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# Nitric Oxide Production and Thermoregulation in *Paramecium caudatum*

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**Summary.** We investigated nitric oxide (NO) production in *Paramecium caudatum*, and the role of NO production in population growth in culture and thermoregulation. Nitrite ($NO_2^-$) concentration in media containing *P. caudatum* [6350 ± 390 (SD) paramecia/ml] was 2.37 ± 0.53 µM after 6 h, compared to 0.16 ± 0.06 µM in media alone ($p<0.005$), and the NO synthase (NOS) inhibitor, $N^{\omega}$-nitro-L-arginine methylester (L-NAME), reduced [$NO_2^-$] to 0.81 ± 0.24 µM ($p<0.02$). Media containing *P. caudatum* produced [$^3$H]L-citrulline from [$^3$H]L-arginine, and the [$^3$H]L-citrulline production was inhibited by L-NAME. Addition of A23187, a calcium ionophore, to the media resulted in greater [$NO_2^-$] (1.49 ± 0.28 µM with no A23187, 2.51 ± 0.23 µM with 0.1 µM A23187 added, $p<0.05$). Western blot analysis revealed a 155 kDa protein that reacted with mouse NOS1 antibody. Paramecia concentration increased from 51 ± 9 per ml on day 0 to 943 ± 53 per ml on day 7. L-NAME decreased paramecia concentration at day 7 (0.1 mM, 720 ± 70 per ml; 1.0 mM, 761 ± 49 per ml; and 10 mM, 132 ± 32 per ml; $p<0.05$ compared to control for all 3 concentrations). In a thermal gradient, *P. caudatum* selected an environmental temperature (Ts) of 32.9 ± 0.3°C, addition of 10 mM L-NAME reduced Ts to 24.3 ± 0.3°C ($p<0.05$). These data suggest that *P. caudatum* produce NO *via* a calcium dependent NOS similar to mammalian NOS1, and inhibition of NO production reduced paramecia number in culture and decreased Ts.

**Key words:** calcium ionophore, environmental temperature, nitric oxide synthase, nitrite, selected temperature, thermal gradient.

**Abbreviations:** D-NAME - $N^{\omega}$-nitro-D-arginine methylester, L-NAME - $N^{\omega}$-nitro-L-arginine methylester, NO - nitric oxide, $NO_2^-$ - nitrites, NOS - nitric oxide synthase, SNAP - S-nitroso-N-acetylpencillamine, Ts - selected temperature.

## INTRODUCTION

Nitric oxide synthase (NOS) was first described in macrophages and endothelial cells isolated from mammals (Nathan 1992). NOS metabolizes L-arginine to NO and L-citrulline. Mammals have three isoforms of NOS; NOS1 and NOS3 are constitutively expressed and are calcium dependent, while NOS2 expression is up-regulated by stimuli including, inflammation, flow and environmental stress. NOS activity can be competitively inhibited using analogs of L-arginine such as $N^{\omega}$-nitro-L-arginine methylester (L-NAME) (Nelin *et al.* 1996). Since its description in mammals, NOS has been described in invertebrates (Regulski and Tully 1995, Nighorn *et al.* 1998, Luckhart and Rosenberg 1999), and recently NOS has been described in Protists (Basu *et al.* 1997,

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Tao *et al.* 1997, Goldstein *et al.* 2000, Piacenza *et al.* 2001). Paramecia express several targets of NO, including guanylyl cyclase, potassium channels and voltage-gated calcium channels (Thiele and Schultz 1981, Preston *et al.* 1992, Prajer *et al.* 1997, Imada and Oosawa 1999, Linder *et al.* 1999). Although, NOS appears to be widespread among taxa, its function in Protists remains unclear. In this study we examined NO production and the possible physiologic effects of its inhibition in *P. caudatum*.

We first assessed whether *P. caudatum* produce NO. NO is highly reactive and therefore short-lived in oxygenated media (Nathan 1992, Nelin *et al.* 1996). Therefore, we measured nitrite ($NO_2^-$) concentration, the stable end product of NO oxidation, in the media after a 6 h incubation period. To further confirm that the $NO_2^-$ was due to NOS activity we also measured the conversion of [$^3$H]L-arginine to [$^3$H]L-citrulline. The effects of L-NAME and the calcium ionophore A23187 on [$NO_2^-$] were also determined.

Thermoregulation is a basic physiologic process that is widespread among taxa (Wood and Malvin 1992). Although, paramecia have only limited internal means to thermoregulate, they and other ectotherms actively select an environmental temperature (Ts) that best supports their physiological processes (Malvin and Wood 1992, Malvin *et al.* 1994, Malvin 1998). Ectotherms can thermoregulate with considerable precision (Vaughn *et al.* 1974, Wood and Malvin 1992). Adaptive changes in thermoregulatory "set-point" in ectotherms include response to infection and hypoxia. For example, in a thermal gradient, infection increases Ts (Kluger 1991), and hypoxia decreases Ts (Malvin and Wood 1992, Malvin *et al.* 1994). The increase in Ts in response to infection aids in immunological function and survival (Kluger *et al.* 1975); while the decrease in Ts in response to hypoxia decreases metabolic needs and improves survival (Malvin and Wood 1992, Malvin *et al.* 1994). Thermoregulation in *P. caudatum* was first described by Mendelssohn (1895) who demonstrated that *P. caudatum* accumulate around their Ts in a thermal gradient. Jennings (1906) demonstrated that *P. caudatum* actively avoided temperatures above and below their Ts. In a thermal gradient, it has been demonstrated that paramecia change swimming direction more frequently when moving away from Ts than when moving toward Ts (Nakaoka and Oosawa 1977). The cellular mechanisms responsible for the changes in swimming direction, or avoiding reactions, have been found to involve membrane depolarization leading to activation of voltage-gated $Ca^{2+}$ channels (Hennessey *et al.* 1983, Imada and Oosawa 1999). The precise mechanisms leading to this thermoregulatory response in *P. caudatum* remains unknown (Malvin 1998). Since temperature selection in *P. caudatum* involves ciliary motion, and ciliary motion involves activation of guanylate cyclase and calcium channels (Erxleben *et al.*1997, Schultz *et al.* 1997, Malvin 1998), and in mammalian cells, NO activates soluble guanylate cyclase and calcium channels (Clementi 1998), we hypothesized that *P. caudatum* produces NO, which is involved in the thermoregulatory responses of *P. caudatum*. To determine the role of NO production in thermoregulation, the effect of L-NAME, and its non-active stereoisomer D-NAME, and the NO donor S-nitroso-N-acetylpencillamine (SNAP) on selected temperature (Ts) were studied in *P. caudatum* in a thermal gradient.

