Nitric Oxide Involvement in *Hydra vulgaris* Very Primitive Olfactory-Like System

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Hydra feeding response is a very primitive olfactory-like behavior present in a multicellular organism. We investigated the role of nitric oxide (NO) in the induction and control of hydra feeding response. Under basal conditions, hydra specimens produce detectable amounts of nitrite (NO₂⁻), the breakdown product of NO. When hydra were incubated with reduced glutathione (GSH), the typical activator of feeding response, an increase of basal NO production was observed. This effect was inhibited by glutamic or α -aminoadipic acids, two GSH antagonists, which block GSH-induced feeding response, and by the NO synthase (NOS) inhibitor L-NAME. Moreover, we found that hydra possess a calcium-dependent (but calmodulin-independent) NOS

isoform. By using exogenous NO donors and NOS inhibitors, we demonstrated that NO stimulus can participate both in triggering tentacular movements and in recruiting neighbor tentacles during hydra feeding response. By using dbt²-cGMP, an analog to cGMP, we observed that the NO effect was independent of cGMP pathway. Our results strongly implicate NO involvement in hydra very primitive feeding behavior, thus confirming its preservation throughout evolution.

Key words: nitric oxide; NO synthase; cyclic GMP; hydra; feeding response; primitive olfactory-like model; chemosensory system

Nitric oxide (NO) is an unstable nitrogen radical spontaneously degrading into nitrites and is generated by the conversion of L-arginine (L-Arg) into citrulline, through the NO synthase (NOS) enzyme. In mammalian neurons, a constitutive Ca²⁺-dependent (cNOS) isoform is activated by the glutamatergic pathway stimulation, as after and consequent to a raised cytosolic Ca²⁺ influx (Garthwaite, 1991; Snyder and Brecht, 1991). NO can exert its biological activity via the stimulation of soluble guanylate cyclase (Garbers, 1992), thus leading to an increase in cGMP.

More recently, we have observed for the first time that, surprisingly, the NO–cGMP pathway is present in the freshwater coelenterate *Hydra* (Colasanti et al., 1995), the most primitive organism possessing a nervous system. Hydra is a sessile predator whose tentacles are armed with the typical, characteristic stinging capsules of the coelenterates called nematocysts. When a prey accidentally touches a tentacle, a typical feeding response is activated. Hydra feeding response, which can be considered the most primitive olfactory-like behavior present in a multicellular organism, is a complex behavioral phenomenon consisting of tentacle writhing and mouth opening. Loomis (1955) determined that the reduced glutathione (GSH) outflow from the prey when pierced by tentacle nematocysts is the physiological activator of hydra feeding response. GSH is thought to interact with a specific receptor, the presence of which in hydra tissues was demonstrated by radioligand binding methods (Venturini, 1987) and has been recently characterized, solubilized, and partially purified (Bellis et al., 1991, 1992, 1994). However, no data are available concerning the cellular localization of GSH receptor. Hydra feeding response

occurs a few seconds after GSH addition, reaches its peak within few minutes, and gradually disappears in ~10 min. Different structural GSH analogs, i.e., glutamic or α -aminoadipic acids, which bind to but do not activate GSH receptors, are able to competitively inhibit GSH activity in eliciting the feeding response (Lenhoff, 1981).

Despite the obvious interest for the study of a primitive chemosensory system as a simple olfactory-like model, little is known about the molecular mechanisms regulating hydra feeding response. Previous reports have indicated that the cytosolic Ca²⁺ influx (Lenhoff, 1981) and the glutamatergic system (Venturini, 1987) are both involved in the induction of feeding response and that calcium ionophore A23187 enhances this response (Venturini et al., 1988). Recently, it has been reported that the inter-neuronal messenger NO seems to play a central role in the processing of olfactory information in invertebrates (Gelperin, 1994). In particular, a behavioral role for NO in chemosensory activation of feeding in a mollusk has been demonstrated (Elphick et al., 1995). In this paper, we have investigated on NO involvement in both induction and control of hydra feeding response.

