Time dependent phosphorylation in eNOS linked signal transduction pathways in endothelial cells reveals feedback mechanisms

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ABSTRACT

Within mammalian cells, developmental and metabolic states are controlled by a complex signal transduction network. By examining behavior of multiple phosphorylation sites of cultured endothelial cells in response to stimuli, patterns of time dependent phosphorylation appear. At these sites oscillatory behavior is seen, indicating the presence of negative feedback loops that include time delays from various sources. Original models allowed for the simulation of results using an explicit time delay with implicit intermediates. By applying fourth order runge kutta to sets of differential equations, we were able to create a program that added explicit intermediates with an implicit time delay to model the data, and to provide information for future experimental design. By expanding upon the initial model to include up to six intermediates, a much closer fit to the data was obtained, strengthening the hypothesis that phosphorylation at the Ser1179 site in eNOS is controlled by a complex series of intermediates (with AKT being the principal regulator). The model suggests rate constants for the various intermediates involved, confirms the oscillatory behavior proposed in our initial hypothesis, shows the limited set of conditions under which the system is stable, and suggests that the system may be subject to bifurcation.

INTRODUCTION

Endothelial nitric oxide synthase (eNOS) is the principal signal generator of NO in endothelial cells. Endothelial NO serves as a paracrine and autocrine messenger in vital biological functions such as vasodilation and relaxation of smooth muscle tissue, angiogenesis, and insulin secretion(1-3). Understanding how eNOS is controlled and how NO serves as a messenger have been areas of intense study for two decades.

Research has shown that the primary regulator of the signal generating NOS isoforms, neuronal (nNOS) and endothelial NOS (eNOS) is calcium/calmodulin (Ca^{2+}/CaM) (4); eNOS is also regulated by an array of protein-protein interactions and covalent modifications that include the effects of kinases, phosphatases, scaffolds, and protein inhibitor (5). The phosphorylation sites of eNOS are an area of particular importance in understanding its regulation.

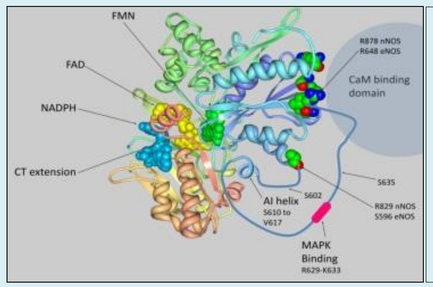


Figure 1: Structural cartoon of the eNOS reductase domains (based on nNOS reductase (15)). The FMN-binding domain is in blue, FAD-binding domain green, and the NADPH-binding domain tan. Cofactors are shown in solid render, as are residues at the N terminal edge of the FMN-binding domain and the ends of the autoinhibitory insertion (AI) (R878/R648 and R829/S596 for nNOS/eNOS). The approximate extent and position of the disordered regions of the AI are indicated by a drawn coil. The AI has the the MAP kinase binding site (shown in red). The C terminal extension carries the S1179 phosphorylation site. T495 is adjacent to the CaM binding site to the right.

Human eNOS is phosphorylated at sites Ser1177, and Thr4[illegible]rme) Ser1179 and Thr497 in bovine eNOS (the form most often s[illegible]rme) (6-8). The phosphorylation site at Ser1179 serves to increas[illegible] phosphorylation (primarily via AKT) near the C-terminal and contributes to the activity of eNOS by reducing the effect of the inhibitory C-terminal extension, whereas Thr495 phosphorylation by PKC inhibitse NOS via prevention of CaM binding to its canonical binding site (9). These phosphorylation sites serve as additional regulators of eNOS and are part of the signal transduction network essential to its activation and control.

