**Viral hemorrhagic septicemia virus alters turbot**

*Scophthalmus maximus* **macrophage nitric oxide production**

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**ABSTRACT:** The effect of viral hemorrhagic septicemia virus (VHSV) *in vitro* infection on the nitric oxide (NO) production by turbot *Scophthalmus maximus* kidney macrophages has been addressed in the past. Previously, we had determined that only a small fraction of turbot possess head kidney macrophages that respond to a single exposure of lipopolysaccharide (LPS) with NO production (LPS-responsive macrophages), whereas macrophage cultures from other individuals were not activated by LPS alone and needed a combination of stimuli to respond (LPS-non-responsive macrophages). In the current work, we examined the effect of VHSV on NO production by macrophages characterized as LPS-responsive macrophages or LPS-non-responsive macrophages. Combinations of LPS and tumor necrosis factor α (TNF-α) and macrophage-activating factor (MAF) were also used to stimulate the cells for NO production. The effect of VHSV on NO production depends on the response to LPS alone. When a low multiplicity of infection was used (1.78 × 10⁻³), the NO production in response to LPS in LPS-responsive macrophages was significantly decreased. However, LPS-non-responsive macrophage cultures produced NO when a combination of LPS and VHSV was used. In the case of a higher VHSV multiplicity of infection (1.78), no significant change was observed in LPS-non-responsive animals. Combinations of LPS with TNF-α, LPS with MAF, and TNF-α with MAF were used to induce NO production in LPS-non-responsive macrophages. In all these cases, VHSV suppressed NO production, although at a significant level only when a combination of TNF-α and MAF was used for the induction of NO.

**KEY WORDS:** Nitric oxide · Lipopolysaccharide · Macrophage · Viral hemorrhagic virus · Turbot *Scophthalmus maximus* · Macrophage activating factor · Tumor necrosis factor α

**INTRODUCTION**

Macrophages inactivate microorganisms by different mechanisms. This microbicidal activity is, in some cases, mediated by the secretion of reactive nitrogen intermediates (Nathan & Hibbs 1991). The molecules produced are nitrite, nitrate and nitric oxide (NO) (Marletta et al. 1988). Macrophage NO is produced via induction of inducible nitric oxide synthase (iNOS) in response to cytokines, bacterial lipopolysaccharide (LPS) or parasites (Nathan 1992). NO produced by macrophages is known to play an important role in cellular defenses against specific viral infections (Croen 1993, Pertile et al. 1996, Lin et al. 1997, Tafalla et al. 1999), although there are other viruses against which NO has no effect (Kreil & Eibl 1996, López-Guerrero & Carrasco 1998). Moreover, in many cases, NO mediates the viral pathogenesis *in vivo* (Kreil & Eibl 1996, Adler et al. 1997). This pathogenesis may in some cases be mediated through the suppression of T lymphocyte proliferation, a role that has been recently attributed to NO (Allione et al. 1999).

In fish, NO production has been observed in goldfish *Carassius auratus* and catfish *Clarias gariepinus* macrophages using LPS induction (Neumann et al. 1999).
1995, Yin et al. 1997). In the case of gilthead seabream, Sparus aurata L., induction of NO production required both macrophage-activating factor (MAF) and LPS (Mulero & Meseguer 1998). In the turbot Scophthalmus maximus, only about one-third of macrophage cultures (30.2%) were significantly stimulated to produce NO by LPS alone (LPS-responsive macrophages), whereas others required a combination of LPS and tumor necrosis factor α (TNF-α), supernatants with MAF activity or supernatants with interferon (IFN)-αβ activity (LPS-non-responsive macrophages) (Tafalla & Novoa 2000). In the presence of LPS, responsive macrophages generate NO more than 2 times higher than non-stimulated controls, while the non-responsive macrophages, in the presence of LPS alone, do not generate NO concentrations significantly higher than non-stimulated controls. A combination of TNF-α and supernatants with MAF activity also stimulated NO production in LPS-non-responsive macrophages.