At present, however, no data are available concerning the interaction between GSH receptors and the NO pathway. Therefore, we decided to approach this problem using GSH antagonists in a study aimed at clarifying the role of the NO–cGMP pathway in the feeding response induced by either GSH or food using NOS inhibitors, exogenous NO donors, and a cGMP analog.

MATERIALS AND METHODS

Materials. Oxyhemoglobin (oxyHb) was prepared from commercial bovine hemoglobin (Hb; Sigma, Milan, Italy) by reduction with a 10-fold excess sodium hydrosulphite (Aldrich, Milan, Italy), followed by gel filtration on prepacked G-25 columns (Pharmacia, Uppsala, Sweden) and equilibration in air; authentic NO solution (NOsol) was obtained by a 30 min bubbling of distilled and deoxygenated water with >99.5% pure NO gas at 4°C. This stock solution is in the low millimolar range (~2 mM at 25°C). GSH was from Merck Italia (Milan, Italy). Sulfanilamide, *N*-(1-

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naphtyl)ethylenediamine (NEDA), *N*^ω-nitro-L-arginine methylester (L-NAME), *N*^ω-nitro-D-arginine methylester (D-NAME), L-Arg, sodium nitroprusside (SNP), 3-morpholino-sydnominine (SIN-1), *N*-(6-aminohept-5-yl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W7), trifluoperazine (TFP), NADPH, *N*²,2'-*O*-dibutylguanosine-3',5'-cyclic monophosphate (dbt²-cGMP), α-amino adipic acid, and glutamic acid were from Sigma. L-2,3,4,5-[³H]arginine was from Amersham Italy (Milan, Italy).

Hydra cultures and feeding response assay. *Hydra vulgaris* (formerly *Hydra attenuata*) from a strain originally provided by P. Tardent (University of Zurich, Zurich, Switzerland) were grown in 1 mM CaCl₂ + 1 mM NaHCO₃ (Lenhoff and Brown, 1970) at 18°C and fed daily with nauplia of *Artemia salina*. Specimens were kept unfed for 72 hr before experiments.

Behavioral observations were performed using a blind procedure in which treatments and doses were unknown. For each observation, 10 specimens were placed in a Petri dish (3.5 cm diameter) containing the tested drug, dissolved in 3 ml of 1 mM CaCl₂ + 1 mM NaHCO₃. Feeding response was induced with GSH (2.5 μM) or nauplia (~300). The effect of the added drugs and food was studied by recording the number of specimens per minute showing the typical feeding response (tentacle curling and mouth opening) under a stereomicroscope. For each experiment, one group of 10 specimens treated with GSH alone was used as control. Each point represents mean ± SEM of 10 experiments.

Analysis of nitrite levels. Nitrite (NO₂⁻) was determined by the Griess reaction, according to the method of Tracey (1992). Briefly, 170 μl of a solution consisting in 1 mM CaCl₂ + 1 mM NaHCO₃ and containing 100 hydra either untreated or treated with GSH or nauplia for 60 min (3 pulses every 10 min) was mixed with 10 μl of sulfanilamide (1 mM final concentration) and 10 μl of HCl (0.1N final concentration). The final reaction step was accomplished by the addition of 10 μl of NEDA (1 mM final concentration). After a 10 min incubation at room temperature, the absorbance was measured at 548 nm, and nitrite concentration was determined using sodium nitrite as a standard. Results are expressed for NO₂⁻ as nmol · ml⁻¹ · 60 min⁻¹.

Spectroscopic analyses. NO production in hydra supernatants was monitored both optically, after the NO-mediated conversion of oxyhemoglobin to methemoglobin (Feilisch et al., 1996), and by revealing nitrosyl-hemoglobin formation by ESR spectroscopy (Kosaka and Shiga, 1996). Optical spectra were recorded on a Perkin-Elmer 330 spectrometer (Emeryville, CA). ESR spectra were measured at X band (~9 GHz) and at 100 K on a Varian E-109 spectrometer interfaced to a Stellar Prometheus Data Handling System.

