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Fenfluramine-induced immunosuppression: an in vivo analysis

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Abstract

We examined the immunomodulatory potential of acute fenfluramine administration, by measuring production of the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α in response to an in vivo challenge with bacterial lipopolysaccharide in rats. Fenfluramine (2.5–10 mg/kg) suppressed tumor necrosis factor-αproduction, but only fenfluramine (5 and 10 mg/kg) suppressed interleukin-1β production. Fenfluramine (10 mg/kg)-induced suppression of interleukin-1β and tumor necrosis factor-α production persisted for 6 and 24 h, respectively. Using in vitro analyses, we demonstrated that the immunosuppressive effect of fenfluramine was not due to a direct effect on immune cells. As fenfluramine activates the hypothalamic pituitary adrenal axis, we examined the ability of the glucocorticoid receptor antagonist mifepristone to block fenfluramine-induced immunosuppression. However, mifepristone (10 mg/kg) failed to attenuate the suppressive effect of fenfluramine on interleukin-1β and tumor necrosis factor-α production, indicating that glucocorticoids do not mediate fenfluramine-induced immunosuppression. We also assessed the effect of fenfluramine on production of the anti-inflammatory cytokine interleukin-10, as interleukin-10 can suppresses pro-inflammatory cytokine production. Fenfluramine (10 mg/kg) increased interleukin-10 production following an in vivo lipopolysaccharide challenge. However, the ability of fenfluramine to suppress tumor necrosis factor-αproduction cannot be accounted for by increased interleukin-10 production, as pretreatment with the β-adrenoceptor antagonist nadolol completely blocked the increase in interleukin-10 without altering the suppression of tumor necrosis factor-α induced by fenfluramine. Taken together, these data demonstrate that fenfluramine promotes an immunosuppressive cytokine phenotype in vivo. The suppression of pro-inflammatory cytokines is not due to a direct effect the drug on immune cells, and also occurs independently of glucocorticoid receptor activation. In addition, whilst fenfluramine increases production of the anti-inflammatory cytokine interleukin-10, this cannot account for the suppression of the pro-inflammatory cytokine tumor necrosis factor-α induced by fenfluramine.

Keywords: Fenfluramine; Lipopolysaccharide; Interleukin-1β; TNF-α (tumor necrosis factor-α); Interleukin-10, glucocorticoid; Catecholamine

1. Introduction

Fenfluramine is a non-psychostimulant amphetamine derivative that was widely used as an anorectic agent (Curzon and Gibson, 1999; McTavish and Heel, 1992). In addition, fenfluramine was commonly used as a probe of the serotonergic system in neuroendocrine studies in psychiatric patients (Connor and Leonard, in press). However, fenfluramine when taken in combination with another amphetamine derivative phenteramine is associated with an abnormally high incidence of primary pulmonary hypertension and heart valve disease in patients being treated with the drug (Vivero et al., 1998). Moreover, studies have demonstrated that fenfluramine is capable of producing serotonergic neurotoxicity in a similar manner to the related drug methylenedioxymethamphetamine (MDMA; ''Ecstasy'') (McCann et al., 1994; Molliver et al., 1990). As well as the cardiotoxic and neurotoxic effects associated with amphetamines, it is known that psychostimulant amphetamines such as D-amphetamine and MDMA have potent immunosuppressive properties following in vivo administration to rodents (Connor et al., 1998, 2000a,b, 2001; Freire-Garabal et al., 1991; Nunez-Iglesias et al., 1996; Pezzone et al., 1992) and humans (Pacifici et al., 2001). Interestingly, we recently observed that fenfluramine also suppresses a number of aspects of cellular immunity such as concanavalin A-stimulated lymphocyte proliferation and T helper 1 and T helper 2 type cytokine production, and also lipopolysaccharide-induced tumor necrosis factor-α pro-

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duction in an ex vivo diluted whole blood culture system (Connor et al., 2000a). In these studies, we examined ex vivo measures of immune function. Whilst such determinations of immune function are useful, and yield important information concerning the status of the immune system, it is important to confirm that they have functional relevance to the whole animal. Therefore, in the present study, we assessed the ability of animals to respond to an in vivo challenge with bacterial lipopolysaccharide. Essentially an in vivo lipopolysaccharide challenge mimics the initial immune response to a bacterial infection. When lipopolysaccharide is injected systemically, it binds to CD14 on monocyte membranes (Jiang et al., 2000) and stimulates the production of pro-inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α, which are important signalling molecules in initiating and coordinating a large range of immune responses against invading pathogens (Hamblin, 1994; Henderson, 1994). Interleukin-1 is a pro-inflammatory cytokine released from activated macrophages. During inflammation, injury, immunological challenge or infection interleukin-1 is produced and because of its multiple biological properties, this cytokine is of primary and strategic importance to the outcome of disease, particularly inflammatory and infectious disease (see Dinarello, 1994). Tumor necrosis factor-α, like interleukin-1, is also a pro-inflammatory cytokine released from activated macrophages. Tumor necrosis factor-α is a vital component of the cellular immune response (see Beutler, 1995), and is a key mediator of inflammation and the mammalian host response to neoplasia, injury or invasion by bacteria, viruses and parasites (Pasparakis et al., 1996; Tracey, 1994; Wong and Goeddel, 1986). Thus an inability to produce interleukin-1β and tumor necrosis factor-α can result in defective host resistance to infectious disease (Beutler, 1995; Denis and Ghadirian, 1994; Pasparakis et al., 1996). Conversely excessive production of these cytokines plays a causal role as inflammatory disorders such as rheumatoid arthritis and inflammatory bowel disease (Feldmann et al., 2001; Gabay, 2002; McClane and Rombeau, 1999; Rogler and Andus, 1998).