Analysis of the effects of viral hemorrhagic septicemia virus (VHSV) on NO production is of particular interest since this virus is known to replicate in macrophages (Estepa et al. 1992, Tafalla et al. 1998). VHSV is one of the most devastating viruses in aquaculture, producing great losses in fish production. In recent years, cultured and wild populations of turbot Scophthalmus maximus have experienced significant mortalities due to VHSV outbreaks (Schlotfeldt et al. 1991, Ross et al. 1994). VHSV is known to replicate in turbot kidney macrophages; however, secretion of oxygen dependent radicals (respiratory burst activity) is not significantly affected by in vitro infection with the virus (Tafalla et al. 1998).

We have studied the effect of VHSV in vitro infection on turbot macrophage LPS induced NO production in LPS-responsive macrophages, as well as on NO induced by combinations of LPS, TNF-α or MAF in LPS-non-responsive macrophages. We studied the effect of these stimuli on VHSV replication to determine whether alterations in the NO production by the virus could be correlated with changes in the susceptibility of macrophages to VHSV due to these substances used for NO induction.

**Virus and cell lines.** VHSV (strain 0771) was used and propagated in TV-1, a fibroblastic cell line derived from turbot Scophthalmus maximus (Fernandez-Puentes et al. 1993), cultured at 18°C in Eagle’s minimal essential medium with Earle’s salts (MEM, Gibco) supplemented with 10% fetal calf serum (FCS, Gibco), containing 100 IU of penicillin and 100 µg of streptomycin ml⁻¹. The virus was inoculated on TV-1 grown in MEM with antibiotics and 2% FCS at 14°C. When the cytopathic effect was complete, the supernatant was harvested and centrifuged to eliminate cell debris. Clarified supernatants were used for the experiments. The virus stock was titrated according to Reed & Muench (1938) in TV-1 96 well plates.

**Isolation of head kidney macrophages.** Macrophages were isolated following the method described by Chung & Secombes (1988). The anterior kidney was removed aseptically and passed through a 100 µm nylon mesh using Leibovitz medium (L-15, Gibco) supplemented with penicillin (100 IU ml⁻¹), streptomycin (100 µg ml⁻¹), heparin (10 units ml⁻¹) and 2% FCS. The resulting cell suspension was placed on a 34/51% Percoll density gradient. The gradients were centrifuged at 500 × g for 30 min at 4°C. The interface cells were collected and washed twice at 500 × g for 5 min in L-15 containing 0.1% FCS. The viable cell concentration was determined by trypan blue exclusion. Cells were resuspended in L-15 with 0.1% FCS and dispensed into 96 well plates at a concentration of 1 × 10⁶ cells ml⁻¹ (1 × 10⁵ cells well⁻¹). An aliquot of the macrophage cultures was placed on a slide and stained with Hema-col (Merck, Darmstadt, Germany). The percentage of macrophages (always higher than 80%) was determined by means of size, shape and staining properties.

After 3 h, the non-adherent cells were removed by washing and the resulting monolayers were exposed to the different treatments or maintained with L-15 with 5% FCS in the case of controls.

**Production of MAF from turbot kidney leucocytes.** Supernatants containing turbot MAF were prepared as described by Graham & Secombes (1988) by stimulation with phorbol myristate acetate (5 ng ml⁻¹) and concavalin A (10 µg ml⁻¹). Supernatants were tested for activity on the respiratory burst of macrophages, assayed through the reduction of ferricytochrome C by the released superoxide, following stimulation of the cells with 1 µg ml⁻¹ of phorbol myristate acetate (Sigma) (Novoa et al. 1996). The supernatants were frozen at –80°C until used. The working dilutions of supernatants with known MAF activity were the dilutions 1:8 and 1:16 in L-15 with 5% FCS.

**Effect of VHSV on macrophage NO production.** To study the effect of VHSV in vitro infection on LPS-induced nitrite production, macrophages were incubated with different concentrations of Escherichia coli.