Determination of NOS activity. NOS activity was measured by evaluating the conversion of [³H]arginine to [³H]citrulline (Rengasamy, 1992). Briefly, either 200 hydra specimens or 160 mg of mouse brain was homogenized in 800 μl of buffer containing 50 mM HEPES, 0.5 mM EDTA, 1 mM dithiothreitol, and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), pH 7.5, and centrifuged at 39,000 × g for 30 min. NOS activity was assayed incubating 340 μl of the supernatant, 1 mM NADPH, 0.45 mM CaCl₂, 100 μM L-Arg, 1 μCi/ml [³H]arginine monohydrochloride in a total volume of 400 μl. In some control samples, L-NAME (100 μM), D-NAME (100 μM), and excess L-Arg (200 μM) were used. After 5, 15, 30, and 60 min incubation at 37°C, the reaction was stopped by mixing 150 μl of the reaction mixture with 2 ml of 20 mM HEPES, pH 5.5, containing 2 mM EDTA. The mixture was loaded on a 1 ml Dowex AG50WX-8 (Na⁺ form) column and eluted with 5 ml of bidistilled H₂O, and [³H]citrulline so obtained was measured by a beta counter. The ratio between labeled citrulline (cpm × 10⁻³) and mg of protein assayed in the homogenate according to the Lowry's method (Lowry et al., 1951) was taken as NOS activity.

Determination of cGMP. Pools of 10 specimens were sonicated in 600 μl of ice-cold trichloroacetic acid (5% w/v). After centrifugation at 10,000 rpm for 10 min, supernatants were washed five times with ethyl ether and then lyophilized. cGMP contents were measured, after reconstitution with 0.05 M sodium acetate buffer (pH 5.8), by using a commercial specific enzyme immunoassay (EIA) system (Amersham, Buckinghamshire, UK). Changes of absorbance at 450 nm were calculated by a microtiter plate photometer, and a standard curve ranging from 2 to 500 fmol/well was used to calculate unlabeled cGMP in each well. Data are expressed as fmol of cGMP per 10 specimens per well.

Statistics. Student's unpaired *t* test was used for significant differences between means, except in the feeding response assay, in which a two-way ANOVA test was used for significant differences between treatments.

Figure 1. Nitrite (NO₂⁻) release in hydra supernatants. Reduced glutathione (GSH; 2.5 μM) treatment increases basal NO₂⁻ levels, as measured by the Griess reaction. A 1 hr pretreatment of hydra by injecting the specific NOS inhibitor L-NAME (L-NAME; 100 μM) into the gastric cavity abolishes GSH-induced NO₂⁻ levels. This effect is reversed by excess L-Arg (L-ARG; 200 μM), whereas the D-isomer of NAME (D-NAME; 100 μM) is inactive. Also, glutamic acid (GLU; 10 μM) or α-amino adipic acid (AAD; 10 μM) reduces GSH-induced NO₂⁻ production. Finally, nauplia increase basal NO₂⁻ levels, this effect being abolished by L-NAME (100 μM). Results are expressed for NO₂⁻ in nmol · ml⁻¹ · 60 min⁻¹, and each bar represents mean ± SEM of three experiments. *p* ≤ 0.001 between basal and GSH or nauplia, between GSH and GSH + L-NAME or GSH + GLU, and between nauplia and nauplia + L-NAME; *p* ≤ 0.01 between GSH and GSH + AAD.