In the present investigation, we sought to extend our preliminary ex vivo data (Connor et al., 2000a) by examining the effects of acute fenfluramine administration on the ability of the immune system to respond to an in vivo challenge with lipopolysaccharide. Production of the pro-inflammatory cytokines interleukin-1β and tumor necrosis factor-α was measured as an index of immune responsiveness following lipopolysaccharide challenge as previously described (Connor et al., 2000b). As fenfluramine-induced immunological changes may demonstrate differential times of onset, as well as differential response duration, pro-inflammatory cytokine production was examined at different time-points following acute fenfluramine administration. In addition, dose-related effects of fenfluramine were examined. In order to examine the possibility that the immunosuppressive effect of fenfluramine could be due to a direct effect of the drug on immune cells, the effect of in vitro fenfluramine exposure on interleukin-1β and tumor necrosis factor-α production in lipopolysaccharide-stimulated diluted whole blood was evaluated. Also as fenfluramine is a potent activator of the hypothalamic pituitary adrenal axis and increases circulating corticosterone concentrations (McGarvey et al., 1995; Van de Kar et al., 1985), we examined the effect of pretreatment with the glucocorticoid receptor antagonist mifepristone (Agarwai, 1996) on fenfluramine-induced immunosuppression. Finally the potential role of the anti-inflammatory cytokine interleukin-10 in fenfluramine-induced immunosuppression was assessed.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing approximately 250–350 g were obtained from a departmental breeding colony and housed four per cage. The rats were maintained on a 12-h light/12-h dark cycle (lights on at 0800 h) in a temperature controlled room (22±2 °C), and food and water were available ad libitum. The experimental protocols were in compliance with the European Communities Council directive (86/609/EEC).

2.2. Study I. Effect of fenfluramine on lipopolysaccharide-induced pro-inflammatory cytokine production: a time-course study

Rats received fenfluramine (Sigma, Poole, Dorset, UK) at a dose of 10 mg/kg i.p. either 1, 6, 12 or 24 h prior to lipopolysaccharide challenge. NaCl (0.89%) was administered alone as a vehicle to the control group 1 h prior to lipopolysaccharide.

The dose of fenfluramine used in this study was chosen based on a pilot study where we demonstrated 10 mg/kg fenfluramine administered 1 h prior to lipopolysaccharide suppressed interleukin-1β and tumor necrosis factor-α production (unpublished data).

2.3. Study II. Effect of fenfluramine on corticosterone secretion and lipopolysaccharide-induced pro-inflammatory cytokine production: a dose–response study

Fenfluramine (2.5, 5 or 10 mg/kg) was administered 1 h prior to lipopolysaccharide challenge. NaCl (0.89%) was administered alone as a vehicle to the control group.

Fenfluramine was administered 1 h prior to lipopolysaccharide in this and subsequent studies, as the results of the time-course study demonstrated that a maximal suppression pro-inflammatory cytokines was observed when fenfluramine was administered 1 h prior to lipopolysaccharide.

2.4. Study III. Effect of pretreatment with the glucocorticoid receptor antagonist mifepristone on fenfluramine-induced immunosuppression

Mifepristone (Exelgyn Laboratories, Paris, France) was dissolved in 20% (v/v) dimethyl sulphoxide (DMSO) to give a concentration of 10 mg/ml, and 20% DMSO was administered alone as a vehicle. Mifepristone was administered in an injection volume of 1 ml/kg using the subcutaneous (s.c.) route 30 min prior to fenfluramine. Lipopolysaccharide was administered 1 h following fenfluramine. The dose of mifepristone used was chosen based on a previous study indicating that mifepristone (10 mg/kg) produces maximal blockade of glucocorticoid receptors (Fleshner et al., 1996).

2.5. Study IV. An investigation of the effect of fenfluramine on lipopolysaccharide-induced interleukin-10 secretion

In this study, fenfluramine (1.25, 2.5, 5 or 10 mg/kg) was administered 1 h prior to lipopolysaccharide challenge. In this experiment, the animals were killed 1 h following lipopolysaccharide administration in order to facilitate measurement of interleukin-10. Other studies from our laboratory have shown that drug-induced changes in interleukin-10 are best observed 1 h post lipopolysaccharide administration (Connor and Kelly, 2002).

2.6. Study V. Effect of the β-adrenoceptor antagonist nadolol on lipopolysaccharide-induced interleukin-10 and tumor necrosis factor-α production

In this study, nadolol (0.3 mg/kg) was administered 30 min prior to fenfluramine and lipopolysaccharide, and rats were killed 1 h following the lipopolysaccharide challenge.

2.7. Lipopolysaccharide challenge and serum preparation

Lipopolysaccharide from *Escherichia coli* serotype 0111:B4 (Sigma) was dissolved in sterile 0.89% NaCl at a concentration of 100 μg/ml. Animals were challenged with either 0.89% NaCl or lipopolysaccharide (100 μg/kg) administered in a 1 ml/kg injection volume by the intraperitoneal (i.p.) route. Two hours following the lipopolysaccharide challenge, animals were killed by decapitation and trunk blood was collected. We have previously found that this dose and route of administration of lipopolysaccharide produces quantifiable increases in circulating interleukin-1β and tumor necrosis factor-α concentrations, and is optimal for simultaneous sampling of these two cytokines (Connor et al., 2000b). However, when assessing the effect of fenfluramine on lipopolysaccharide-induced interleukin-10 production, animals were killed 1 h following lipopolysaccharide administration in order to facilitate measurement of interleukin-10. We reliably find this dose and route of administration of lipopolysaccharide to produce a large increase in circulating interleukin-10 concentrations in rats (Connor and Kelly, 2002; Shen et al., 1999).

Following collection, blood samples were centrifuged (800×*g* at 4 °C for 15 min) and aliquots of serum were removed for cytokine determinations. Serum samples were frozen immediately on dry ice and then stored at −80 °C until the assays were performed.