## MATERIALS AND METHODS

**Organisms**. *Paramecium caudatum* (Carolina Biological Supply, Burlington, NC) were cultured as described previously (Malvin and Wood 1992, Malvin *et al.* 1994, Malvin 1998). Briefly, *P. caudatum* were cultured at 19-21°C in cerophyl media (Sigma Chemicals, St Louis, MO) inoculated with *Enterobacter aerogenes*. *P. caudatum* in mid-log phase growth were isolated by filtration (5 µm pore size) and then centrifuged at 100 *g* for 10 min, and washed three times in fresh cerophyl media before experimentation.

**Nitrite measurement.** The samples of medium were assayed for $NO_2^-$ using a chemiluminescence analyzer (Sievers Instruments, Boulder, CO) as previously described (Nelin *et al.* 2001). The samples were injected into a reaction chamber containing NaI in 1N acetic acid through which a steady stream of $N_2$ gas flowed. The NaI mixture reduced $NO_2^-$ to NO and the stream of $N_2$ gas carried the NO gas into the chemiluminescence analyzer. $NaNO_2$ (Sigma Chemicals, St Louis, MO) was used for a standard curve.

**Production of [$^3$H]L-citrulline.** To determine if the nitrites measured above represented NO produced from NOS, we measured the production of [$^3$H]L-citrulline from [$^3$H]L-arginine in similar experimental conditions. In other words, we did not strictly measure NOS activity in these paramecia, but rather measured the appearance in the medium of the NO co-product, L-citrulline to further demonstrate that *P caudatum* produce NO *via* NOS. Paramecia were concentrated to ~6500/ml in a final volume of fresh cerophyl medium of 25 ml in a 50 ml test tube. Then L-[2,3,4,5-$^3$H]arginine monohydrochloride (25 µCi; specific activity 59 Ci/mmol; Amersham Life Sciences, Arlington Heights, IL) was added to the medium. After 1 h, 3 ml of cell suspension was removed and centrifuged at 500 *g* for 5 min. Then 2 ml of the supernatant was passed over a Dowex

AG50W-X8 ($Na^+$ form) column. [$^3$H]L-citrulline was eluted with 2 ml of distilled water. The radioactivity was measured using a liquid scintillation counter.

**Western blots.** Basu *et al.* (1997) and Goldstein *et al.* (2000) found that *Leishmania donovani* and *Trypanosoma cruzi*, respectively, express a protein that cross-reacts with mammalian NOS1 on Western blots. Therefore, Western blots were done on *P caudatum* protein as previously described (Nelin *et al*. 2001). Paramecia were concentrated to ~15000/ml by filtration and centrifugation, and then centrifuged at 1500 *g* for 5 min at room temperature. The pellet containing the paramecia was then resuspended in 2 ml of sonication buffer (pH 7.5) containing 0.32 M sucrose, 10 mM Tris HCl, 1 mM EDTA, 1 mM dithiothreitol, 0.01 mg/ml aprotinin, 0.01 mg/ml leupeptin, 0.01 mg/ml soybean trypsin inhibitor and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The paramecia were then sonicated for 10 s. The paramecia protein extracts were centrifuged at 1500 *g* at 4°C for 10 min to remove cellular debris. The protein was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 10% acrylamide. In addition to samples, each gel included molecular weight standards (Bio-Rad) and purified NOS1, NOS2 and NOS3 (Transduction Laboratories) standards. The separated proteins were transferred to PVDF membranes and stained with Coomassie brilliant blue to confirm equal protein loading in all lanes. Membranes were blocked overnight with 5% nonfat milk in 20 mM Tris-HCl, 50 mM NaCl (pH 7.5) buffer. The blot was incubated for 4 h with a mouse monoclonal antibody specific for NOS1 (1:1500), NOS2 (1:1000) or NOS3 (1:2500) (Transduction Laboratories) in TBS with 0.02% $NaN_3$. Immunochemical labeling was achieved by incubation for 2 h with biotinylated goat anti-mouse IgG (1:5000) (Bio-Rad) followed by chemiluminescence labeling (Amersham ECL detection assay). NOS1, NOS2 or NOS3 protein bands were detected by exposure to chemiluminescence-sensitive film. Protein concentrations of samples were determined by the Bradford method.