RESULTS

Analysis of nitrite levels

Under basal conditions, hydra specimens produced detectable amounts of NO₂⁻, the breakdown product of NO, in a range of 0.18–0.20 nmol · ml⁻¹ · 60 min⁻¹ (Fig. 1), as measured by the Griess reaction (Tracey, 1992). A treatment of hydra with GSH (2.5 μM), the typical inducer of hydra feeding response, caused a significant (*p* ≤ 0.001) increase in NO₂⁻ levels (0.19 ± 0.01 to 0.52 ± 0.02 nmol/ml). This effect was reduced by the specific NOS inhibitor *N*^ω-nitro-L-Arg methylester (L-NAME). In fact, when hydra were injected with L-NAME (100 μM) into the gastric cavity and preincubated for 1 hr, the GSH-induced NO₂⁻ levels were significantly (*p* ≤ 0.001) decreased (0.52 ± 0.02 to 0.08 ± 0.00 nmol/ml; see Fig. 1), this effect being reversed by excess L-Arg (200 μM; 0.08 ± 0.00 to 0.57 ± 0.03 nmol/ml). On the contrary, the D-isomer of NAME (100 μM) was inactive (Fig. 1). To test the interaction between GSH receptors and NO production, we examined the role of GSH antagonists that inhibited GSH-induced feeding response (see Table 2) on hydra NO₂⁻ release. In fact, when hydra were preincubated with glutamic acid (10 μM) or α-amino adipic acid (10 μM), the GSH-induced NO₂⁻ production was significantly reduced (0.52 ± 0.02 to 0.16 ± 0.01 and 0.32 ± 0.05 nmol/ml, respectively). Finally, nauplia (~300), the physiological activators of hydra feeding response, increased basal NO₂⁻ levels (0.19 ± 0.01 to 0.65 ± 0.04 nmol/ml), as shown in Figure 1. This effect was abolished by preincubation of hydra with L-NAME (100 μM; 0.65 ± 0.04 to 0.05 ± 0.005 nmol/ml; Fig. 1). We verified that nauplia (~300), either whole or injured, in the absence of hydra did not release NO, as determined by measuring nitrite levels (data not shown).

ESR analysis

Both optical and ESR spectroscopy of hemoglobin were used to probe the NO released by hydra. To do this, oxyhemoglobin (25

Figure 2. Spectroscopic detection of NO release in hydra supernatants. Hemoglobin (25 μ M) added to the medium is converted from the oxy (*a*) to the met form (*b*) after treatment of hydra with 2.5 μ M GSH as monitored by optical spectroscopy. Spectra are measured after a fivefold dilution of the sample. The inset shows the corresponding X-band low-temperature ESR curves at $g \approx 2$, which reveal the GSH-induced selective formation of a small amount of nitrosyl-hemoglobin (*a'*, control; *b'*, GSH-stimulated hydra). Arrow corresponds to 5 mT.

μ M) was included in hydra supernatants before the addition of 2.5 μ M GSH. Figure 2 shows the optical spectra of hemoglobin in supernatants from unstimulated (*a*) and GSH-stimulated (*b*) hydra. The spectral variations observed are consistent with the massive conversion of oxyhemoglobin (oxyHb) to methemoglobin (metHb). This conversion is typical of oxyHb reacting with NO (Kelm et al., 1988) and was not observed when hemoglobin was treated with GSH in the absence of hydra (data not shown). The corresponding low-temperature ESR spectra are shown in the inset of Figure 2. As can be seen, no signal is detected in the control sample from unstimulated hydra (*a'*), whereas a resonance centered at $g \approx 2.004$ appears after treatment with GSH (*b'*). This resonance can be safely ascribed to nitrosyl-hemoglobin (Hb-NO), which is formed either from NO reaction with the small fraction of reduced, deoxy-hemoglobin present under our conditions or, more likely, from a complex pathway leading from oxyHb to Hb-NO through formation of metHb (Kosaka and Shiga, 1996).

Ca²⁺-dependent NOS activity

NO production was the result of a basal expression of NOS activity, as measured by a time-dependent [³H]citrulline generation from [³H]arginine (Fig. 3*A*). As shown in Figure 3*B*, in hydra homogenates [³H]citrulline production was dependent on the presence of NADPH (94.6 ± 7.2 cpm $\times 10^{-3}$ /mg protein). Moreover, NOS activity of hydra homogenates incubated with 1 mM NADPH was inhibited by co-incubation with L-NAME (100 μ M; 94.6 ± 7.2 to 44.54 ± 3.6 cpm $\times 10^{-3}$ /mg protein; Fig. 3*B*). This effect was reversed by excess L-Arg (200 μ M; 44.54 ± 3.6 to 84.00 ± 5.93 cpm $\times 10^{-3}$ /mg protein), whereas D-NAME (100 μ M) was inactive, as shown in Figure 3*B*.