2.8. In vitro effect of fenfluramine on cytokine production in lipopolysaccharide-stimulated whole blood

In the present study, a diluted whole blood method was used for the assessment of cytokine production as previously described (Connor et al., 2000a). In diluted whole blood, the natural cell–cell interactions are preserved, whereas the methods used to isolate peripheral blood mononuclear cells modify the lymphocyte/monocyte ratio and eliminate endogenous immunomodulatory agents. Thus, in vivo conditions are better represented using whole blood culture methods. Blood samples were obtained via cardiac puncture from eight male Sprague–Dawley rats (220–250 g) whilst under ether anaesthesia into a sterile heparinized syringe. Heparinized blood was mixed with complete RPMI-1640 medium [RPMI-1640+10% (v/v) heat-inactivated foetal bovine calf serum+2% (v/v) penicillin/streptomycin] (Gibco Life Technologies, Scotland) (1:10 dilution) as previously described. Briefly, 1 ml aliquots of diluted whole blood were pipetted into wells of a sterile flat-bottomed 24-well plate (Starstedt, Ireland). To each well was added 100 μl lipopolysaccharide (Sigma) at a working concentration of 10 μg/ml. To each well was added either 100 μl of RPMI-1640 culture medium alone (control) or 100 μl of fenfluramine at a final concentration of either 0.1, 0.5, 1, 5 or 10 μg/ml dissolved in RPMI-1640. Cultures were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. At the end of the culture period, the contents of each well were harvested, centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatants stored at −80 °C until cytokine assays were performed.

2.9. Measurement of interleukin-1β and tumor necrosis factor-α in serum and culture supernatants

Serum interleukin-1β and tumor necrosis factor-α concentrations were determined using enzyme-linked immunosorbent assays performed using antibodies and standards obtained from Dr. S. Poole (NIBSC, UK) as previously described (Connor et al., 2000b). Briefly, flat-bottomed 96-well Maxisorp microtitre plates (Nunc, Belgium) were coated overnight with sheep anti-rat cytokine antibodies (2 μg/ml in bicarbonate coating buffer; 0.1 M $NaHCO_3$, 0.1 M NaCl, pH 8.2, for 20 h at 4 °C) and were then washed three times with wash/dilution buffer (0.5 M NaCl, 2.5 mM NaH_2PO_4, 7.5 mM Na_2HPO_4, 0.1% Tween 20, pH 7.2). One hundred microliters of a 1% (w/v) ovalbumin (Sigma)

solution in bicarbonate coating buffer was added to each well and incubated at 37 °C for 1 h. Following three washes, 100 μl aliquots of samples and standards (0–1000 pg/ml) were added and plates were incubated at 4 °C for 20 h. After three washes, 100 μl of the specific biotinylated antibody (1:1000 or 1:2000 dilution in wash buffer containing 1% sheep serum, Sigma) was added to each well. A further incubation was carried out for 1 h at room temperature. After three washes, 100 μl avidin–horseradish peroxidase (Dako, UK) (1:5000 dilution in wash buffer) was added to each well and plates were incubated at room temperature for 15 min. Following three washes, 100 μl of tetramethylbenzidine substrate solution (Dako) was added per well and the plates were incubated for 10 min at room temperature. At the end of the incubation period, 100 μl of 1 M H_2SO_4 was added per well to stop the reaction and to facilitate colour development. Absorbance was read immediately at 450 nm on a microtitre plate reader (ELx 800, Bio-Tek instruments). Results are expressed as pg interleukin-1β/ml of serum or ng tumor necrosis factor-α/ml of serum.

2.10. Serum corticosterone analysis

Corticosterone concentrations were measured in serum obtained from the non-lipopolysaccharide-challenged animals in the dose–response study. Animals were killed and serum collected 3 h following fenfluramine administration. Serum corticosterone concentrations were measured using a previously described method (Grealy and O'Donnell, 1991). A corticosterone stock (Sigma) solution (100 μg/dl) was prepared and diluted to produce a range of concentrations (10–80 μg/dl). Serum samples and corticosterone standards were mixed in 600 μl of dichloromethane for 15 s. Five hundred microliters of the resulting dichloromethane (Lab Scan, Dublin, Ireland) extract phase was then transferred into a tube containing 400 μl of concentrated sulphuric acid/absolute ethanol (65:35), and the tubes mixed on a vortex mixer for 15 s. Samples were then placed in the dark for 45 min. A 300-μl aliquot of the lower phase was removed and the fluorescence was measured at excitation 474 nm and emission 518 nm (Perkin Elmer LS-5 spectrophotofluorimeter). The validity of the fluorometric assay was previously checked against a radioimmunoassay employing ^{3}H-corticosterone and rabbit anti-corticosterone 21-thyroglobulin serum where it was demonstrated that the fluorometric method correlated well with the RIA (Grealy and O'Donnell, 1991). The detection limit of the assay is 1–2.5 μg/dl. The intra- and inter-assay variances were 8.3% and 11%, respectively. The results were expressed as μg corticosterone per dl of serum.

2.11. Measurement of interleukin-10 concentrations in serum and culture supernatants

Interleukin-10 concentrations were measured by enzyme-linked immunosorbent assay using antibodies and standards provided in an Op EIA kit (Pharmingen, UK). The assay was performed according to the manufacturer's instructions. Results are expressed as pg/ml interleukin-10.

2.12. Statistical analysis of data

Data were analysed using a one-way or two-way analysis of variance where appropriate. If any statistically significant change was found, post hoc comparisons were performed using Fisher's least significant difference (LSD) test. Data were deemed significant when $P<0.05$. Data are expressed as group mean with standard errors.

