Mediation by nitric oxide formation in the preoptic area of endotoxin and tumour necrosis factor-induced inhibition of water intake in the rat

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1 Drinking was induced in rats by 24 h of water deprivation. Water intake (ml) was evaluated for a 1 h period.
2 N²-nitro-L-arginine methyl ester (L-NAME, 5–10 μg, i.c.v., 50–100 ng into the preoptic area (POA)), an inhibitor of nitric oxide (NO) synthase, and methylene blue (50–100 ng into POA), an inhibitor of guanylate cyclase activation, antagonized the inhibition of drinking induced by E. coli endotoxin (LPS, 640 μg kg⁻¹, i.v.) and tumour necrosis factor (TNFα, 40 ng, i.c.v.) in 24 h water-deprived rats.
3 L-Arginine (25, 50 and 100 ng), the precursor aminoacid of NO, but not the stereoisomer D-arginine (100 ng), inhibited drinking induced by water deprivation when injected into the POA 30 min before water presentation (74.4% of inhibition with the highest dose). A dose of 12.5 ng L-arginine into the POA did not exhibit antidipsogenic effects.
4 TNFα (20 and 40 ng, i.c.v.; 1.25, 2.5 and 5 ng into the POA) showed a dose-dependent and powerful inhibition of drinking behaviour in water-deprived rats (70.4% and 80.8%, i.c.v. and into POA, with the highest doses, respectively). A dose of 10 ng of TNFα given i.c.v. had no effect on the intake of water.
5 LPS and TNFα, given at doses (160 μg kg⁻¹, i.v. and 10 ng, i.c.v., respectively) that did not influence drinking in water-deprived rats, exhibited a strong antidipsogenic effect in water-deprived rats treated with a dose of L-arginine (12.5 ng, into the POA) which did not modify drinking by itself. (LPS + L-arginine; 53.6% of inhibition; TNFα + L-arginine: 52.0% of inhibition).
6 These results suggest that NO into the POA: (1) acts as an inhibitory mechanism on thirst and (2) plays a role in the antidipsogenic effect of LPS and TNFα.

Keywords: Nitric oxide; drinking; LPS; TNFα; preoptic area; N²-nitro-L-arginine methyl ester (L-NAME); methylene blue

Introduction

Intravenous (i.v.) injection of Escherichia coli endotoxin (lipopolysaccharide, LPS) causes a potent, dose-dependent and long-lasting inhibition of the rat’s drinking behaviour induced by water deprivation, as well as by intracerebroventricular (i.c.v.) injection of angiotensin II, carbachol or hypertonic NaCl solution. The inhibitory effect of LPS on water intake is not a mere consequence of behavioural alteration, including a depressed motor activity, since this point has been investigated in previous work (Foca’ et al., 1983) and we concluded that this hypothesis can be excluded.

This antidipsogenic effect becomes evident 30 min after the injection, reaches a maximum after 3 h and is prevented by acetylsalicylic acid injected into the hypothalamic preoptic area (POA; Foca’ et al., 1985; Calapai et al., 1990). Furthermore, LPS injected directly into the POA inhibits drinking induced by water deprivation (Foca’ et al., 1985).

LPS has been shown to induce a release of tumour necrosis factor (TNFα) from neuronal and glial cells (Koenig, 1991) and, in water-deprived rats, a serum, containing antibodies raised against TNFα, antagonizes LPS induced inhibition of thirst (Calapai et al., 1991). Moreover, i.c.v. injection of TNFα in water-deprived rats mimics the antidipsogenic action of LPS (Calapai et al., 1992b).

More recently, we found that nitric oxide (NO) acts as an inhibitory mechanism when thirst is stimulated by water deprivation or by angiotensin II, and we suggested that the POA could be one of the central sites of its antidipsogenic activity (Calapai et al., 1992a).

Nitric oxide synthase in the brain has been shown, characterized, purified, and cloned as a cytosolic and Ca²⁺-dependent enzyme (Knowles et al., 1989; Forstermann et al., 1990; Breit & Synder, 1990; Breit et al., 1990). It is present in the largest concentration in the cerebellum, followed by the hypothalamus, midbrain, striatum, hippocampus, and medulla oblongata (Breit & Snyder, 1992). This enzyme is competitively inhibited by analogues of L-arginine (Schmidt et al., 1989; Knowles et al., 1990a; Forstermann et al., 1990). Nitric oxide activates the soluble guanylate cyclase in the brain and L-arginine can be considered the endogenous stimulator of the soluble guanyl cyclase in the CNS (Knowles et al., 1989).