In addition, the removal of Ca²⁺ by EGTA (2 mM) from the incubation medium greatly affected citrulline formation (94.6 ± 7.2 to 34.54 ± 6.36 cpm $\times 10^{-3}$ /mg protein), showing that NOS isoform was Ca²⁺-dependent (Fig. 3*B*).

Calmodulin-independent NOS activity

Interestingly, we found that the NOS isoform was calmodulin (CaM)-independent. In fact, high concentrations of CaM inhibitors,

Figure 3. NOS activity in hydra homogenates. In *A*, a time-dependent [³H]citrulline production is shown. *B*, Constitutively, hydra express NOS activity in the presence of NADPH, this expression being reduced by L-NAME (100 μ M). Excess L-Arg (200 μ M) reverses the L-NAME effect, whereas D-NAME (100 μ M) is inactive. NOS isoform results in being Ca²⁺-dependent. In fact, when resuspended in EGTA/Ca²⁺-free buffer, NADPH-incubated hydra homogenates show a significant decrease in NOS activity. Data are expressed as the ratio between [³H]citrulline production (cpm $\times 10^{-3}$) and mg protein as assayed in the homogenates. In *C* the isoform of NOS is shown to be CaM-independent. In fact, CaM inhibitors, such as W7 (50 μ M) and trifluoperazine (TFP; 50 μ M), do not alter NOS activity. In contrast, both W7 and TFP are able to strongly inhibit mouse brain Ca²⁺/CaM-dependent [³H]citrulline generation. Results are expressed as percent NOS activity with respect to the basal expression in NADPH-incubated hydra homogenates. Each bar corresponds to mean $\pm$ SEM of samples performed in triplicate; * $p \leq 0.001$.

Table 1. Exogenous NO donors are able to elicit an incomplete feeding response^a

	Contr	SIN-1 (10 μ M)	SNP (20 μ M)	NOsol (30 μ M)	dbt ² -cGMP (100 μ M)
Alone	–	+	+	+	–
OxyHb (10 μ M) ^b	–	–	–	–	–
L-NAME (100 μ M)	–	+	+	+	–

^a The incomplete feeding response is represented by tentacle curlings identical to those elicited by GSH, but no mouth opening.

^b Oxyhemoglobin (OxyHb), a trapping agent for NO, is coincubated with NO donors 30 min before treatments.

such as *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride (W7; 50 μ M) and trifluoperazine (TFP; 50 μ M), either alone or in synergism, did not alter NOS activity (Fig. 3C). In our experimental conditions, the efficiency of the above mentioned CaM inhibitors was tested on mouse brain Ca²⁺/CaM-dependent NOS activity. As shown in Figure 3C, both W7 (50 μ M) and TFP (50 μ M) were able to strongly inhibit mouse brain [³H]citrulline generation.

Effect of exogenous NO donors on hydra feeding response

By using exogenous NO donors, we studied the role of NO in the control of hydra feeding response. In this respect, we found that 3-morpholino-sydnimine (SIN-1) elicited an incomplete feeding response consisting in tentacular movements similar to those of GSH-induced feeding response (Lenhoff, 1981), but without the typical mouth opening (see Fig. 5D). When tested at concentrations of 0.03–100 μ M, the effect of SIN-1 exhibited a bell-shaped profile. Tentacle curlings were absent at low levels of SIN-1 (0.03 μ M). They were observed to gradually occur up to 0.1 μ M, then to rise sharply up to a peak of 1–10 μ M. At higher SIN-1 concentrations, movements were seen to decline.

As shown in Table 1, an induction of an incomplete feeding response similar to the one observed after SIN-1 stimulation was also obtained by using NO donors like sodium nitroprusside (SNP; 20 μ M) or authentic NO solution (NOsol; 30 μ M). The NO donor-induced tentacle contraction was completely inhibited by oxyHb (10 μ M), a trapping agent for NO, but not by L-NAME (100 μ M; Table 1). Like GSH-induced feeding response, NO-stimulated tentacle curlings appeared a few minutes after addition of NO donors and then slowly disappeared. After 15 min, all specimens exhibited a normal behavior.