3. Results

3.1. Effect of fenfluramine on lipopolysaccharide-induced pro-inflammatory cytokine production: a time-course study

3.1.1. Interleukin-1β

There was a significant effect of the lipopolysaccharide challenge [$F(1,68)=154.06$, $P<0.0001$], of fenfluramine treatment [$F(4,68)=4.87$, $P<0.01$] and a significant lipopolysaccharide×fenfluramine interaction [$F(4,68)=5.15$, $P<0.01$] on serum interleukin-1β concentrations. Post hoc analysis revealed that lipopolysaccharide challenge provoked a large increase in circulating interleukin-1β in vehicle-treated rats. Fenfluramine significantly suppressed lipopolysaccharide-induced interleukin-1β production at 1 and 6 h following its administration (Fig. 1A).

3.1.2. Tumor necrosis factor-α

There was a significant effect of the lipopolysaccharide challenge [$F(1,69)=78.37$, $P<0.0001$], of fenfluramine treatment [$F(4,69)=9.21$, $P<0.0001$] and a significant lipopolysaccharide×fenfluramine interaction [$F(4,69)=9.21$, $P<0.0001$] on serum tumor necrosis factor-α concentrations. Post hoc analysis revealed that lipopolysaccharide challenge provoked a large increase in circulating tumor necrosis factor-α ($P<0.01$) in vehicle-treated rats, which was suppressed for up to 24 h following fenfluramine administration (Fig. 1B).

In a second study, we examined lipopolysaccharide-induced tumor necrosis factor-α production in animals that were administered fenfluramine 36 and 48 h prior to the lipopolysaccharide challenge. The results demonstrate that fenfluramine failed to suppress lipopolysaccharide-induced tumor necrosis factor-α production when administered at these intervals prior to the lipopolysaccharide challenge.

Control: 16.09±5.9 ng/ml;
Fenfluramine (36 h): 23.3±7.5 ng/ml;
Fenfluramine (48 h): 16.9±2.6 ng/ml.

Data expressed as means±S.E.M. (n=4).

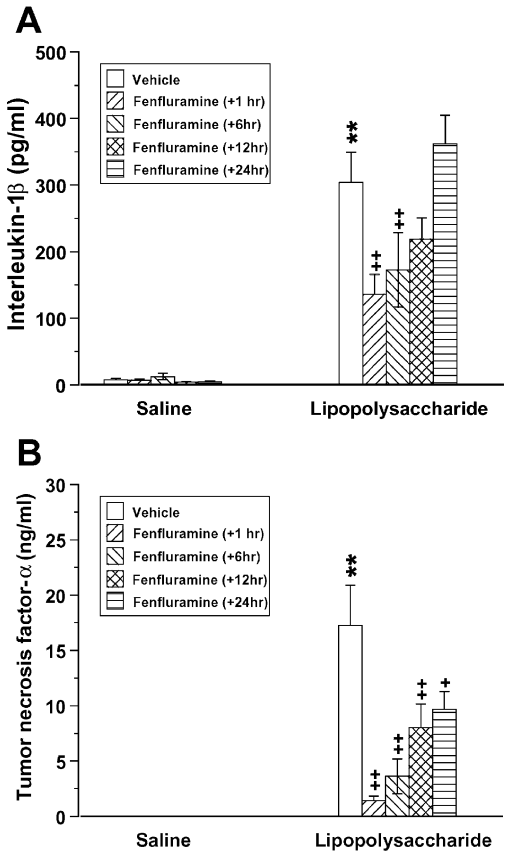

Fig. 1. Effect of acute fenfluramine administration (10 mg/kg; i.p.) on circulating (A) interleukin-1β and (B) tumor necrosis factor-α following an in vivo challenge with lipopolysaccharide (100 μg/kg; i.p.). Fenfluramine was administered 1, 6, 12 or 24 h prior to the lipopolysaccharide challenge and rats were killed 2 h following the lipopolysaccharide challenge. Data expressed as means±S.E.M. (n=7–8). **P<0.01 vs. saline-challenged counterparts; ^{+}P<0.05, ^{++}P<0.01 vs. vehicle+lipopolysaccharide (Fisher's LSD).

3.2. Effect of fenfluramine on lipopolysaccharide-induced pro-inflammatory cytokine production: a dose–response study

3.2.1. Interleukin-1β

There was a significant effect of the lipopolysaccharide challenge [F(1,56)=72.69, P<0.0001], of fenfluramine treatment [F(3,56)=6.77, P<0.001] and a significant lipopolysaccharide×fenfluramine interaction [F(3,56)=5.55, P<0.01] on serum interleukin-1β concentrations. Post hoc analysis revealed that lipopolysaccharide challenge provoked a large increase in circulating interleukin-1β in vehicle-treated rats. Fenfluramine treatment (5 and 10 mg/kg) produced a suppression of lipopolysaccharide-induced interleukin-1β production, whereas the lower dose of fenfluramine did not alter lipopolysaccharide-stimulated interleukin-1β production (Fig. 2A).

3.2.2. Tumor necrosis factor-α

There was a significant effect of the lipopolysaccharide challenge [F(1,56)=57.11, P<0.0001], of fenfluramine treatment [F(3,56)=10.98, P<0.0001] and a significant lipopolysaccharide×fenfluramine interaction [F(3,56)=10.98, P<0.0001] on serum tumor necrosis factor-α concentrations. Post hoc analysis revealed that lipopolysaccharide challenge provoked a large increase in circulating tumor necrosis factor-α concentrations in vehicle-treated rats. All doses of fenfluramine produced a profound suppression of lipopolysaccharide-induced tumor necrosis factor-α production (Fig. 2B).

3.3. Effect of in vitro exposure to fenfluramine on lipopolysaccharide-stimulated interleukin-1β and tumor necrosis factor-α production in diluted whole blood cultures

In vitro exposure to fenfluramine (0.1–10 μg/ml) did not significantly alter lipopolysaccharide-stimulated interleukin-1β or tumor necrosis factor-α production in diluted whole blood (Fig. 3).