Recently it has been demonstrated that NO could also play a role in food intake and in the regulation of body weight (Morley & Flood, 1991; 1992; Squadrito et al., 1993), as well as in the regulation of drinking behaviour (Calapai et al., 1992a).

Several effects, induced by both LPS and TNFα, are mediated, at least in part, by an overproduction of NO, which is caused by the induction of the Ca²⁺-independent NO synthase in endothelial and smooth muscle cells, in macrophages and in other cells and tissues (Moncada et al., 1992). Moreover, recent data indicate that a NO synthesis-like activity can be induced in microglia and astrocytes by LPS (Simmons & Murphy, 1992).

In the light of the above findings we have investigated whether NO in the POA could be involved in drinking behaviour and water intake inhibition induced by LPS and TNFα in water-deprived rats.

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Methods

Animals

Adult male Sprague-Dawley rats weighing 280–320 g were used. The animals were housed at a constant temperature of 22 ± 2°C under a 12/12 h light-dark cycle (lights on at 6 h 00 min), with free access to Purina rat chow pellets and tap water, unless otherwise stated.

Water intake evaluations

Drinking was elicited by 24 h water deprivation. The rats had free access to food during this period. Water intake following deprivation was monitored for a 1 h period and expressed as ml/rat. Water was provided in graduated burettes, with drinking spouts, allowing direct volumetric measurement of intake to the nearest 0.1 ml. In order to avoid appearance of tolerance to LPS, each animal was treated only once with this substance and not used for other experiments.

Intracerebral and intravenous injections

Stainless steel guide cannulae (o.d. = 0.66 mm) were inserted 7 days before the experiments. The rats were anaesthetized with chloral hydrate (400 mg kg⁻¹, i.p.) and placed in a stereotaxic instrument. For i.c.v. injections, cannulae were implanted in the left lateral ventricle (stereotaxic coordinates: AP = 1 mm behind the bregma, L = 2.5 mm from the mid-sagittal suture and V = 2 mm from the dura). For injections into the POA, the cannulae were implanted, aimed 2 mm above the left medial preoptic area, at the following stereotaxic coordinates: AP = 7.9 mm anterior to the interaural line, L = 0.7 mm from the mid-sagittal suture, V = 3.4 mm up from the interaural line. The incisor bar of the stereotaxic was elevated 5 mm above the interaural line. In a group of animals cannulae were implanted both in the lateral ventricle and into the POA. Injections into the lateral ventricle (5 μl) or into the POA (1 μl) were made by a 30 gauge injector temporarily inserted into the guide-cannula and protruding 2 mm beyond the cannula tip. Injections were carried out over a period of 1–2 min. I.v. injections were made through the tail vein under light ether anaesthesia.

LPS was injected i.v. in 24 h water-deprived rats 3 h before water presentation, in order to obtain its maximum inhibitory antidiapogenic effect (Foca et al., 1983), while naive water-deprived animals received saline solution (0.1 ml 100 g⁻¹, wt.).

TNFα was injected i.c.v. or into the POA in 24 h water-deprived rats 20 min before water presentation. Animals treated i.c.v. (5 μl) or into the POA (1 μl) with bovine serum albumin (BSA; 125 μg rat), the solvent of TNFα, served as controls.

L-Arginine, D-arginine, N⁵-nitro-L-arginine methyl ester (L-NAME) and methylene blue were injected into the POA (1 μl) of water-deprived rats, 30 min before water presentation. Control animals received saline solution.

In order to study the effects on water intake for 24 h, four groups of animals were treated at 10 h 00 min with a single injection into the preoptic area (1 μl) of only one of the following drugs: L-NAME (100 ng), L-arginine (100 ng), D-arginine (100 ng), methylene blue (100 ng). The intake of water was evaluated 24 h after drug administration.