***Paramecium caudatum* population growth in culture.** *P. caudatum* were washed and placed in fresh Erlenmeyer flasks containing cerophyl medium. Then the number of paramecia per ml was determined by counting the number of paramecia in 20, 50-µl aliquots of media treated with 20-µl of 1 mM NiCl under a stereo microscope. NiCl inhibited ciliary motion, facilitating cell counting. Paramecia were counted daily for seven days.

***Paramecium caudatum* temperature selection (Ts).** Ts was determined as previously described (Malvin and Wood 1992, Malvin *et al.* 1994, Malvin 1998). Briefly, *P. caudatum* were washed and resuspended in cerophyl medium at a concentration of 100 per ml. One ml of the suspension was placed in a plexiglass aquatic thermal gradient (80 mm x 4 mm x 2 mm). The thermal gradient was cooled at one end by a copper tube beneath the gradient carrying chilled polyethylene glycol, and was warmed using heating tape at the other end. Sixteen thermal couples were placed 5 mm apart along the gradient for measuring temperature. A microscope was positioned above the gradient to visualize the paramecia in the gradient, and the magnified images were recorded by video microscopy. One hour after the paramecia were added to the thermal gradient, the distribution of Ts values was determined by video recording for 10 s at each of the 16 gradient positions containing a thermocouple. From the tapes the number of paramecia at each location was determined. Thermocouple temperatures were recorded immediately before videotaping. The temperature at each thermocouple is shown in Fig. 1.

## Experimental protocols

**Nitric oxide production.** To determine if paramecia produce NO, *P. caudatum* were isolated as described above and concentrated (6350 ± 390 per ml). In the first set of experiments, the final volume of medium was 4 ml. Paramecia were incubated for 6 h in either cerophyl medium or cerophyl medium with 1 mM L-NAME. Cerophyl medium without paramecia, and cerophyl medium without paramecia but with 1 mM L-NAME were also incubated at room temperature for 6 h. 400-µl of cell suspension was then removed and centrifuged at 500 *g* for 5 min to remove paramecia, and the supernatant was assayed for $NO_2^-$ as described above. In a second set of experiments, the final volume of medium was 25 ml. Paramecia were incubated at room temperature in either cerophyl medium or cerophyl medium containing 1 mM L-NAME. Cerophyl medium without paramecia was also incubated at room temperature. After 1 h 3 ml of the cell suspension was removed and processed for [$^3$H]L-citrulline measurement as described above. In a third set of experiments, to determine the calcium sensitivity of paramecium NO production, cells were incubated for 6 h in cerophyl medium or cerophyl medium with either 0.01 µM A23187 (Sigma Chemicals, St Louis, MO) or 0.1 µM A23187. Then 400 µl of the cell suspensions were removed and centrifuged at 500 *g* for 5 min to remove paramecia, and the supernatant was assayed for $NO_2^-$.

To determine if *P. caudatum* contains a protein that cross-reacts with mammalian NOS1 antibody Western blot analysis was done. *P. caudatum* were isolated and concentrated to ~15000 per ml in a final cell suspension volume of 8 ml. Four cultures of *P. caudatum* were then processed as described above for Western blot analysis for NOS1, NOS2 or NOS3.

**NO production and *P. caudatum* population growth in culture.** To determine the effect of blocking NO production on population growth in culture, *P. caudatum* were isolated as described above and placed in fresh cerophyl medium [50 ± 9 (SD) paramecia per ml]. The paramecia concentration was determined daily for 7 days as described above in cerophyl medium, cerophyl medium with 0.1, 1.0 or 10 mM L-NAME, or in cerophyl medium with 0.1, 1.0 or 10 mM D-NAME.

**NO and selected temperature in *P. caudatum*.** Finally, to determine the effect of blocking NO production on Ts, Ts was determined for paramecia in cerophyl medium (control), cerophyl medium with either 1 or 10 mM L-NAME, or in cerophyl medium with either 1 or 10 mM D-NAME. In addition, to determine if the L-NAME effect was due to inhibition of NO production, Ts was determined for paramecia in cerophyl medium (control), cerophyl medium with 10 mM L-NAME and either 0, 1, 10 or 100 µM of the NO donor S-nitroso-N-acetylpencillamine (SNAP). Finally, to determine the effect of SNAP on Ts, Ts was determined for paramecia in cerophyl medium (control), or cerophyl medium with 1, 10 or 100 µM SNAP added. In our laboratory, the control Ts varied ~10% depending on the conditions, i.e. the temperature in the laboratory, the time of season, etc. Therefore in the studies examining Ts in different experimental conditions, each control and experimental condition was carried out in random order and on the same day.

**Statistics.** Data are shown as mean ± SE unless otherwise specified. Groups were compared using one-way analysis of variance (ANOVA). Differences between groups were identified using a Newman-Keuls post-hoc test. A p-value of $<0.05$ was used to determine statistical significance.

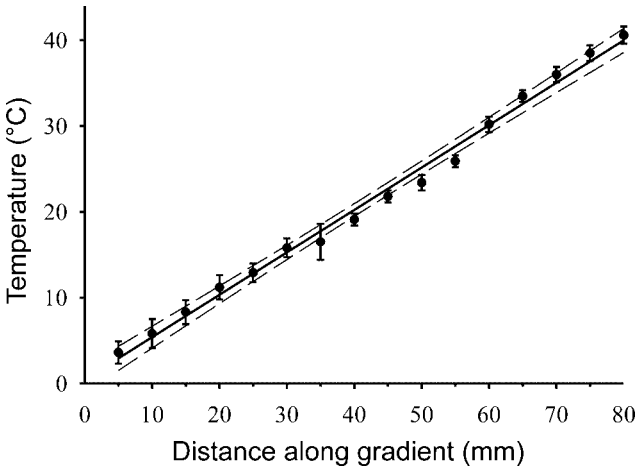


**Fig. 1.** Temperature range of aquatic thermal gradient. The temperatures (°C) at each of the 16 thermocouples along the 80 mm length of the aquatic thermal gradient (mean ± SD). The solid line is the linear regression fit of the data, and the dashed line is the 95% confidence intervals, $y = 0.49x + 0.44$, $r^2 = 0.99$.