3.4. Effect of fenfluramine on serum corticosterone concentrations

There was a significant effect of fenfluramine administration on serum corticosterone concentrations [F(3,28)=

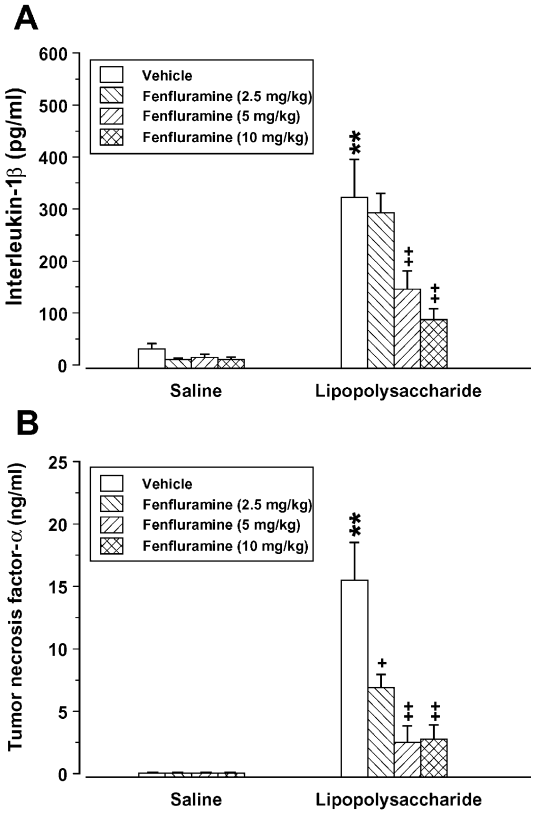

Fig. 2. Effect of acute fenfluramine administration (2.5, 5 and 10 mg/kg; i.p.) on circulating (A) interleukin-1β and (B) tumor necrosis factor-α following an in vivo challenge with lipopolysaccharide (100 μg/kg; i.p.). Fenfluramine was administered 1 h before lipopolysaccharide and rats were killed 2 h following the lipopolysaccharide challenge. Data expressed as means±S.E.M. (n=8). **P<0.01 vs. saline-challenged counterparts; ^{+}P<0.05, ^{++}P<0.01 vs. vehicle+lipopolysaccharide (Fisher's LSD).

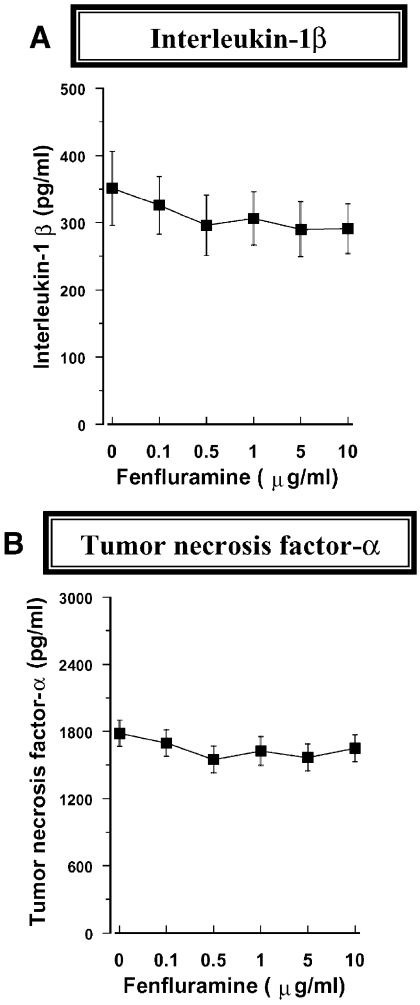

Fig. 3. In vitro exposure to fenfluramine does not alter lipopolysaccharide-induced interleukin-1β and tumor necrosis factor-α production. Diluted whole blood was incubated with fenfluramine for 1 h prior to stimulation with lipopolysaccharide (10 μg/ml) and cell free supernatants were harvested 24 h later for measurement of interleukin-1β and tumor necrosis factor-α. Data expressed as means±S.E.M. (n=8).

7.78, P<0.001]. Post hoc analysis indicated that fenfluramine (5 and 10 mg/kg) significantly increased circulating corticosterone concentrations (Fig. 4).

3.5. Effect of pretreatment with the glucocorticoid receptor antagonist mifepristone on fenfluramine-induced immunosuppression

3.5.1. Interleukin-1β

There was a significant effect of fenfluramine treatment [F(1,34)=75.63, P<0.0001] on serum interleukin-1β concentrations. Post hoc analysis revealed that fenfluramine suppressed lipopolysaccharide-induced interleukin-1β production and that this effect was not altered by pretreatment with mifepristone (Fig. 5A).

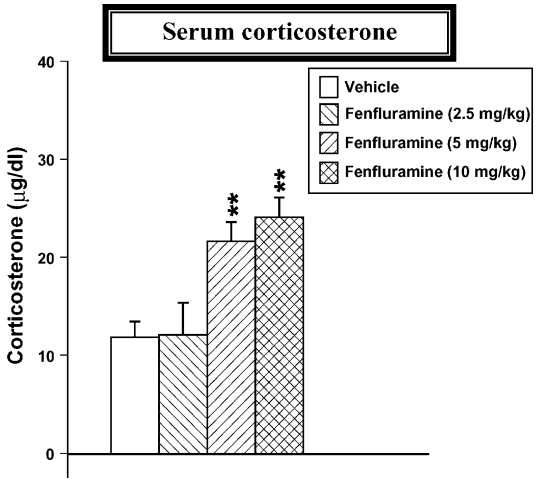

Fig. 4. Effect of acute fenfluramine administration (2.5–10 mg/kg; i.p.) on circulating corticosterone concentrations. Rats were killed 3 h following the fenfluramine administration. Data expressed as means±S.E.M. (n=). **P<0.01 vs. vehicle+lipopolysaccharide (Fisher's LSD).