**MATERIALS AND METHODS**

**Fish.** Turbot Scophthalmus maximus of 40 to 60 g were obtained from a commercial fish farm. The animals were acclimatized to laboratory conditions for 3 wk, maintained at 18°C and fed daily with a commercial diet (Trow, Burgos, Spain). The health status of the animals was checked daily, and they never presented clinical symptoms and none died.
serotype O111:B4 LPS (Sigma), known to induce NO production in LPS-responsive macrophages (0.1 and 0.01 µg ml⁻¹), and 2 doses of VHSV (multiplicity of infection [MOI] of 1.78 × 10⁻³ and 1.78) in L-15 with 5% FCS, which were added simultaneously to the cultures. The LPS doses used have been determined to be optimal for stimulation of turbot macrophages (Tafalla & Novoa 2000). The response to the different doses of LPS by the various turbot was used to characterize their macrophages as LPS-responsive or non-responsive. Non-infected controls and controls without LPS were also included. To assure that iNOS activity was being measured, the iNOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; Sigma) was added in some experiments at a final concentration of 5 mM (Tafalla & Novoa 2000). After 72 h of incubation at 14°C, the nitrite production was assayed in the cultures by the Griess reaction (Green et al. 1982) using a modification of the method described by Neumann et al. (1995). The Griess reaction quantifies the nitrite content of the macrophages supernatants, since NO is an unstable molecule and degrades to nitrite and nitrate. After incubation of macrophages at 18°C, 50 µl of the macrophage supernatants was removed from individual wells and placed in a separate 96 well plate. One hundred microliters of 1% sulfanilamide (Sigma) in 2.5% phosphoric acid was added to each well, followed by 100 µl of 0.1% N-naphthyl-ethylenediamine (Sigma) in 2.5% phosphoric acid. Optical density was determined using a multiscan spectrophotometer (Labsystems, Helsinki) at 540 nm. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite.

We also determined whether VHSV altered the response of turbot LPS-non-responsive macrophages stimulated for NO production with combinations of stimuli. The optimal concentrations for NO induction of the different stimuli used had been previously determined (Tafalla & Novoa 2000). Macrophages isolated as described above were infected with the low VHSV dose (MOI 1.78 × 10⁻³) and immediately the different treatments were added: LPS (0.01 µg ml⁻¹) and human recombinant TNF-α (Sigma, 10 ng ml⁻¹), LPS (0.01 µg ml⁻¹) and MAF-containing supernatants (dilution 1:8) or a combination of MAF (dilution 1:8) and TNF-α (10 ng ml⁻¹). After 3 and 5 d of incubation, the NO production was assayed as described above.

**Determination of VHSV titers.** To determine whether the different cytokines or combinations of cytokines altered the susceptibility of turbot macrophages to VHSV, the viral replication (viral titer) was assayed 72 h after infection with VHSV (MOI 1.78 × 10⁻³). The experiments were conducted as described above and, after 72 h at 14°C, clarified supernatants were collected for titration. The virus present in the supernatants was titrated in TV-1 96 well plates as described before and the log TCID₅₀ was calculated.

**Statistical analysis.** The data were compared using Student’s t-test. Results are expressed as mean ± standard deviation and differences were considered statistically significant at p < 0.05. All treatments were assayed by triplicate in each fish.

**RESULTS**

**Effect of VHSV on NO production induced by LPS**

To classify the macrophages as responsive or non-responsive, in each experiment different concentrations of LPS were added to some wells and the NO production was determined in parallel in order to determine whether macrophages responded to LPS alone (data not shown). The effect of the low VHSV dose (MOI 1.78 × 10⁻³) on the macrophage response to LPS in both types of macrophages is shown in Fig. 1A,B. Fig. 1A shows the effect of the virus on those