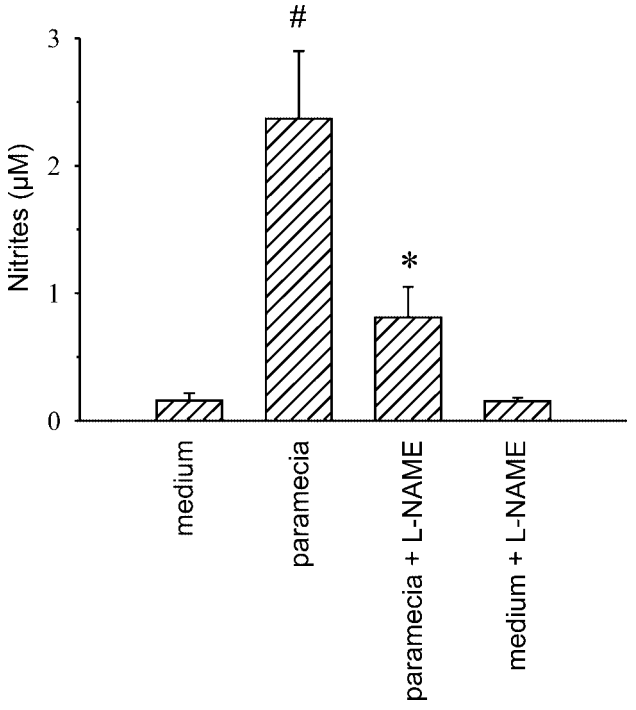


**Fig. 2.** Paramecia and production of nitrites. Paramecia produced NO as measured by nitrite concentration [$NO_2^-$] in the medium after a 6 h incubation, and NO production was inhibited by L-NAME. The first bar is cerophyl medium without paramecia (n = 6), the second bar is cerophyl medium with ~6500 paramecia/ml (n = 6), the third bar is cerophyl medium with paramecia (~6500 paramecia/ml) and 1 mM L-NAME added (n = 6) and the fourth bar is cerophyl medium without paramecia with 1 mM L-NAME added (n = 6). Note that L-NAME had no effect on [$NO_2^-$] in the medium. # signifies paramecium different from medium, $p<0.005$. * signifies paramecium + L-NAME different from both paramecium and medium, $p<0.05$.

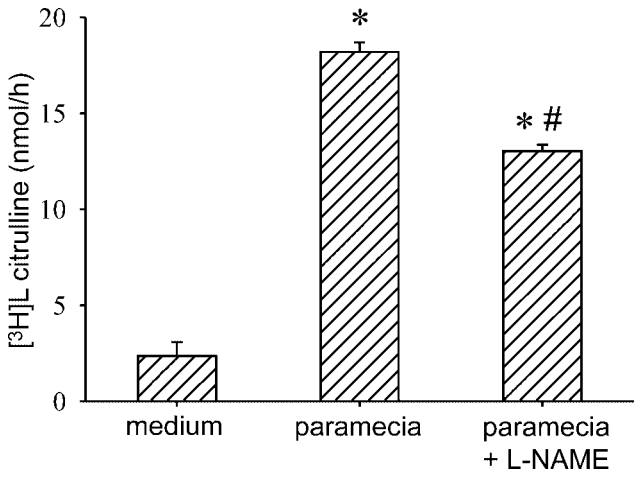


**Fig. 3.** Paramecia and L-citrulline production. Paramecia produced NO as measured by [$^3$H]L-citrulline production from [$^3$H]L-arginine, and the production of [$^3$H]L-citrulline was inhibited by L-NAME. [$^3$H]L-citrulline production 1 h after adding [$^3$H]L-arginine to 25 ml of cerophyl medium containing ~6500 paramecia/ml. The first bar is cerophyl medium without paramecia (n = 4), the second bar is cerophyl medium with ~6500 paramecia/ml (n = 4), and the third bar is cerophyl medium with paramecia (~6500 paramecia/ml) and 1 mM L-NAME added (n = 4). * different from medium, $p<0.001$. # paramecia + L-NAME different from paramecia, $p<0.005$.

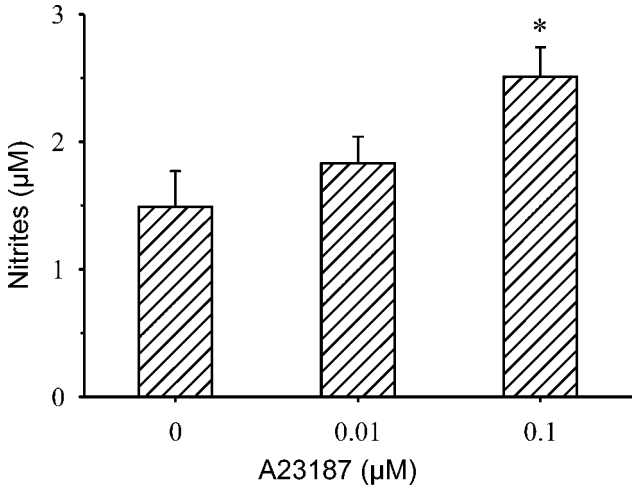


**Fig. 4.** Paramecia NO production was calcium sensitive. The bars are the [$NO_2^-$] after a 6 h incubation in cerophyl medium with ~6500 paramecia/ml containing either 0 (n = 6), 0.01 (n = 8) or 0.10 (n = 8) µM A23187. * signifies 0.10 µM different from both 0 µM and 0.01 µM, $p<0.05$.

