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Cyclic nitric oxide release by human granulocytes, and invertebrate ganglia and immunocytes: nano-technological enhancement of amperometric nitric oxide determination

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Various tissues from vertebrates and invertebrates respond to external signal molecules by rapid release of nitric oxide (NO) mediated by constitutive nitric oxide synthase.

Material/Methods: Invertebrate immunocytes were collected from maintained stock and human granulocytes were isolated from leukocyte-enriched blood obtained from the Long Island Blood Services. The invertebrate ganglionic tissue was either extracted or exposed for *ex vivo* and *in vivo* evaluation. Nitric oxide release was measured using a newly developed NO-selective nanoprobe, exhibiting enhanced sensitivity.

Results: Evaluation of NO release from the pedal ganglia of the marine bivalve, *Mytilus edulis*, demonstrated *in vitro* release of NO that fluctuated from 969 to 1003 pM, with a mean change in NO of 35 pM/cycle and a mean cycle time of approximately 4 minutes. Basal release of NO/cycle from the ganglia *in vivo* was increased significantly to approximately 65 pM ($P<0.05$) with an increase in cycle time to approximately 7 minutes. Exposure of the ganglia to morphine *in vivo* resulted in a significant increase in NO release and a lack of NO pulsations. The fluctuation in NO release from immunocytes of *Mytilus edulis* was approximately 27 pM per cycle with a cycle time of 4 minutes whereas human granulocytes release fluctuated approximately 23 pM with a cycle time of 6 minutes.

Conclusion: These data demonstrate that basal release of NO from various tissues is released in a cyclic manner and the cycle time and magnitude is subject to regulation by external stimuli.

key words: **nitric oxide • granulocytes • immunocytes • cyclic**

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BACKGROUND

The release of NO mediated by cNOS activation has been reported to stimulate as well as inhibit tissue responsiveness to external stimulation [1]. We have demonstrated previously that NO can inhibit cell activation and subsequent responsiveness to stimulatory signal molecules [1]. Alternatively, a period of hyper-responsiveness to stimulatory signal molecules was observed following the period of inhibition [2]. We surmise that the cyclic release of NO is autoregulatory in nature and may allow enhancement and inhibition of cellular responsiveness [3].

The current report suggests that basal release of NO in the absence of stimulatory molecules may also provide dynamic autoregulation of activation at the cellular level. The enhanced sensitivity of a stabilized nano-amperometric probe allowed for the evaluation of basal cNOS production previously estimated to be approximately 1 nM. Increased sensitivity of the stabilized probe confirms the previous estimates for basal NO release and demonstrates, for the first time, that human granulocytes and invertebrate neural and immune tissue produce basal levels of NO that is cyclic in nature with pulses of NO release observed with a repetition cycle of approximately 4 to 7 minutes. Our data implies that modulation in the magnitude or cycle time of basal NO release may provide dynamic regulation of cellular responsiveness to signal molecules.

MATERIAL AND METHODS

Invertebrate and Human tissue

Mytilus edulis, a marine bivalve mollusk, was harvested from the Long Island Sound, Montauk, New York and maintained in the laboratory for three weeks prior to their use in these studies [4]. Invertebrate immunocytes were collected and processed for use exactly as described previously [5,6]. Immunocytes were aspirated by insertion of a needle through the posterior adductor muscle of *Mytilus edulis* and suspended in incubation medium, 50% by volume Instant Ocean and cell-free hemolymph supplemented with streptomycin (50 mg/100 ml), penicillin (0.3 mg/ml) and gentamycin (50 mg/ml) to minimize bacterial growth during the study [7] until use in experiments. For *ex vivo* evaluation of pedal ganglia, tissue was isolated and removed exactly as described [7] and placed in incubation media. For *in vivo* evaluation of pedal ganglia, the pedal ganglion was exposed at the base of the animal's foot by cutting the connective tissue just above its superior surface and bathed with incubation media just prior to use in experiments. Human granulocytes were isolated from leukocyte-enriched blood obtained from the Long Island Blood Services (Melville, New York). Granulocytes were isolated following dextran sedimentation and Ficoll-Hypaque density gradient centrifugation (1.077–1.080 g/ml) as described in detail [8–10]. Cells were stained with Wright's stain to assess purity by microscopic examination which was ranged from 94 to 97% with a viability of > 95% as assessed by trypan blue exclusion.