![Fig. 1. Effect of the low viral hemorrhagic septicemia virus (VHSV) dose (multiplicity of infection [MOI] 1.78 × 10⁻³) on nitric oxide (NO) production by lipopolysaccharide (LPS)-responsive (A) (n = 5) and LPS-non-responsive macrophages (B) (n = 3). Cells were incubated at 14°C for 72 h. LPS was added at a concentration of 0.01 µg ml⁻¹. The effects of the high VHSV dose (MOI 1.78) on NO production by LPS-responsive (C) (n = 5) and LPS-non-responsive macrophages (D) (n = 3) are also shown. LPS was also added at a concentration of 0.01 µg ml⁻¹. Data are expressed as mean concentration of nitrite (µM). *NO production significantly different from that of macrophages treated with LPS alone (p < 0.05)](image-url)
animals that responded to LPS. These macrophages were significantly stimulated for NO production with 0.01 µg ml⁻¹ of LPS. In this case, the virus in combination with LPS decreased the production of NO compared with that observed when macrophages were incubated with LPS alone. However, macrophages that did not respond to LPS (Fig. 1B) when incubated with the virus and LPS had a significantly increased NO production upon infection. These 2 responses were always observed when the experiments were repeated.

In LPS-responsive macrophages, the effect of the high VHSV dose (MOI 1.78) was the same as that observed with the low viral dose (Fig. 1C). However, when the high dose was used, no significant effect on NO production was observed in LPS-non-responsive macrophages (Fig. 1D).

In all cases, the VHSV alone never significantly altered the NO production of macrophages after 72 h of incubation.

**Effect of L-NAME on NO production**

To determine whether the iNOS activity was really being measured, L-NAME was added. The results obtained when L-NAME was added to LPS-non-responsive macrophage cultures are shown in Fig. 2. The inhibitor significantly decreased the nitrite production observed with and without LPS. In this experiment, again the nitrite production observed with LPS and the low VHSV dose was significantly higher than that observed with LPS alone, and L-NAME partially reversed this NO induction. This decrease was only significant when individual data and not mean values were compared. In the case of LPS-responsive macrophage cultures, the inhibitor significantly decreased the LPS-induced nitrite production (Fig. 3).

**Effect of VHSV on the response to combinations of stimuli in LPS-non-responsive macrophages**

The effect of VHSV (low MOI) on NO induced by LPS combined with TNF-α or with MAF, and with TNF-α combined with MAF was assayed. The results observed after 3 d of incubation are shown in Fig. 4A. All the combinations induced NO production in individual macrophage cultures; however, when mean values were compared, only TNF-α and MAF were found to significantly induce NO. In this case, VHSV significantly suppressed this NO induction. The same response was observed after 5 d of incubation (Fig. 4B). In the non-infected controls, after 3 d of incubation, the NO produced in response to TNF-α and MAF was significantly higher than that observed with LPS and TNF-α; however, after 5 d of incubation, the NO production observed with the different combinations was not significantly different.

**Effect of the different stimuli on viral replication in turbot macrophages**

To determine whether the differences in NO production induced by the various inducers in the presence of VHSV were due to an altered replication of VHSV in macrophages treated with these substances, the viral replication with and without stimulation was determined. No significant differences were found between the VHSV titer obtained in macrophages treated with any of the substances or combinations and the viral titer observed with VHSV alone. After 3 d of incubation, the log TCID₅₀ ml⁻¹ in the presence of LPS was 3.8 ± 0.37 and 4 ± 0.5 in cultures treated with only VHSV. In all cases, the virus replicated, since the viral titer increased with time.
DISCUSSION

It has been shown that VHSV replicates in turbot macrophages, although only a small percentage of the cells support active replication (1.7%) (Tafalla et al. 1998). Concerning turbot macrophage NO production induced by LPS, we have previously found that macrophages isolated from individual turbot can exhibit 2 different responses to LPS: about one-third of the animals yielded macrophages that produced NO in response to a single stimulation with LPS (30.2%) (LPS-responsive macrophages) whereas the others required a combination of LPS and TNF-α, IFN-γ or IFN-αβ (LPS-non-responsive macrophages) (Tafalla & Novoa 2000). Some authors reported that, in mice, macrophages do not respond to LPS alone but need a previous exposure to IFN-γ (Krei & Eibl 1995) but other authors have reported stimulation of NO production with LPS alone (López-Collazo et al. 1998). In the case of bovine macrophages and neutrophils, it has been shown that, in some individuals, both cell types respond to individual stimulants like IFN-γ or TNF-α, while other individuals require a combination of IFN-γ and TNF-α to produce NO (Goff et al. 1996).