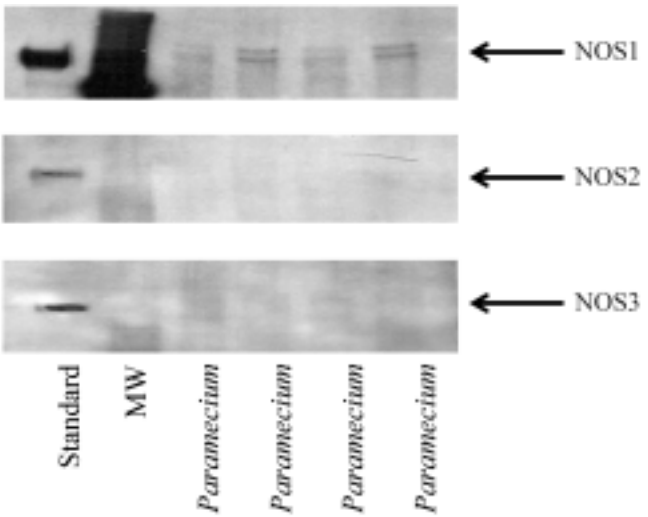


**Fig. 5.** Paramecia protein cross-reacts with mammalian NOS1. The top blot demonstrates that *P. caudatum* had a protein that cross-reacted with mammalian NOS1 antibody, but not mammalian NOS2 (middle blot) or NOS3 (bottom blot) antibodies. The first lane in each blot is the standard (NOS1, NOS2 or NOS3, respectively), the second lane is the molecular weight (MW) marker shown in the NOS1 blot, and the next 4 lanes in all three blots are paramecia protein extract.

## RESULTS

**Nitric oxide production.** Nitrite concentration in medium containing paramecia was much higher than in medium alone (Fig. 2). This demonstrates that *P. caudatum* produced the majority of the $NO_2^-$ in the medium. The mean medium [$NO_2^-$] concentration of 2.37 µM suggests that a single paramecium produces NO at ~60 femtomoles per h. L-NAME (1 mM) reduced mean medium $NO_2^-$ concentration, although the medium [$NO_2^-$] was still greater than in medium without paramecia, and L-NAME had no effect on medium [$NO_2^-$] since medium + L-NAME was not different from medium alone (Fig. 2). [$^3$H]L-citrulline production in medium containing paramecia was much higher than in medium alone (Fig. 3). L-NAME (1 mM) reduced mean medium [$^3$H]L-citrulline production, although the medium [$^3$H]L-citrulline production was still greater than in medium without paramecia (Fig. 3).The mean medium [$^3$H]L-citrulline production of 18.20 nmol/h suggests that a single paramecium produces NO at ~120 femtomoles per h. The appearance of [$^3$H]L-citrulline in medium alone without added paramecia, may represent [$^3$H]L-arg that passed through the column as a function of the L-arg binding efficiency of the column. The binding efficiency of columns in our laboratory are ~95%. Thus, both methods, $NO_2^-$ and [$^3$H]L-citrulline, give good agreement for NO production rates in paramecium, and the fact that measurement of 2 different products of NOS by 2 different methods resulted in similar results demonstrates that *P. caudatum* produces NO from NOS. Furthermore, these results demonstrate the utility of the measurement of medium $NO_2^-$ concentration as a marker of NO production, and the ability of L-NAME to inhibit NO production by *P. caudatum*.

Nitric oxide production by *P. caudatum* was calcium sensitive (Fig. 4). Addition of the calcium ionophore, A23187 significantly increased medium [$NO_2^-$] by *P. caudatum* at the highest dose tested (0.1 µM A23187). Furthermore, *P. caudatum* contain a protein that cross-reacts with mammalian NOS1 on Western blotting, but not with mammalian NOS2 or NOS3 (Fig. 5).

**NO production and *P. caudatum* population growth in culture**. Figure 6A is the mean 7 day growth curve for *P. caudatum* in cerophyl medium in our laboratory. The concentration of paramecia per ml increased from 51 ± 9 to 943 ± 53 in seven days. The majority of the increase in paramecia number occurred between days 2 and 5. Figure 6B is the mean 7 day growth curve for paramecia in cerophyl medium with 0.1 mM L-NAME or D-NAME added, Fig. 6C is the mean 7 day growth curve for paramecia in cerophyl medium with 1.0 mM L-NAME or D-NAME added, and Fig. 6D is the mean 7 day growth curve for paramecia in cerophyl medium with 10 mM L-NAME or D-NAME added. Paramecia concentrations were not different between L-NAME and D-NAME at the same concentration on days 1 through 5 (Fig. 6). However, on day 7 L-NAME treated cultures had fewer paramecia than did D-NAME treated cultures (Fig. 6). The 10 mM concentration of D-NAME had a small effect on paramecia number on day 7, however the effect of 10 mM L-NAME was much greater than that of D-NAME on paramecia number (Fig. 6D).