3.5.2. Tumor necrosis factor-α

There was a significant effect of fenfluramine treatment [F(1,34)=68.29, P<0.0001] and a significant fenfluramine×mifepristone interaction [F(1,34)=6.76, P<0.05] on

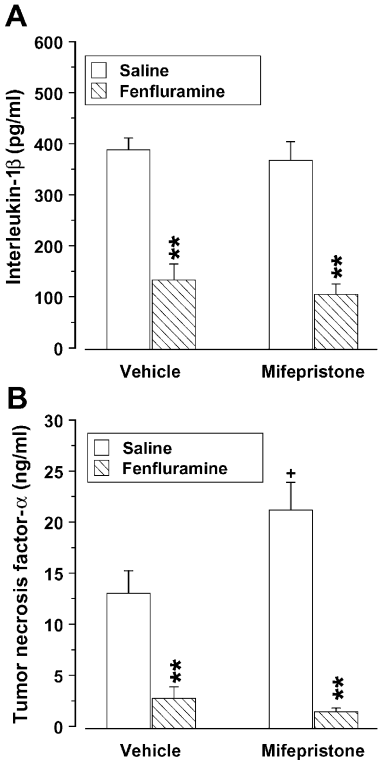

Fig. 5. Effect of pretreatment with mifepristone (10 mg/kg; s.c.) on fenfluramine-induced suppression of circulating (A) interleukin-1β and (B) tumor necrosis factor-α following an in vivo challenge with lipopolysaccharide (100 μg/kg; i.p.). Mifepristone was administered 30 min prior to fenfluramine. Fenfluramine was administered 1 h before lipopolysaccharide and rats were killed 2 h following the lipopolysaccharide challenge. Data expressed as means±S.E.M. (n=8–12). **P<0.01 vs. saline-treated controls, ^{+}P<0.05 vs. vehicle-pretreated control group (Fisher's LSD).

serum tumor necrosis factor-α concentrations. Post hoc analysis revealed that fenfluramine suppressed lipopolysaccharide-induced tumor necrosis factor-α production and that this effect was not altered by pretreatment with mifepristone. In addition, mifepristone pretreatment increased lipopolysaccharide-induced tumor necrosis factor-α production in the vehicle-treated control group (Fig. 5B).

3.6. Evaluation of the role of interleukin-10 in fenfluramine-induced immunosuppression

There was a significant effect of fenfluramine administration on lipopolysaccharide-induced tumor necrosis factor-α [$F(4,27)=23.44$, $P<0.0001$] and interleukin-10 [$F(4,27)=5.15$, $P<0.01$] production. Post hoc analysis indicated that fenfluramine (2.5, 5 and 10 mg/kg) significantly suppressed lipopolysaccharide-induced tumor necrosis factor-α production and fenfluramine (10 mg/kg) significantly increased lipopolysaccharide-induced interleukin-10 production (Fig. 6).

3.7. Effect of in vitro exposure to fenfluramine on lipopolysaccharide-stimulated interleukin-10 production in diluted whole blood cultures

In vitro exposure to fenfluramine (0.1–10 μg/ml) did not significantly alter lipopolysaccharide-stimulated interleukin-10 production in diluted whole blood (Fig. 7).

3.8. Effect of pretreatment with the β-adrenoceptor receptor antagonist nadalol on interleukin-10 and tumor necrosis factor-α production following an in vivo lipopolysaccharide challenge

3.8.1. Interleukin-10

There was a significant effect of fenfluramine treatment [$F(1,25)=98.15$, $P<0.0001$] and a significant fenfluramine × nadalol interaction [$F(1,25)=96.49$, $P<0.0001$] on serum interleukin-10 concentrations. Post hoc analysis revealed that fenfluramine increased lipopolysaccharide-induced interleukin-10 production and that this effect was completely blocked by pretreatment with nadolol (Fig. 8A).

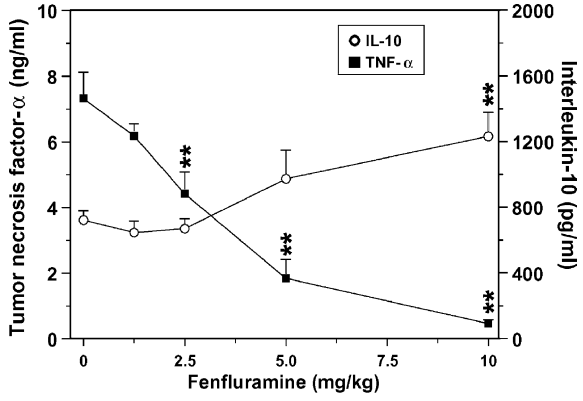

Fig. 6. Effect of acute fenfluramine administration (1.25–10 mg/kg; i.p.) on circulating interleukin-10 and tumor necrosis factor-α concentrations following an in vivo challenge with lipopolysaccharide (100 μg/kg; i.p.). Fenfluramine was administered 1 h before lipopolysaccharide and rats were killed 1 h following the lipopolysaccharide challenge. Data expressed as means±S.E.M. ($n=6–8$). **$P<0.01$ vs. vehicle+lipopolysaccharide (Fisher's LSD).

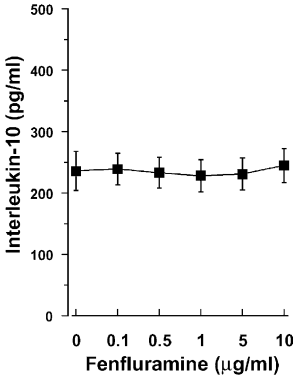

Fig. 7. In vitro exposure to fenfluramine does not alter lipopolysaccharide-induced interleukin-10 production. Diluted whole blood was incubated with fenfluramine for 1 h prior to stimulation with lipopolysaccharide (10 μg/ml) and cell free supernatants were harvested 24 h later for the measurement of interleukin-10. Data expressed as means±S.E.M. ($n=8$).