Involvement of cGMP

Because NO exerts its biological activity by stimulating the soluble guanylate cyclase, the role of cGMP in this event was studied. As shown in Figure 4, a basal level of cGMP (117 $\pm$ 22 fmol per 10 specimens) was present. Treatment of hydra with GSH (2.5 μ M) increased production of cGMP levels, a peak being observed after a 1 min treatment (from 117 $\pm$ 22 to 297 $\pm$ 27 fmol per 10 specimens; Fig. 4). This maximal effect was abolished by preincubation of hydra with L-NAME (100 μ M) (from 297 $\pm$ 27 to 144 $\pm$ 31 fmol per 10 specimens), indicating that cGMP generation is attributable to NO synthesis. To test the role of cGMP in the induction of hydra feeding response, *N*²,2'-*O*-dibutylguanosine-3',5'-cyclic monophosphate (dbt²-cGMP), a cGMP analog, was used. When hydra were incubated with 100 μ M dbt²-cGMP alone, neither tentacle curlings nor mouth opening was observed (Table 1).

Figure 4. cGMP production in hydra. GSH (2.5 μ M) induces an increase in hydra levels of cGMP, and the maximal effect is observed after 2 min of treatment. This effect is inhibited by a 24 hr preincubation of hydra with L-NAME (L-NAME; 100 μ M), showing that the production of cGMP depends on NO release. Data are expressed as fmol of cGMP per 10 specimens per well. Each point represents mean $\pm$ SEM of three experiments performed in triplicate; **p* $\leq$ 0.01.

Effect of NO inhibitors on hydra feeding response

In a typical GSH-induced feeding response, all tentacles were simultaneously curled, as shown in Figure 5B. A pretreatment of hydra with the NOS inhibitor L-NAME (100 μ M) or with oxyHb (10 μ M) for 1 hr did not alter the induction of tentacular movements of GSH-induced feeding response (Table 2). However, when prey such as nauplia of *Artemia salina* are pierced by a tentacle nematocyst, the hydra feeding response begins with activation of the same tentacle touched by nauplia and then spreads to the neighboring tentacles (Fig. 5E). When hydra were preincubated with L-NAME (100 μ M) for 1 hr, nauplia were able to induce curlings only in the tentacles directly touched by nauplia, whereas all the other untouched tentacles were at complete rest (Fig. 5F). The same effects were also obtained by co-incubating hydra specimens with oxyHb (10 μ M) for 1 hr (Table 2).

DISCUSSION

NO production in hydra

In this study, we report that NO is involved in hydra feeding response, the most primitive olfactory-like behavior present in a multicellular organism. Hydra are able to release basal levels of NO, as determined by measuring nitrite, the NO breakdown product (Tracey, 1992). Nitrite production was also verified by fluorimetric assay according to the method of Misko (Misko et al., 1993) (data not shown). Optical and ESR spectroscopies further proved NO production. The conversion of oxyHb to metHb (see Fig. 2) provided strong evidence that NO had been selectively released by GSH-treated hydra, because NO is known to act as an oxidant on oxyHb (Kelm et al., 1988). The ESR result was even more clear-cut, in that a signal with features typical of nitrosyl-Hb appeared in Hb-containing hydra supernatants after the GSH stimulus. Note that the intensity of this latter signal was lower than the massive phenomenon observed by optical spectroscopy. This is not surprising because, under aerobic conditions, Hb is mostly in the oxy form, which converts to metHb in the presence of NO, and only a very small fraction escapes oxidation forming the nitrosyl adduct. This adduct, on the other hand, can also be

Figure 5. Hydra feeding behaviors. Hydra at rest (*A*). GSH ($2.5\ \mu\text{M}$) induces a typical feeding response consisting of tentacle-simultaneous curlings and mouth opening (*B*). In *C* a mouth-opening detail (arrowhead) is shown. When hydra are co-incubated with $10\ \mu\text{M}$ SIN-1, tentacle curlings are elicited, but not mouth opening (see arrowhead in *D*). Nauplia (arrowhead in *E*) elicit a typical feeding response: the tentacles piercing nauplia are initially activated, and the response successively spreads to the neighboring tentacles (*E*). When hydra are preincubated with L-NAME ($100\ \mu\text{M}$), the tentacle piercing nauplia is curled (see arrowhead), whereas all nauplia-untouched tentacles are completely at rest (*F*). Magnification, $10\times$.

