. A 15 µg dose was associated with a reliable 50–60% reduction of behavior which emerged within 120 min after injection. Complete recovery of this behavior was detected by 5 h after LPS administration. These findings are consistent with previous reports of LPS-induced deficits in rats (Bluthe et al. 1994), pigs (Johnson and von Borell 1994), birds (Johnson et al. 1993a), and mice (Bret-Dibat et al. 1994) and provide systematic characterization of dose and time-course in mouse. LPS administration increases IL-1 mRNA and immunoreactivity in brain, endocrine, and immune tissues (Koenig et al. 1990; Ban et al. 1992). There is a substantial body of evidence that many of the behavioral and physiological consequences of endotoxicity are orchestrated by IL-1 (e.g. Quan et al. 1994). IL-1 administered centrally and peripherally induces sickness responses in rats similar to LPS (Kent et al. 1992; Bluthe et al. 1995), and mice (Bluthe et al. 1991), and LPS-induced sickness behavior can be reduced by IL-1ra (Bluthe et al. 1992b).

Pre-treatment with doses of amphetamine that had no effect when administered alone enhanced LPS effects on social investigation. Enhancement may be related to a specific interaction between noradrenergic and cytokine systems. Interleukin-1 release has been frequently and reliably associated with increased NE release (Shintani et al. 1993), turnover (Terao et al. 1993; Shintani et al. 1995) and depletion (Fleshner et al. 1995). Consistent with the current findings, incubation of astrocytes with NE enhanced the abilities of both IL-1β and TNF-α to induce IL-6 secretion in vitro (Maimone et al. 1993), and depletion of rat hypothalamic and brain NE with 6-OHDA reduced IL-1α associated elevations of plasma corticosterone (Chuluyan et al. 1992) and blocked IL-1 induced analgesia (Bianchi and Panerai 1995). The amphetamine effects described here suggest a synergistic interaction between noradrenaline and cytokines on the disruption of social investigation by LPS. The results also demonstrate the importance of studying sub-maximal effects of LPS on social investigation to reveal synergy as well as antagonism by pre-treatment conditions.

Pre-treatment of mice with the cyclooxygenase inhibitors and adrenocortical steroid drugs significantly reduced LPS-induced deficits in social investigation. These findings replicate and extend similar findings in rats (Bluthe et al. 1992a), mice (Bret-Dibat et al. 1994), birds (Johnson et al. 1993b), and pigs (Johnson and von Borell 1994). The relative potency of these compounds compares well with potencies measured against other inflammatory stimuli or indices (Otterness and Bliven 1985; Tsurufuji and Ohuchi 1989). The antagonism of LPS effects on social behavior by these compounds may reflect interference with central IL-1 mediated stimulation of the arachidonic acid cascade. Katsuura et al. (1990) report that peripheral IL-1β may penetrate the blood-brain barrier at the organum vasculosum of the lamina terminalis specifically to stimulate prostaglandin E_2 synthesis and release from the pre-optic area of the hypothalamus. Cyclooxygenase inhibitors and adrenocortical steroids interfere with the production of prostaglandins during the arachidonic cascade by competitively binding to synthetic enzymes or by inhibiting prostaglandin H synthase gene transcription (Wolf and Horrocks 1994). In the current studies cyclooxygenase inhibitors produced complex dose-effects with low doses efficacious and higher doses either ineffective or in the case of the high dose of ibuprofen, enhancing LPS-induced deficits. In a previous report, ibuprofen at comparable doses also enhanced endotoxin

mortality and plasma TNF-α in mice administered a suboptimal dose of LPS (Pettipher and Wimberly 1994).

Flavonoids form a group of naturally occurring phenoylbenzo-γ-pyrones present in plants commonly found in human and animal diet (Gabor 1979). Members of this group have been found to have significant anti-inflammatory properties in several rodent paradigms (see for reviews Gabor 1979; Havsteen 1983; Middleton and Kandaswami 1992). The mechanism for the anti-inflammatory activity of flavonoids remains unclear (Middleton and Kandaswami 1992). For example, flavones are reported to be potent antioxidants (Limasset et al. 1993) and potently interfere with leukocyte adhesion protein upregulation (Gerritsen et al. 1995). Flavonoids, like indomethacin and ibuprofen, are also effective cyclooxygenase inhibitors (Abad et al. 1995) and this latter activity is a potential mechanism of action for the findings reported here. In particular, the flavones chrysin and rohitukine (Lakdawala et al. 1988) significantly reduced the effects of LPS on social investigation. The potency of chrysin was comparable to and rohitukine approximately 10 times more potent than indomethacin or ibuprofen. These findings compare well with reports of activity in other animal models of inflammation (Middleton and Kandaswami 1992). Rohitukine has previously been reported to have significantly improved gastric tolerance compared to indomethacin (Lakdawala et al. 1988) and may consequently represent an important new class of anti-inflammatory drugs.

In summary, olfactory investigation of a conspecific is a highly motivated, spontaneous behavioral activity expressed by most rodent species. The topography of behavior is remarkably similar between species and offers a comparable background for interspecific characterization of drug effects against a common behavioral endpoint. The current findings in mice replicate and extend comparable studies in rats. LPS produced systematic, dose-dependent decreases in mouse social investigation. The dosage and latency to onset of this effect was comparable to that described in rats. Interaction studies using an LPS dose of 15 mg/kg IP, revealed potentiation of deficits by amphetamine, and complex dose-effect interaction with cyclooxygenase inhibitors, corticosteroids, and flavones. These findings are: (1) consistent with a postulated role for adrenergic modulation of cytokine mediated changes in behavior, and (2) demonstrate the complex dose-effect activity of anti-inflammatory compounds on endotoxin sickness which can be detected after administration of low doses of LPS.

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