NO Determination

Invertebrate Pedal Ganglia

Release of NO from pedal ganglia of *M. edulis ex vivo* was evaluated using a NO-selective nanoprobe (ISO-NOPNM) [11] and NO meter (ISONO-MARK II), manufactured by World Precision Instruments (Sarasota, FL). The system was calibrated daily as described [12] using the NO donor, SNAP, in presence of Cu(I), to liberate known quantities of NO. The probe was allowed to polarize for 30 min in incubation media prior to transfer to an Eppendorff tube containing a single ganglion in 1 ml incubation media. The nanoprobe tip (100 nm diameter) was positioned 10 μm above the tissue surface using a micromanipulator (Zeiss-Eppendorff) attached to the stage of an inverted microscope (Nikon Diaphot). Constitutive NO release was then evaluated for an additional 15 minutes. Data acquisition was accomplished using the computer interfaced DUO-18 software (World Precision Instruments, Sarasota, FL.) and experimental values were transferred to Sigma-Plot and Sigma-Stat (Jandel, CA) for graphic representation and evaluation. For determination of the specificity of the NO response, experiments as described above were repeated in the presence of 100 mM N-omega-nitro-L-arginine methyl ester (L-NAME), a selective NOS inhibitor. Evaluation of NO release from pedal ganglia *in vivo* was performed exactly as described above for ganglia *ex vivo* except that ganglia were not removed from the animal. NO measurements were performed using ganglia surgically exposed and bathed in incubation media prior to positioning of the nanoprobe 10 µm above the ganglia.

Invertebrate Immunocytes

Immunocytes from *Mytilus edulis* were suspended in incubation medium to a final concentration of 10^3 immunocytes per ml incubation media. 1 ml of this suspension was added to an Eppendorff tube and gently centrifuged for 10 min at 800 xg to create a cell-free zone that allowed placement of the nanoprobe approximately 10 µm above the soft cell pellet. Detection of NO release from the immunocytes was then performed exactly as described above for pedal ganglia.

Human Granulocytes

Detection of NO release from the human granulocytes was performed exactly as described above for invertebrate immunocytes except that the cells were resuspended in phenol-free RPMI to a final concentration of 10^3 cells per 1 ml.

Specificity of NO release

To determine the specificity of the NO production and exclude the impact of experimental drift or noise, experiments in pedal ganglia and immunocytes of *Mytilus edulis* and human granulocytes were repeated in the presence of the 100 mM omega-nitro-L-arginine methyl ester (L-NAME), a potent NOS inhibitor.

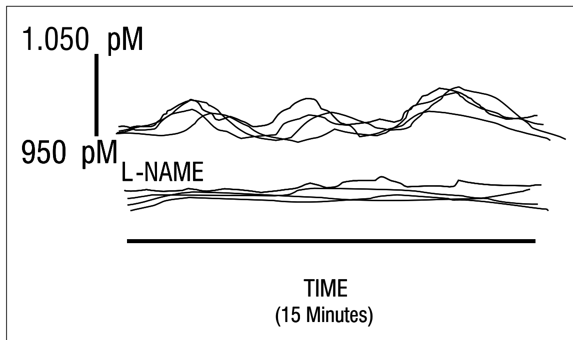

Figure 1. Real-time nitric oxide release from excised pedal ganglia from *M. edulis*. Release of NO was determined using a nanoamperometric probe. Basal release of NO fluctuated from a minimum of 968.6±2.7 pM to maximum of 1002±7.7 pM. Basal fluctuations in NO release were abrogated in tissue pretreated L-NAME 100mM and cyclic release was abolished.

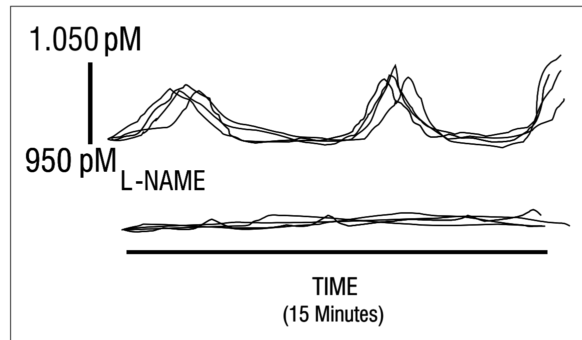

Figure 2. Real-time nitric oxide release from *Mytilus edulis* pedal ganglia *in vivo*. Basal release of NO fluctuated from a minimum of 960.1±0.1 pM to a maximum of 1025.3±1.7 pM. Basal fluctuations in NO release were abrogated in tissue pretreated L-NAME 100mM and cyclic release was abolished.