In turbot, we have found that when macrophages are infected in vitro with a low VHSV dose, responses are strikingly different in LPS-responsive versus LPS-non-responsive macrophages. In LPS-responsive macrophages, the virus caused a decrease in NO production, whereas in LPS-non-responsive macrophages a significant increase in NO production with a combination of LPS and VHSV was observed. When combinations of stimuli were used to induce NO, we also found a suppression of the NO production in macrophages infected with VHSV. This suppression was significant when TNF-α and MAF were combined, probably because this was the combination that induced the higher NO production. This suppression was observed after 3 and 5 d of incubation.

The suppression of NO production seen in both cases (LPS-responsive macrophages stimulated with LPS and LPS-non-responsive macrophages stimulated with a combination of MAF and TNF-α) could be due to intrinsic interactions between macrophages and the virus that usually alter macrophage functions (Morahan et al. 1985). We determined that these substances used in NO induction do not alter the replication of VHSV in turbot macrophages (viral titer after 3 d), so this decrease was not due to an increased susceptibility but rather to an interaction between the macrophages and the virus. It is known that viral infections usually produce a depression of macrophage functions, even when only a subpopulation is infected, although no effect and even enhanced activity have also been reported (Lussenhop et al. 1982, Mogesen 1982). Macrophages are known to be heterogeneous for viral infection, as frequently a subpopulation is infected and the outcome of the infection is dependent on a great number of host-related factors, including the state of differentiation or activation of the immunocompetent cells or even genetic determinants (Morahan et al. 1985).

The up-regulation of NO production observed in the case of the LPS-non-responsive macrophages in the presence of VHSV could be explained as a result of an antiviral defense mechanism being triggered in macrophages, since NO is known to have an antiviral effect on many viruses (Croen 1993, Pertile et al. 1996, Lin et al. 1997), including antiviral activity toward VHSV (Tafalla et al. 1999). It is known that viral infection of macrophages leads to the production of IFN-αβ (de Maeyer & de Maeyer-Guignard 1991) and that in some cases this IFN has a priming effect on macrophages for further LPS activation (Fujihara et al. 1994, Krei & Eibl 1995). In mice, it was determined that macrophages were not stimulated to produce NO with LPS alone and required another activating factor, while virus-infected
macrophages produced NO in response to LPS alone due to the secretion of IFN-αβ in infected macrophages (Kreil & Eibl 1995). In turbot, it has been previously determined that a combination of LPS and IFN-αβ significantly stimulates otherwise non-responsive macrophages for NO production (Tafalla & Novoa 2000). It could be possible that these LPS-non-responsive macrophages produce IFN-αβ in response to the virus. This IFN-αβ secretion would also make them less susceptible to VHSV infection, since IFN-αβ is known to have antiviral activity (de Maeyer & de Maeyer-Guignard 1991). It is known that constitutive viral resistance is usually mediated through high NO production (López-Guerrero et al. 1997). This up-regulation in response to VHSV disappeared when the high VHSV was used, suggesting that if this response constitutes some type of antiviral activity, it is significant only at low viral doses.

In conclusion, turbot macrophages that differ in their response to LPS also differ in their response to VHSV infection, which is probably related to the outcome of the infection. Whenever NO production was induced, VHSV suppressed this induction, whereas when NO was not induced, VHSV acted as a second stimulating signal or co-stimulator to induce NO production.

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LITERATURE CITED