Drugs

Escherichia coli lipopolysaccharide (LPS) (055: B5 phenol extract) was purchased from the Sigma Chemical Company, U.S.A.; recombinant human tumour necrosis factor-α (TNF-α) from Nuclear Laser Medicine (Milan, Italy); L-arginine and D-arginine and N⁵-nitro-L-arginine methyl ester (L-NAME) from the Sigma Chemical Company; methylene blue from Hoechst, Italy. RhTNFα was dissolved in bovine serum albumin (BSA). Lipopolysaccharide (LPS), L-arginine, D-arginine, L-NAME and methylene blue were dissolved in 0.9% NaCl solution warmed to 37°C before the injection.

Statistical analysis

Data are expressed as the means ± s.d. Statistical analysis of data was performed by ANOVA (one way analysis of variance). Statistical significance was set at P < 0.05.

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Treatment (i.v.)</th>
<th>Pretreatment</th>
<th>Water intake (ml/rat)</th>
<th>% inhibition water intake vs Sal + Sal</th>
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<tr>
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<td>(i.c.v.; μg)</td>
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<td></td>
<td></td>
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<tr>
<td>6</td>
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<td>L-NAME 5</td>
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<tr>
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<td>11.3 ± 2.0**</td>
<td>9.6</td>
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<tr>
<td>6</td>
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<td>L-NAME 10 (into POA; ng)</td>
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<td>4.0</td>
</tr>
<tr>
<td>6</td>
<td>Sal</td>
<td>Sal</td>
<td>12.0 ± 1.3</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
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<td>Sal</td>
<td>4.1 ± 0.7***</td>
<td>65.8</td>
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<td>L-NAME 50</td>
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<td>Sal</td>
<td>L-NAME 100</td>
<td>10.6 ± 2.0</td>
<td>16.6</td>
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</tbody>
</table>

LPS was injected 3 h before water presentation.

*P<0.05 vs Sal + Sal i.c.v.;

**P<0.05 vs LPS 640 + Sal i.c.v.;

***P<0.05 vs Sal + Sal into POA;

****P<0.05 vs LPS 640 + Sal into POA.
Results

Effect of L-NAME on LPS inhibition of thirst (Table 1)

L-NAME, given i.c.v. (5 and 10 ng) or into the POA (50 and 100 ng), 30 min before water presentation in water-deprived rats, significantly inhibited the antidipsogenic effect induced by i.v. injections of LPS (640 μg kg⁻¹). Lower doses (2.5 μg, i.c.v.; 10 ng into the POA) were without effect. L-NAME, either i.c.v. (10 μg) or into the POA (100 ng), by itself, did not modify drinking behaviour induced by 24 h water-deprivation.

Effect of L-arginine and D-arginine on water intake induced by water deprivation and on its inhibition induced by LPS

L-arginine (25, 50 and 100 ng), given into the POA 30 min before water presentation in 24 h water-deprived rats, inhibited drinking in a dose-dependent way (Table 2). A dose of 12.5 ng of L-arginine, as well as a dose of 100 ng of D-arginine did not modify water deprivation-induced drinking (Table 2).

A significant inhibition of water intake (Figure 1a) was observed in water-deprived rats when animals were treated with LPS (160 μg kg⁻¹, i.v.) together with L-arginine (12.5 ng, into the POA). Both doses were without inhibitory effects, by themselves. L-Arginine (12.5 ng) was injected 30 min before water presentation and 150 min after LPS. When D-arginine (100 ng) was injected into the POA, the LPS dose of 160 μg kg⁻¹ did not inhibit drinking (Figure 1a).

Effect of TNFα on drinking behaviour induced by water-deprivation

TNFα given i.c.v. (20 and 40 ng) or into the POA (1.25, 2.5 and 5 ng) inhibited the intake of water deprived rats. Lower doses were without effect (Figure 2).

Effect of the injection of L-NAME and L-arginine into the preoptic area on the antidipsogenic effect induced by TNFα injected i.c.v.

For these experiments rats were prepared with two cerebral cannulae. Injections into the POA were made 10 min before TNFα (40 ng) i.c.v. injection.