Table 1. Characteristics of basal NO release from invertebrate and human tissue.

Tissue	NO release per cycle (pM)	P value	Cycle Time Minutes	P value
Pedal Ganglia *ex vivo*	34.0±5.2	–	4.1±0.2	
Pedal Ganglia *in vivo*	65.1±1.5	0.001*	6.9±0.4	0.05*
Immunocytes	26.8±2.5	–	4.2±0.1	–
Granulocytes	23.1±3.3	NS**	6.4±0.2	0.05***

*Untreated invertebrate pedal ganglia *ex vivo* compared to *in vivo*;
**NS, not significantc;
***Untreated invertebrate immunocytes compared to human granulocytes

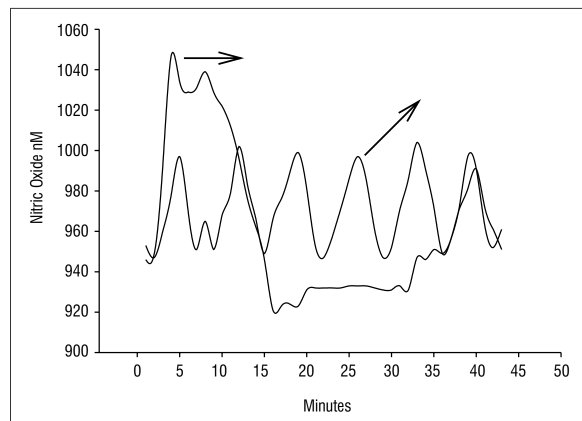

Figure 3. Modulation of nitric oxide release from *Mytilus edulis* pedal ganglia *in vivo* by morphine. Basal release of NO in untreated ganglia fluctuated from a minimum of 944.5±1.4 pM to a maximum of 997.8±1.8 pM. Exposure of the ganglia to morphine resulted in a marked increase in NO release with maximal fluctuation of approximately 107 pM over unstimulated levels. The increase in NO release was observed for 15 minutes followed by a 20 minute period of reduced NO release whereupon a release of NO comparable to untreated ganglia was observed.

Statistical analysis

All experiments, as described above, were repeated n=4 times using *Mytilus edulis* tissue (pedal ganglia and immunocytes) from different animals. Experiments using human granulocytes were repeated n=4 times from granulocytes extracted from different blood donors. Data shown is the mean ± SEM of NO release observed in the absence or presence of L-NAME. Data was subjected to paired ANOVA for evaluation of statistical significance where a p value less than 0.05 was considered to be statistically significant. In all experiments, data gatherers were blinded to the experimental conditions to avoid bias.

RESULTS

NO release from invertebrate ganglia

Excised pedal ganglia released NO at low levels in a cyclic manner which varied from 970 to 1002 pM (Figure 1). The fluctuation in NO per cycle ranged from 20 to 46 pM with a mean of 34±5 pM and cycle time of 4.1±0.2 minutes. Basal NO release from pedal ganglia evaluated *in vivo* ranged from 960 to 1025 pM (Figure 2). The fluctuation in NO release and mean cycle time *in vivo* were increased significantly to 65±2 pM and 6.9±0.4 min, respectively (P<0.001) compared to ganglia evaluated *ex vivo* (Table 1). Basal NO levels detected in ganglia *ex vivo* (Figure 1) or *in vivo* (Figure 2) in the presence of L-NAME were diminished to undetectable levels and fluctuations in NO release were abolished.

The effect of external stimuli on fluctuations in NO release and cycle time was evaluated using pedal ganglia *in vivo* in the absence or presence of morphine. Release of NO from untreated ganglia fluctuated from approximately 950 pM to 995 with a mean cycle time of 7 minutes (Figure 3). Treatment of the ganglia with exogenous morphine resulted in an increase in NO release that was markedly greater than that observed in unstimulated preparations. Approximately 15 min following morphine exposure NO release was reduced relative to basal release with ablation of the cyclic fluctuations in