**NO and selected temperature in *P. caudatum*.** The addition of 1 mM of either L-NAME or D-NAME had no significant effect on Ts (Fig. 7A). However, the addition of 10 mM L-NAME to the medium reduced Ts (Fig. 7A). The addition of 10 mM D-NAME to the medium also reduced Ts. However, the Ts of the 10 mM D-NAME treated *P. caudatum* was greater than Ts of the 10 mM L-NAME treated paramecia (Fig. 7A). When the NO donor, SNAP, was added to the medium containing 10 mM L-NAME there was a dose-dependent increase in Ts, although the Ts at 100 µM SNAP remained less than the Ts without added L-NAME and

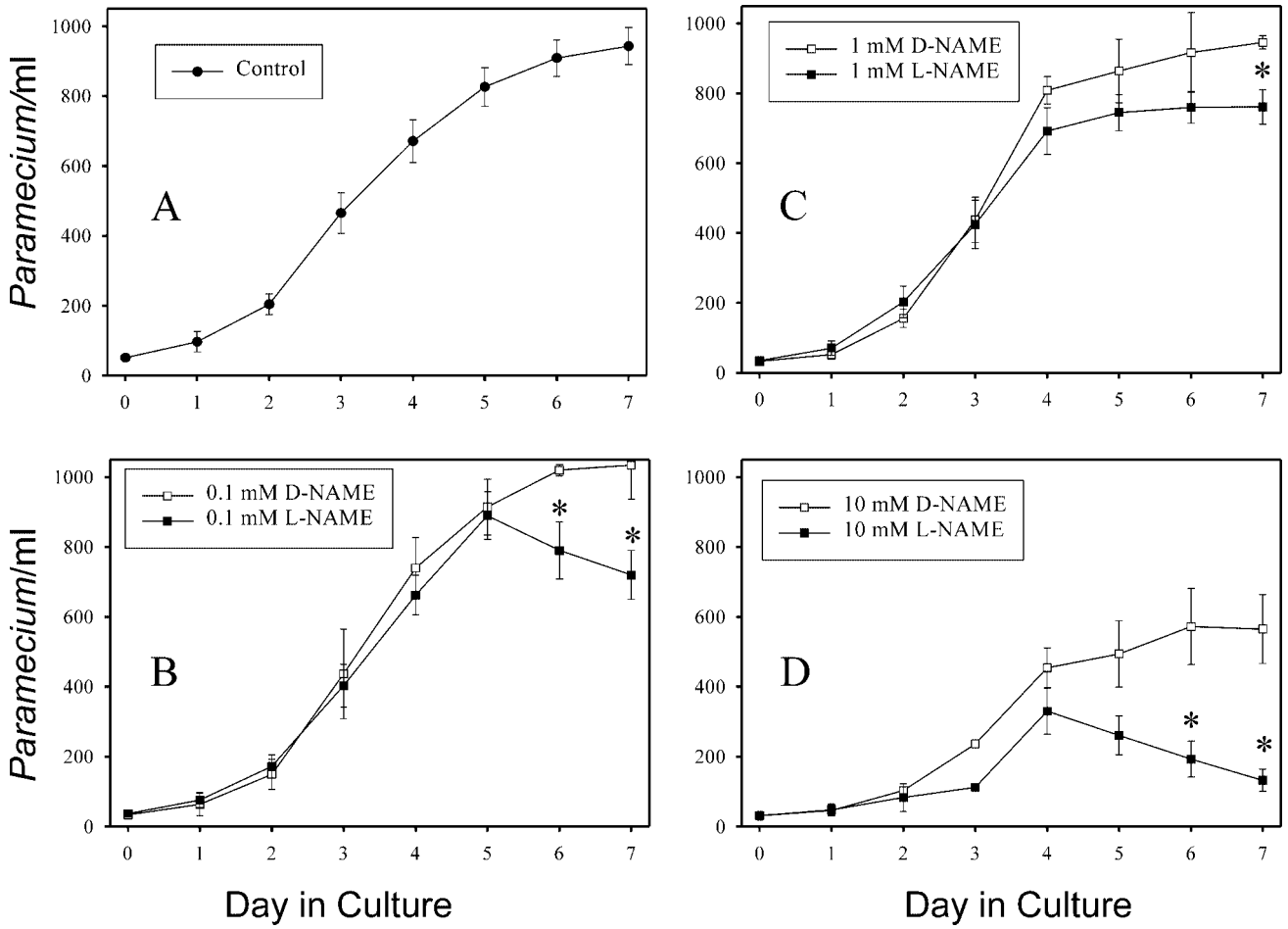


**Fig. 6.** L-NAME decreased the number of paramecia in culture. Inhibiting NO production with L-NAME resulted in a dose-dependent decrease in number of paramecia in culture. Panel 6A is the paramecia concentration (per ml) during the 7 day culture period (n = 11). **Panel 6B** is the paramecia concentration during the 7 day culture period with either 0.1 mM L-NAME (black squares; n = 6) or D-NAME (opened squares; n = 5) added to the medium. **Panel 6C** is the paramecia concentration during the 7 day culture period with either 1 mM L-NAME (black squares; n = 6) or D-NAME (opened squares; n = 5) added to the medium. **Panel 6D** is the paramecia concentration during the 7 day culture period with either 10 mM L-NAME (black squares; n = 4) or D-NAME (opened squares; n = 4) added to the medium. * signifies L-NAME different from D-NAME, $p<0.05$.

SNAP (Fig. 7B). Interestingly, the addition of SNAP alone to cerophyl medium resulted in a small reduction in Ts, which did not appear to be dose-dependent (Fig. 7C).