In water-deprived rats, L-NAME (100 ng) given into the POA inhibited (by 70%) the antidipsogenic effect induced by the i.c.v. injection of TNFα (Figure 1b). In water-deprived rats, injection into the POA of L-arginine, 12.5 ng (not antidipsogenic, by itself) given 10 min before the i.c.v. injection of TNFα, 10 ng, (not antidipsogenic, by itself), showed a powerful antidipsogenic effect in 24 h water-deprived rats. D-Arginine (100 ng into the POA) did not inhibit drinking when associated with TNFα (10 ng, i.c.v.).

Table 2 Effect of injections of L-arginine and D-arginine into the preoptic area (POA) on 1 h water intake (ml/rat) of rats stimulated by 24 h water deprivation

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Treatment (i.c.v.; μg)</th>
<th>Water intake (ml/rat)</th>
<th>% inhibition water intake vs Sal + Sal</th>
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<tr>
<td>4</td>
<td>Sal</td>
<td>12.5 ± 2.0</td>
<td>–</td>
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<td>L-Arg 25</td>
<td>10.4 ± 2.0</td>
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<tr>
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<td>L-Arg 50</td>
<td>7.8 ± 1.5*</td>
<td>37.6</td>
</tr>
<tr>
<td>5</td>
<td>L-Arg 100</td>
<td>5.7 ± 1.0*</td>
<td>44.4</td>
</tr>
<tr>
<td>8</td>
<td>L-Arg 100</td>
<td>3.2 ± 0.6*</td>
<td>74.4</td>
</tr>
<tr>
<td>4</td>
<td>D-Arg 100</td>
<td>11.5 ± 2.3</td>
<td>8.0</td>
</tr>
</tbody>
</table>

*P < 0.05 vs Sal

Figure 1 (a) Effect of injection of saline (Sal, into the preoptic area), L-arginine (L-Arg, 12.5 ng i.c.v.), LPS (160 μg kg⁻¹, i.v.), L-Arg + LPS (12.5 ng, POA + 160 μg kg⁻¹, i.v.) and D-arginine (100 ng, POA) on 1 h water intake in animals deprived of water for 24 h. *P < 0.01 vs Sal. Number of rats used in parentheses. (b) Water intake in 24 h water-deprived rats treated as following: BSA i.c.v. + Sal into POA (n = 4); tumour necrosis factor (TNFα, 10 ng, i.c.v.) + Sal into POA (n = 6); TNFα (10 ng, i.c.v.) + Sal TNFα + L-Arg + L-NAME into POA (100 ng; n = 6); TNFα (40 ng, i.c.v.) + L-NAME into POA (12.5 ng); TNFα (10 ng, i.c.v.) + D-Arg (12.5 ng) into POA. **P < 0.01 vs saline.

Figure 2 Effect of injection i.c.v. or into the preoptic area (POA) of bovine serum albumin (BSA) (solid columns) or tumour necrosis factor (TNFα; ng) on 1 h water intake in animals deprived (open column) of water for 24 h. Each column represents the average of 5–6 animals. *P < 0.01 and **P < 0.01 vs BSA.

Effects of methylene blue on LPS and TNFα inhibition of drinking behaviour induced by water-deprivation

Methylene blue (50 and 100 ng) injected into the POA produced a reduction of the inhibition of drinking induced by LP (640 μg kg⁻¹, i.v.) of TNFα (40 ng, i.c.v.) in water-deprived rats. (Figure 3). Methylene blue (100 ng) into the POA did not modify, by itself, drinking stimulated by water-deprivation (data not shown).
induced by inhibition and inhibitor of (saline).

**Figure 3** Effect of injection into the preoptic area (POA) of methylene blue (MB, ng) or saline (Sal) on 1 h water intake in animals deprived of water for 24 h and i.v. injected 3 h before testing with (a) saline ( ), or LPS (640 ng kg$^{-1}$), or tumour necrosis factor (TNFα, 40 ng; ), or (b) i.c.v. with saline ( ), or LPS ( ), or TNFα ( ), or tumour necrosis factor. Each column represents the average ± s.d. of five animals.

**Effect of injection into the preoptic area of L-arginine, D-arginine, L-NAME and methylene blue of 24 h water intake**

A single administration of L-arginine when injected into the preoptic area of non-deprived rats produced a significant decrease (43.53%) of the intake of water compared to the controls (saline). The intake of water during 24 h was unaffected by the other drugs (Figure 4).