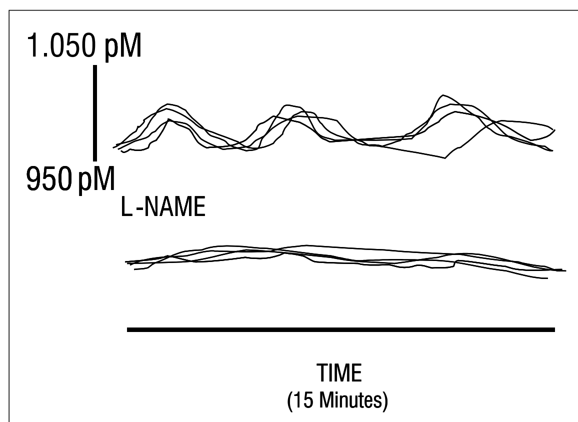

Figure 4. Real-time nitric oxide release from *Mytilus edulis* immunocytes. Basal NO release was 957±12 pM with pulses reaching levels of 977±6.7 pM of NO.

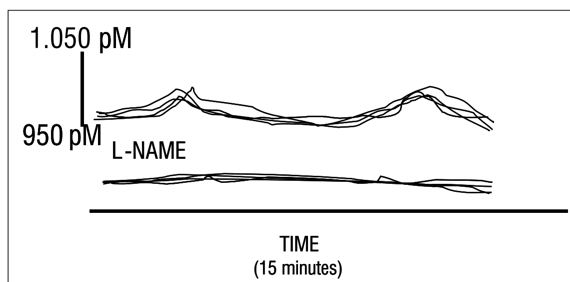

Figure 5. Representative real-time nitric oxide release from human granulocytes determined by a nano-amperometric probe. Basal release occurs at the 951±5.3 pM level with pulses reaching levels of 978±3.0 pM of NO ($P>0.05$ comparing both values). In this regard, L-NAME 100mM diminished basal NO release as well as the wave pattern.

NO release. A recovery in the basal-type release was observed approximately 40 minutes following the initial morphine exposure.

NO release from inflammatory tissue

Evaluation of basal NO release from invertebrate immunocytes was comparable to that obtained for the pedal ganglia except that it occurred with increased frequency; about once every 4.3±0.1 min (Figure 4). Evaluation of NO release from human granulocytes ranged from 953 to 976 pM (Figure 5). The fluctuation in NO release per cycle was 23.1±2.2 pM compared to 26.8±2.5 pM in invertebrate immunocytes. The cycle time was 6.4±0.2 minutes. Basal NO levels detected in invertebrate (Figure 4) or human inflammatory tissue (Figure 5) in the presence of L-NAME was diminished to undetectable levels and fluctuations in NO release were abolished.

DISCUSSION

General mechanism

Improved sensitivity and increased signal to noise ratio of the nano-amperometric NO probe allowed first time evaluation of basal NO release from invertebrate and human tissue. The current report demonstrates that basal release of NO from human and invertebrate inflammatory cells and invertebrate pedal ganglia was approximately 950 to 1100 pM, consistent with that estimated in our previous reports [8,13,14]. In the current report we demonstrate for the first time that invertebrate neural and invertebrate and human immune tissue release basal levels of NO in a pulsatile manner with a magnitude and cycle time that is subject to external regulation.

NO autoregulation

Recent data has emerged from the literature demonstrating that NO, either exogenously applied or produced as a result of cNOS stimulation, can attenuate the induction of iNOS in vascular smooth muscle, neutrophils, microglia, astrocytes and hepatocytes [15–21]. In this regard, we have also demonstrated that NO can diminish cNOS derived NO production [22] as well as iNOS derived NO release [23]. Furthermore, cNOS derived NO can inhibit iNOS derived NO induction by inhibiting iNOS expression in human endothelial cells [23,24]. Thus, NO can autoregulate its own production [3].

NO effects on cell activation

Neural vascular endothelial and inflammatory cells are constitutively activated and can respond to microenvironmental changes by changing from a low level to a increased level of activation [1]. We hypothesize that basal NO production may provide a critical pathway to dampen microenvironmental 'noise' that would otherwise nonspecifically and inappropriately lead to increased activation [1]. NO modulates the threshold required for cell activation [1] and the magnitude of the subsequent response [2]. A diminished level of NO would represent a disinhibition process that could overwhelm inhibitory signals and allow increased influence of excitatory signal required for cellular activation [1]. In this regard, we have demonstrated that exposure of cells to lipopolysaccaride (LPS) triggers an excitatory signal that reduces the constitutive production of NO and subsequent activation of these cells occurs [23,25]. However, 8 to 24 hours following LPS administration, NO production mediated by iNOS is observed and cell activation is maintained.