produced in a sequence as follows: after a binding of NO to metHb; autooxidation of the complex to Hb(II)NO^+ ; dissociation of NO^+ ; and binding of a second molecule of NO to reduced iron (Kosaka and Shiga, 1996). The basal production of NO was enhanced in the presence of GSH, the activator of hydra feeding response. This effect was abolished by the specific NOS inhibitor L-NAME. Excess L-Arg reversed the L-NAME effect, whereas the D-isomer of NAME was inactive, thus demonstrating that NO_2^- production was dependent on L-Arg metabolism. In addition, GSH-induced NO production was also decreased in the presence of glutamic or α -aminoadipic acids, two GSH antagonists, which bind to but do not activate GSH receptors (Lenhoff, 1981), showing that NO synthesis was attributable to GSH receptor activation. It is worth noting that the same compounds are able to competitively inhibit the activity of GSH in eliciting hydra feeding response (see Table 2). Finally, nauplia caused an increase of basal nitrite release, whereas nauplia alone did not produce nitrite. This

effect was inhibited by L-NAME, thus demonstrating that also a physiological stimulus of hydra feeding response (Table 2) was able to activate the L-Arg-NO pathway.

NOS expression in hydra

We have observed that hydra constitutively express a NADPH/ Ca^{2+} -dependent NOS activity, as verified by evaluating the conversion of $[^3\text{H}]$ arginine to $[^3\text{H}]$ citrulline. L-NAME was able to reduce significantly the *in vitro* NOS activity, whereas D-NAME was inactive. In addition, excess L-Arg reversed the effect of L-NAME, demonstrating that $[^3\text{H}]$ citrulline production was attributable to L-Arg metabolism. Because Ca^{2+} ions are not required for the binding of GSH to its receptor (Venturini, 1987), GSH may stimulate NOS by activating a receptor-linked calcium channel. Interestingly, hydra NOS appears to be CaM-independent. It should be pointed out that Ca^{2+} -binding protein(s) other than CaM in hydra may account for NOS regulation.

Table 2. Effect of NO inhibitors on hydra feeding response induced by reduced GSH and nauplia

Inhibitor	Treatment		
	Contr	GSH ^a (2.5 μ M)	Nauplia
None	–	+	+ ^b
OxyHb (10 μ M)	–	+	– ^c
L-NAME (100 μ M)	–	+	– ^c
Glutamic ac. (10 μ M)	–	–	–
α -aminoadipic ac. (10 μ M)	–	–	–

^a In the GSH-induced feeding response, tentacle curlings are simultaneously elicited.

^b In the nauplia-elicited feeding response, only the directly touched tentacles are activated, and then the stimulus spreads to the neighboring tentacles.

^c Oxyhemoglobin (OxyHb), a trapping agent for NO, and the NOS inhibitor L-NAME prevent the activation of all tentacles except those directly touched by nauplia.

It is very intriguing to note that a CaM-independent/Ca²⁺-dependent NOS has been described already in the catfish taste organ (Huque and Brand, 1994) as well as in rat neutrophils (Yui et al., 1991). Although no evidence is available concerning the molecular evolution of NOS, our results suggest the hypothesis that the primitive NOS isoform as appearing throughout evolution may be a CaM-independent isoform.

Role of the NO–cGMP pathway in hydra feeding response

By using exogenous NO donors like SIN-1, SNP, or authentic NO solution, we observed that an incomplete feeding response was elicited, consisting of tentacular movements similar to those of GSH-induced feeding response, but without the typical mouth opening. This effect was completely inhibited by oxyHb, a trapping agent for NO, but not by L-NAME, thus demonstrating that the effect was attributable to exogenous NO.