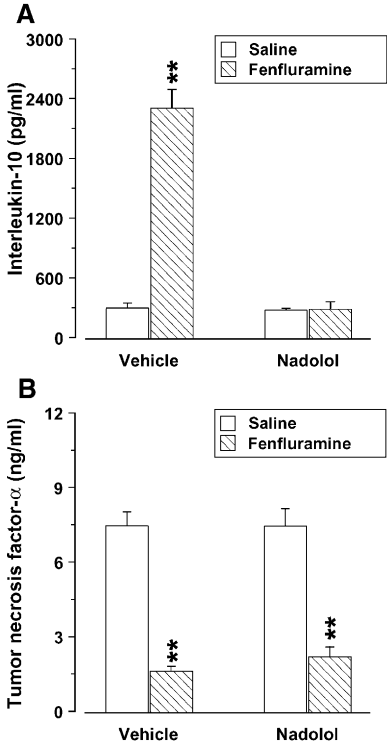

Fig. 8. Pretreatment with the β-adrenoceptor antagonist nadolol blocks fenfluramine-induced increase in interleukin-10 without altering fenfluramine-induced suppression of tumor necrosis factor-α production. Nadolol (0.3 mg/kg) was administered 30 min before fenfluramine and lipopolysaccharide and rats were killed 1 h following the lipopolysaccharide challenge. Data expressed as means±S.E.M. ($n=7–8$). **$P<0.01$ vs. saline-treated counterparts (Fisher's LSD).

3.8.2. Tumor necrosis factor-α

There was a significant effect of fenfluramine treatment [$F(1,25)=118.18$, $P<0.0001$] on serum tumor necrosis factor-α concentrations. Post hoc analysis revealed that fenfluramine suppressed lipopolysaccharide-induced tumor necrosis factor-α production and that this effect was not altered by pretreatment with nadolol (Fig. 8B).

4. Discussion

In the present series of studies, we observed that lipopolysaccharide produced a characteristic increase in circulating concentrations of the pro-inflammatory cytokines interleukin-1β and tumor necrosis factor-α in which is concurrent with previous observations (Connor et al., 2000b; Givalois et el., 1994). Acute administration of fenfluramine significantly suppressed lipopolysaccharide-induced production of these cytokines. Whilst the effect of fenfluramine on interleukin-1β persisted for up to 6 h following administration, suppression of tumor necrosis factor-α secretion persisted for up to 24 h following administration. Although interleukin-1β and tumor necrosis factor-α are both macrophage-derived pro-inflammatory cytokines, the results demonstrate a differential temporal sensitivity of these cytokines to the suppressive effects of fenfluramine, with the suppression of tumor necrosis factor-α persisting for a much longer period than that of interleukin-1β. As maximal suppression of lipopolysaccharide-induced cytokine production was observed 1 h following fenfluramine administration, in the second study we examined the ability of a number of doses of fenfluramine (2.5, 5 and 10 mg/kg) to suppress interleukin-1β and tumor necrosis factor-α production at this time-point. Whilst all three doses of fenfluramine (2.5, 5 and 10 mg/kg) suppressed lipopolysaccharide-induced tumor necrosis factor-α production, only the two higher doses significantly altered interleukin-1β production. Taken together, these data demonstrate that the suppression of lipopolysaccharide-induced tumor necrosis factor-α production, in addition to be longer lasting, is also more sensitive to the suppressive effects of fenfluramine than is the related pro-inflammatory cytokine interleukin-1β. This is consistent with a previous study where we observed that administration of the related substituted amphetamine MDMA produced a longer lasting and more profound suppression of tumor necrosis factor-α than interleukin-1β (Connor et al., 2000b).

In order to determine if fenfluramine suppressed interleukin-1β and tumor necrosis factor-α by a direct action on immune cells, we examined the effect of in vitro incubation of diluted whole blood with fenfluramine (0.1–10 μg/ml) and observed no significant effect on lipopolysaccharide-induced interleukin-1β and tumor necrosis factor-α. The range of fenfluramine concentrations employed in vitro was based on a study reporting a plasma level of 2.6 μM or 696 ng/ml of fenfluramine in rats 1 h following a single 10 mg/kg i.p. injection (Clausing et al., 1998). In the present study, we employed doses of fenfluramine that were both below, and well in excess of this concentration.

In contrast to their in vitro counterpart, in vivo immunopharmacological analyses are more functionally relevant, as they take into account the contribution of circulating endogenous immunomodulatory substances such as glucocorticoids, catecholamines and anti-inflammatory cytokines such as interleukin-10, all of which have the ability to inhibit lipopolysaccharide-induced interleukin-1β and/or tumor necrosis factor-α production (Bogdan et al., 1992; Elenkov et al., 1995; Gerard et al., 1993; Knudsen et al., 1987; Van der Poll et al., 1994; Zuckerman et al., 1989). In this regard, it is well established that fenfluramine activates the hypothalamic–pituitary–adrenal axis resulting in increased circulating concentrations of glucocorticoids (McGarvey et al., 1995; Van de Kar et al., 1985). This was confirmed in the present study where we observed that fenfluramine (5 and 10 mg/kg) significantly increased circulating corticosterone concentrations. Thus, it was possible that the ability of fenfluramine to increase circulating glucocorticoid concentrations may mediate the suppressive effect of fenfluramine on lipopolysaccharide-induced tumor necrosis factor-α and/or interleukin-1β production. In order to test this hypothesis, we examined the effect of pretreatment with the glucocorticoid receptor antagonist mifepristone. However, mifepristone (10 mg/kg; s.c.) failed to attenuate the suppressive effect of fenfluramine on lipopolysaccharide-induced interleukin-1β and tumor necrosis factor-α production, indicating that glucocorticoids do not mediate fenfluramine-induced immunosuppression. It has been previously demonstrated that the dose and route of administration of mifepristone used in the present study produces maximal blockade of glucocorticoid receptors (see Fleshner et al., 1996). Moreover, in the present study, pretreatment with mifepristone facilitated an increase in lipopolysaccharide-induced tumor necrosis factor-α production, probably as a result of reduced feedback inhibition of corticosterone on macrophage cytokine production. This indicates that the dose of mifepristone used elicited expected pharmacological effects. This is consistent with previous reports where treatment with mifepristone (10 mg/kg) increased lipopolysaccharide-induced tumor necrosis factor-α and interleukin-6 production in rats (Hawes et al., 1992).