## DISCUSSION

The main findings of this study were that: (1) *P. caudatum* produced NO, and the NO production was inhibited by L-NAME, (2) inhibiting NO production reduced the number of paramecia after 7 days of culture, and (3) inhibiting NO production altered thermoregulation, as evidenced by a reduction in selected temperature by *P. caudatum*. These data support our hypothesis and demonstrate for the first time that *P. caudatum* produce NO, and that this NO production is physiologically important to *P. caudatum*.

*Paramecium caudatum* NO production was inhibited by the L-arginine analogue, L-NAME, and was sensitive to the calcium ionophore, A23187. This suggests that *P. caudatum* produced NO from L-arginine by a calcium sensitive NO synthase. A role for L-arginine in NO production is confirmed in this study by the ability of *P. caudatum* to produced [$^3$H]L-citrulline from [$^3$H]L-arginine, by an L-NAME inhibitable process. Recently, similar findings have been described in *Trypanosoma cruzi* (Piacenza 2001) and *Dictyostelium discoideum* (Tao 1997). Thus, our results are consistent

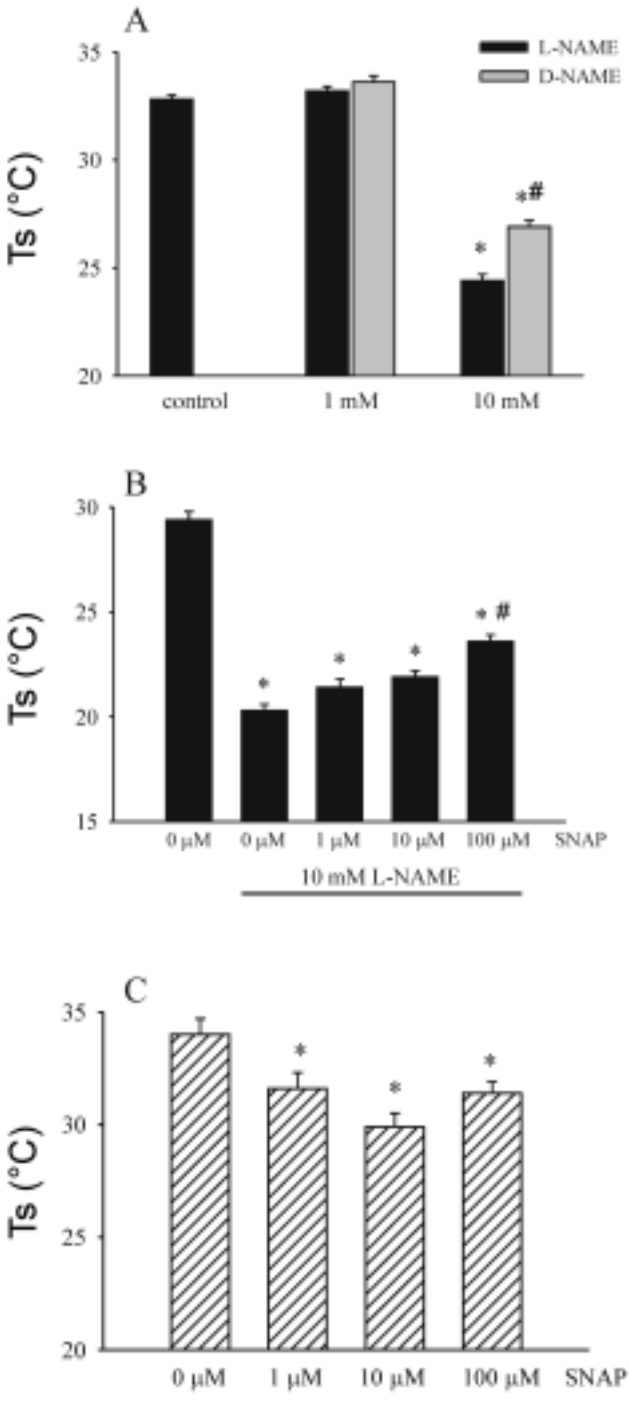


**Fig. 7. Panel A.** L-NAME reduced Ts. Inhibiting NO production with L-NAME resulted in a reduction in the selected temperature (Ts). The first bar is the Ts for paramecia in cerophyl medium (control; n = 4), the second set of bars is the Ts for paramecia in cerophyl medium with either 1 mM L-NAME (black bar; n = 6) or D-NAME (gray bar; n = 3) added, and the third set of bars is the Ts for paramecia in cerophyl medium with either 10 mM L-NAME (black bar; n = 5) or D-NAME (gray bar; n = 6) added. * signifies different from control, $p<0.01$. # signifies different from 10 mM L-NAME, $p<0.05$. **Panel B.** SNAP attenuated the L-NAME-induced reduction in Ts. The addition of the NO donor, SNAP, to cerophyl medium containing 10 mM L-NAME resulted in an increase in Ts. The first bar is the Ts for paramecia in cerophyl medium (0 µM; n = 5), the next four bars are the Ts for paramecia in cerophyl medium with 10 mM L-NAME and 0 (n = 5), 1 (n = 5), 10 (n = 5) or 100 (n = 5) µM SNAP added, respectively. * signifies different from control, $p<0.01$. # signifies different from previous SNAP concentration, $p<0.05$. **Panel C.** SNAP reduced Ts. The addition of SNAP alone to cerophyl medium resulted in a small reduction in Ts. The first bar is the Ts for paramecia in cerophyl medium (control; n = 5), the next 3 bars are the Ts for paramecia in cerophyl medium with 1 (n = 5), 10 (n = 5) or 100 (n = 5) µM SNAP added, respectively. * signifies different from control, $p<0.05$.

with the finding that Protists have a NO synthase, which oxidizes L-arginine to NO and L-citrulline. Furthermore, we found that a 155 kDa protein derived from *P. caudatum* reacted with an antibody against mammalian NOS1 but not with antibodies against mammalian NOS2 or NOS3. *Leishmania donovani* have a protein that reacts with mammalian NOS1 (Basu *et al.* 1997), and a polyclonal antibody against mammalian NOS1 has been utilized to determine the location of *Trypansoma cruzi* nitric oxide synthase (Goldstein *et al.* 2000). Thus, taken together these results suggest that Protists contain an NO synthase that is similar to mammalian NOS1.