Because NO is a potent inducer of soluble guanylate cyclase (Garbers, 1992), the role of cGMP in this event was analyzed. A basal level of cGMP was present in hydra, and GSH treatment was able to significantly increase cGMP production. This effect was abolished by L-NAME, showing that GSH-induced cGMP generation derives from NO synthesis. However, we observed that an analog to cGMP, dbt²-cGMP, was unable to trigger tentacle curlings. It is worth noting that the same amount of dbt²-cGMP was capable to make the GSH-induced hydra feeding response shorter (Colasanti et al., 1995), thereby demonstrating the efficiency of the cGMP analog. Thus, these results show that NO was sufficient to induce tentacular movements and that this process was independent of NO-stimulated cGMP production. The dissociation of the NO and cGMP pathways has been reported elsewhere for many processes (Brunner et al., 1995; Brune et al., 1996; Vallette et al., 1996). In many of these cases, this dissociation appears to be attributable to the redox state of NO (Stamler et al., 1992), even if in our context, other different mechanisms may be involved.

Again, we along with other authors have indicated that factors such as cAMP, IP₃, and/or Ca²⁺ can be important as primary mediators of the GSH-induced hydra feeding response (Cobb et al., 1980; Lenhoff, 1981; Venturini, 1987; Venturini et al., 1988). Our experiments show that NO does not seem to be a primary stimulus in eliciting feeding response. In fact, a pretreatment of hydra with NO inhibitors did not alter the tentacular movements of the GSH-induced feeding response. However, it is interesting

to note that in GSH-induced feeding response, all tentacles are simultaneously curled because of all available receptors for GSH being stimulated. This massive stimulation can induce a cascade of the primary mediators in all tentacles, in a way likely to disguise the NO stimulus-induced effect. On the contrary, when a prey (i.e., nauplia) accidentally touches a tentacle, initially a certain amount of GSH is released locally, thus stimulating only the local GSH receptors present in that tentacle. In fact, the nauplia-induced hydra feeding response begins with the activation of the tentacle touched by nauplia and successively spreads to the neighboring tentacles. In these conditions, the movements of all of the nauplia-untouched tentacles were totally inhibited by the NO inhibitors, whereas the nauplia-touched tentacle still curled, thus demonstrating that NO inhibitors prevent the diffusion of the activator stimulus to the neighboring tentacles.

The present data suggest that in hydra, NO stimulus can participate in the triggering and coordination of tentacular curling, providing for the fast diffusion of a primary stimulus to the neighboring tentacles, regardless of any direct connection through synapses (tentacle recruitment). NO stimulus, however, is not sufficient for complete induction of the complex behavioral phenomenon.

Therefore, we propose that initially GSH induces the feeding response through mediators such as cAMP, IP₃, and/or Ca²⁺, while successively, an increase of GSH-induced Ca²⁺ levels is responsible for NO production, which in turn elicits the recruitment of neighboring tentacles. Finally, on a longer timescale, elevated NO-induced cGMP levels are able to trigger inhibition of the GSH-induced feeding response, as described elsewhere (Colasanti et al., 1995), presumably via cGMP-activated protein kinases. Taken together with data in the literature, our results are consistent with those reported for the mammalian olfactory system (Breer and Shepherd, 1993). In the latter, in fact, the rapid and transient generation of pulses of cAMP and/or IP₃ is considered the primary reaction in olfactory signal transduction. However, high doses of odorant elicit a delayed and sustained elevation of Ca²⁺ that is sufficient to initiate NO formation. NO is thought to induce the recruitment of neighboring cilia. Finally, the rise in NO-induced cGMP levels is supposed to trigger molecular mechanisms leading to olfactory inhibition (e.g., adaptation processes) (Breer and Shepherd, 1993). It should be pointed out that, like NO, carbon monoxide (CO), another activator of soluble guanylate cyclase, may serve as an intercellular messenger in mammalian olfaction (Verma et al., 1993). In the near future, it may be very intriguing to verify the presence of a CO pathway in hydra and to study the role of CO in the hydra feeding response.

In conclusion, our results confirm that the NO pathway is highly preserved throughout evolution and that it is involved in the tentacle control of feeding response, i.e., in the most primitive model of an olfactory-like system present in a multicellular organism. Noteworthy is the conclusion that NO seems to play a similar activation–adaptation role both in a primitive chemoreceptorial mechanism of a coelenterate and in the sophisticated olfactory system of a mammal.

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