In the present study, we also examined the effect of fenfluramine administration on production of the endogenous immunosuppressive agent interleukin-10, an anti-inflammatory cytokine that inhibits several macrophage functions including pro-inflammatory cytokine production in response to lipopolysaccharide (Bogdan et al., 1992; Gerard et al., 1993). We recently observed that the related amphetamine derivative MDMA (1.25–10 mg/kg) increased interleukin-10 production following an in vivo lipopolysaccharide challenge, and that the increase in interleukin-10 was highly correlated to the suppression of tumor

necrosis factor-α production (Connor and Kelly, 2002). In the present study, we observed that fenfluramine (10 mg/kg) provoked a significant increase in lipopolysaccharide-induced interleukin-10 production. Interleukin-10 was also increased by 5 mg/kg of fenfluramine, albeit nonsignificantly. In contrast, in vitro exposure to fenfluramine had no effect on lipopolysaccharide-induced interleukin-10 production, indicating that the increase observed in vivo was not due to a direct effect of fenfluramine on immune cells. However, our results indicate that whilst fenfluramine increases interleukin-10 production in response to an in vivo lipopolysaccharide challenge, the suppression of the pro-inflammatory cytokine tumor necrosis factor-α induced by fenfluramine (10 mg/kg) occurs independently of increased interleukin-10 production. This was demonstrated by the fact that the β-adrenoceptor antagonist nadolol completely blocks the fenfluramine-induced increase in IL-10, yet fails to alter the suppressive effect of fenfluramine on tumor necrosis factor-α production. These data indicate that the ability of fenfluramine to increase interleukin-10 production following and in vivo lipopolysaccharide challenge is mediated by β-adrenoceptor activation. This is consistent a previous report indicating that fenfluramine increases plasma catecholamine concentrations (Chaouloff et al., 1992), and is also consistent with studies demonstrating that stimulation of β-adrenoceptors on macrophages increased lipopolysaccharide-induced interleukin-10 production (Suberville et al., 1996; Wu et al., 2000). In addition to suppressing the production pro-inflammatory cytokines, increased interleukin-10 production has also been reported to down-regulate MHC class II and B7 expression on antigen-presenting cells, thereby inhibiting the antigen-presenting and co-stimulatory capacity of these cells (De Waal Malefyt et al., 1991; Ding et al., 1993). Further studies are required to ascertain whether fenfluramine-induced increases in interleukin-10 production are sufficient to alter MHC II and B7 expression on antigen-presenting cells.

Interestingly, in contrast to our present and past (Connor et al., 2000a) results demonstrating an immunosuppressive effect of fenfluramine, it has been previously reported that repeated treatment with low (sub-anorexic) doses of fenfluramine enhanced the local T lymphocyte response to the opportunistic microbial pathogen, *Candida albicans*, by increasing the number and biological activity of lymphocytes at the site of the infection in rats (Mathews et al., 1996). In addition, the same investigators demonstrated that in vitro incubation of peripheral blood mononuclear cells isolated from human immunodeficiency virus-infected individuals with fenfluramine (5–50 ng/ml) resulted in increased T lymphocyte proliferation and interleukin-2 production, and augmented the capacity of $CD8^+$ lymphocytes to inhibit the growth of *C. albicans* (Mathews et al., 1998). Nonetheless, it is of interest that they simultaneously reported that fenfluramine suppressed T-cell-derived tumor necrosis factor-α production in control subjects but not HIV^+ individuals (Mathews et al., 1998).

In conclusion, the results of this study confirm our previous ex vivo data suggesting an immunosuppressive effect of fenfluramine. Here we demonstrate that acute administration of fenfluramine suppresses the production of the pro-inflammatory cytokines interleukin-1β and tumor necrosis factor-α in response to an in vivo lipopolysaccharide challenge, and that tumor necrosis factor-α is more sensitive to the suppressive effects of fenfluramine than is and interleukin-1β. However, the immunosuppressive effect of fenfluramine could not be attributed to a direct effect on immune cells, nor to increased secretion of glucocorticoids. Whilst fenfluramine increases production of the anti-inflammatory cytokine interleukin-10 following in vivo administration, this increase in interleukin-10 does not mediate the suppression of tumor necrosis factor-α induced by fenfluramine. Further studies are underway in order to elucidate the exact mechanisms that underlie the ability of fenfluramine to suppress pro-inflammatory cytokine production. It is possible that fenfluramine-induced suppression of interleukin-1β and tumor necrosis factor-α could lead to an abnormal immune response at times of infection or illness. In this regard, it has been demonstrated that tumor necrosis factor-α knockout mice have reduced host resistance to *Listeria monocytogenes* infection (Pasparakis et al., 1996). Similarly, administration of the interleukin-1 receptor antagonist interferes with host resistance to infection with *Mycobacterium avium* (Denis and Ghadirian, 1994).

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