Paramecia express many of the proteins necessary for NOS activity and NO effects. For example, paramecia express calmodulin (Chan *et al.* 1999). In mammals, the best characterized target for NO is guanylate cyclase, and NO activates guanylate cyclase to increase cyclic guanosine monophosphate (cGMP) production (Nathan 1992). In insects, the presence of soluble guanylyl cyclase in the nervous system has been used to infer the function of insect NOS (Nighorn *et al.* 1998). Paramecia have guanylyl cyclase in their cilia, although it is a particulate guanylyl cyclase (Linder *et al.* 1999). In mammalian cells, it has been demonstrated that NO also activates voltage-gated calcium channels to increase the influx of calcium (Clementi 1998). Paramecia also have voltage-gated calcium channels in their cilia (Thiele and Schultz 1981, Preston *et al.* 1992, Schultz *et al.* 1997), and cilia are involved in thermoregulatory behavior (Tominaga and Naitoh 1992, Imada and Oosawa 1999). Thus, one possible role for NO production in *P. caudatum* may be involvement in ciliary function and thermoregulation.

We found that inhibition of NO production reduced Ts by ~30%, or ~9°C in *P. caudatum*, and that this response could be partially reversed by administration of exogenous NO during inhibition of NO production. We know of no studies examining the role of NO production in

temperature regulation in *P. caudatum*, however NO has been found to be important in the central thermoregulatory response in mammals (Steiner and Branco 2002). For example, in rats inhibiting NO production with L-NAME resulted in a reduction in body temperature, whereas administration of D-NAME had no effect on body temperature (DePaula *et al.* 2000, Nakano *et al.* 2001). It is of interest to note that when we administered the NO donor, SNAP, alone there was also a reduction in Ts by ~5% or 2°C, which was much smaller than with NO inhibition. This finding is compatible with studies in mammals, wherein administration of SNAP caused either no significant change in body temperature (Eriksson *et al.* 1997) or a slight decrease in body temperature (Mathai *et al.* 1997). Thus, it may be that the influence of exogenous NO on Ts may be dependent on the starting Ts, i.e. if the starting Ts is low, as in the L-NAME treated paramecia, then exogenous NO will raise Ts. On the other hand, if the starting Ts is closer to the normal range, then exogenous NO may cause a slight reduction in Ts.

Inhibition of NO production with L-NAME resulted in a dose-dependent decrease in the number of paramecia at 7 days in culture. Since calcium oscillations have been implicated in cell division (Prajer *et al.* 1997), and NO activates voltage-gated $Ca^{2+}$ channels (Clementi 1998), one might speculate that inhibition of NO production affects the population of *P. caudatum* in the culture flask by decreasing these $Ca^{2+}$ oscillations, and thereby decreasing the number of cell divisions. However, Fig. 6 shows that the paramecia increased in number normally until approximately day 5 in 0.1 and 1 mM L-NAME, then paramecia number began to fall when compared to control paramecia number. One possible explanation for this is that inhibition of NO production increased paramecia death. The addition of 10 mM D-NAME decreased paramecia number suggesting that a change in osmolality or pH may have contributed to the reduction in paramecia number in the culture flasks with the highest concentrations of NAME (either stereoisomer). However, 10 mM L-NAME reduced paramecia number to a greater extent than did 10 mM D-NAME, demonstrating that even at the highest concentrations of L-NAME and D-NAME employed in these studies, L-NAME-induced inhibition of NO production decreased paramecia number in culture. Again, Fig. 6D suggests that the number of paramecia in the 10 mM D-NAME and 10 mM L-NAME cultures increased at approximately the same rate for the first 4 days and then in the L-NAME treated group paramecia numbers began to fall. This is consistent with the concept that inhibiting NO production hastened cell death. Alternatively, NO inhibition may have interfered with later fission, as has been observed in aging paramecia (Smith-Sonnenborn 1981), resulting in more rapid decline in paramecium number in the L-NAME treated cells. Thus, inhibition of NO production clearly decreased the number of paramecia in culture, although further studies will be needed to elucidate the exact mechanism of the decreased number of paramecia in culture.

In summary, this study demonstrates that $NO_2^-$ accumulated in the media containing paramecia, and that the $NO_2^-$ accumulation was inhibited by L-NAME and was calcium sensitive. Confirmation that the changes in $[NO_2^-]$ reflected changes in NO production by NOS in *P. caudatum* comes from the finding that [$^3$H]L-arginine was converted to [$^3$H]L-citrulline, in a process that was inhibited by L-NAME. Paramecia had a protein that cross-reacted with mouse NOS1 antibody, but not with mouse NOS2 or NOS3 antibody. Inhibition of NO production resulted in reduced numbers of paramecia after 7 days in culture. Finally, inhibition of NO production resulted in a reduced selected temperature for paramecia in a thermal gradient. Thus, these data demonstrate for the first time that *P. caudatum* produce NO, that *P. caudatum* NO production is calcium dependent, and that NO production by *P. caudatum* is involved in basic physiologic processes including population growth in culture and thermoregulation.

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