**Discussion**

The results of the present experiments strongly support the role of nitric oxide in the POA in the antidipsogenic effect of LPS and TNFα. In fact, the peripheral administration of LPS and the intracerebroventricular injection of TNFα produced inhibition of drinking behaviour, which was partially abolished by the injection into the POA of L-NAME, a competitive inhibitor of NO synthase. Antagonism of L-NAME of inhibition of drinking behaviour was stronger when inhibition was induced by i.c.v. TNFα than i.v. LPS.

The experiments carried out with the injection of methylene blue (an inhibitor of guanylate cyclase activation by NO, Murad et al., 1978) into the POA indicate that the enzyme in this area is involved in LPS and TNFα inhibition of drinking behaviour. Since biological effects of NO are accompanied by enhanced cyclic GMP levels, these data further support the idea that LPS and TNFα inhibit water intake through formation of NO in the preoptic area.

These results indicate that the inhibition of the enzymes (NO synthase and guanylate cyclase) antagonizes the effects of LPS and TNFα. Even when this inhibition was produced in the absence of LPS or TNFα or in non-deprived rats, there were no changes in the intake of water. Therefore, when rats were water-deprived, the stimulation of NO production by LPS or TNFα inhibited drinking, while the inhibition of the enzymes NO synthase or guanylate cyclase was not effective. We have already suggested that water deprivation, for 24 h, which produces intra- and extracellular dehydration, could be a stimulus able, by itself, to modify the activity of the enzymes (Calapai et al., 1992a).

Experiments carried out with L-arginine and TNFα injected into the preoptic area confirm the role of this area in the regulation of water intake. Finally, L-arginine, but not D-arginine, when injected into the POA at doses not antidipsogenic by themselves, made doses of LPS and TNFα antidipsogenic which did not otherwise impair water intake in thirsty rats. Therefore, both LPS and TNFα require NO formation in the preoptic area to elicit their inhibitory effect.

In the brain, astrocytes or microglia synthesize or secrete cytokines, such as interleukin-1 (IL-1), IL-6 and TNF, following infection or injury (Hauser et al., 1990; Morganti-Kossmann et al., 1992). In culture, glial cells and microglia release TNF following LPS stimulation (Morganti-Kossmann et al., 1992). The injection of LPS into experimental animals has been demonstrated to produce TNFα (Carswell et al., 1975). A trace amount of TNFα can be detected in serum 15 to 20 min after injection of endotoxin and the concentration of TNFα peaks after 1 or 2 h (Beutler et al., 1985; Waage, 1987).
The fact that LPS is a very large molecule, which does not cross the blood brain barrier (Calapai et al., 1990), raises the question of how peripherally administered LPS might exert its effect at the level of NO-synthase in the POA. Several hypotheses may be proposed: (1) TNFα, synthesized in peripheral cells following LPS, could enter the brain at specific sites and reach the POA, where it stimulates NO-synthase; (2) the synthesis of TNFα may be induced directly into the brain by LPS acting on astrocytes lying on the blood brain barrier; (3) LPS could stimulate some neural afferents, outside the brain, which in turn trigger the release of TNF in the preoptic area.

LPS and TNFα stimulate NO production, through the induction of a calcium-independent NO synthase, in endothelial and smooth muscle cells, in macrophages and in other cells and tissues (Knowles et al., 1990b; Moncada et al., 1991). Furthermore, treatment of glial cells for 16–18 h with LPS induces a NO synthase-like activity that is L-arginine dependent and Ca2+-independent. The induction is evident after 4 h and depends on the dose of LPS and requires protein synthesis (Simmons & Murphy, 1992). It might be hypothesized that TNFα could induce this NO synthase in the glial cells or, alternatively, it could increase L-arginine uptake into neuronal cells, thus increasing NO production through a calcium-dependent and constitutive NO synthase.

In conclusion, the results of the present study indicate that nitric oxide in the preoptic area is an inhibitory mechanism of drinking behaviour stimulated by water-deprivation and is one of the mediators of the antidiipsogenic effects of TNFα and LPS.

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References