We have reported previously that cNOS coupled NO release may modulate cell activation and the observations in the current report suggests that basal NO production may also participate in the regulation of the activation state of a cell. The demonstration that NO release from the pedal ganglia of *Mytilus edulis* could be modulated by signal molecules, such as morphine, demonstrates that basal production of NO is subject to external regulation (Table 1). Morphine stimulation of human monocytes, granulocytes and vascular endothelial cells as well as invertebrate immunocytes has been previously demonstrated to trigger a cNOS-mediated

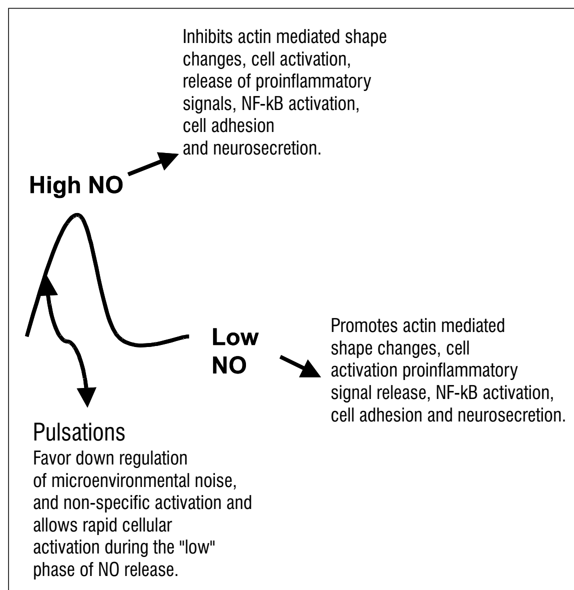

Figure 6. Physiological and biochemical significance of naturally occurring fluctuations in NO release. During the ascending phase of NO release the basal levels of NO may inhibit cellular activity by reducing changes in cell conformation by inhibition of actin polymerization, inactivation of NF-κB, inhibition of cell adhesion and hyperpolarization of post-synaptic membranes. The descending phase of NO release may promote cell shape changes by allowing actin polymerization, depolarization and NF-kB signaling [1–3,8].

NO production-dependent period of cellular inactivity where NO release is reduced to undetectable levels [8,14,26–28]. Following this period of inhibition, cellular activation is increased over basal levels and responsiveness to stimulatory molecules is significantly increased despite restoration of cNOS-coupled NO production [25,29]. In the current report, we demonstrated morphine inhibition of basal NO production for approximately 20 minutes followed by restoration of basal NO production (Figure 3). Should inhibition of basal NO release act in a manner similar to that previously demonstrated for inhibition of cNOS-coupled NO production, a period of hypersensitivity to external signal molecules could be observed following restoration of basal NO production. We have previously hypothesized that such a mechanism could function as a filter to regulate cellular responsiveness to inappropriate environmental noise and would allow the rapid responsiveness to signal molecules that may have been generated during the period of inhibition [1,2,25,29,30]; our observations with basal NO release in the current report are consistent with this hypothesis.

Conservation of NO regulation across evolution

The presence of ganglionic NO pulsations suggest that NO may be an innate mechanism for regulation of constitutive NO release as well as cellular activity as shown in Figure 6. In this regard, we surmise that the upward phase, generating increased NO release would dampen potential excitatory processes via hyperpolarization of the post synaptic membranes [1] and minimize microenvironmental free radical levels [3]. During the decline phase where decreasing levels of NO are observed, NO mediated inhibition would be reduced allowing for up regulation of cellular excitatory processes (Figure 5). Indeed, this decline phase would represent a critical time period where critical signal molecule activity could exert increased effects on physiological processes, i.e, immune activation to meet a proinflammatory situation. Cyclic elevation and decline in NO release was also observed in human cells as well, where it appears to be involved with hypothalamic neurosecretion [31]. The release of NO in a cyclic manner appears to have been conserved during evolution since they occur in evolutionarily diverse animals.

Conclusions

In summary, NO release in *Mytilus edulis* ganglionic tissues and immunocytes and human granulocytes occurs under basal conditions and is cyclical in nature. The data in the current report, taken together with our previous observations with cNOS-coupled NO production, suggest that changes in basal NO production by external signal molecules may modulate cellular activity. In this regard, the cyclical nature of NO release may represent a dynamic autoregulatory process that results in discreet control of the cellular responsiveness resulting in varying degrees of alertness and a graded response to microenvironmental changes.

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