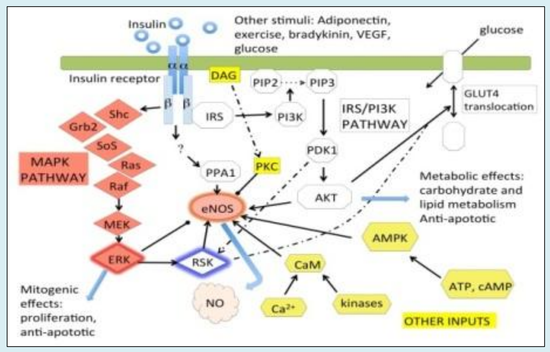
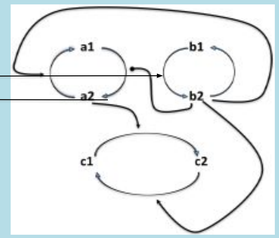
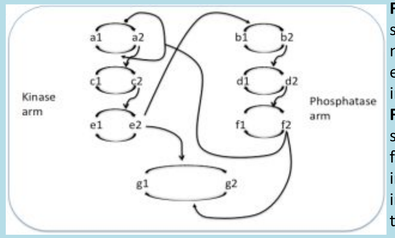


Figure 2: Scheme of eNOS regulation by various inputs including our hypothesized RSK connection.

ACKNOWLEDGEMENTS

This work was supported by NSF 1020261 and Research Corporation CC6942 (to C.A.C.), NIH 1R15GM083317 (J.L.M.) and NIH 3R15GM080701-01 and NSF 0950920 (J.C.S.).





Figures 3(Left): Figure 1 shows the initial proposed model of feedback with explicit delays and implicit intermediates.
Figure 4(Right): Figure 2 shows the current proposed feedback network, that incorporates explicit intermediates with an implicit time delay

$$\frac{\partial b1}{\partial t} = \frac{-b1}{Kbn+b1}Kb1 - \frac{b1}{Kbm+b1}Kb2a2 + \frac{b2}{Kbo+b2}Kb3$$

$$\frac{\partial b2}{\partial t} = \frac{-\partial b1}{\partial t}$$

$$\frac{\partial a1}{\partial t} = \frac{-a1}{Kan+a1}Ka1 - \frac{a1}{Kam+a1}Ka2b2 + \frac{a2}{Kao+a2}Ka3b2 + \frac{a2}{Kap+a2}Ka4$$

$$\frac{\partial a2}{\partial t} = \frac{-\partial a1}{\partial t}$$

Figure 5: Ka1, Ka2, Ka3, Ka4, Kb1, Kb2, and Kb3 are the rate constants for the forms of A and B, and a1, a2, b1, and b2 correspond to the respective fractional occupancies of A1, A2, B1, and B2. The parameters of the form Kam through Kap and Kbm through Kbo are effective Michaelis constants for the enzymes that interconvert the species.

Results

The program was able to replicate the oscillatory behavior seen in the phosphorylation of Ser1179. Serum starved endothelial cells treated with glucose showed a loss of phosphorylation at 30 seconds, and oscillated over the next 15 minutes, eventually reaching a steady state as seen in Figure 6. The oscillations produced by the program (Figures 10, 11), provided insight into additional time points to be examined. By using the model as a guide we examined additional time points at 1 and 4 minutes, which accurately validated the model predictions.

The program supported the presence of a negative feedback loop involved in the phosphorylation of Ser1179 and Thr495. By building on the initial two system program, we were able to model the system without time delays, suggesting that intermediates are in fact present, and provide the groundwork for future research as to their identities. We identified one of the intermediates involved in this cascade pathway as PKC, likely fitting into the phosphate arm of the model (Figure 4). The Western blots showed the phosphorylation of Ser1179 and Thr495 were out of phase, with one being on while the other was off and vice-versa (Figure 8). This suggests that though PKC is not directly involved in phosphorylation of Ser1179, that its activity and subsequent phosphorylation of Thr495 affect Ser1179, possibly through similar, or linked, pathways.

NO production also oscillated, with an increase in production corresponding to the phosphorylated state of Ser1179 by AKT (Thr495 dephosphorylated), and a decrease in NO production to the phosphorylated state of Thr495 (Ser1179 dephosphorylated). This activity further validated the role that the sites Ser1179 and Thr495 play in activation and inhibition, respectively.

The program also provided insight to the sensitivity of the system, initial states of the various intermediates, and the potential for the system to undergo bifurcation. The model obtained falls in line with Nyquists' asymptotic stability guidelines, in that the fractional occupancy of the respective intermediates approaches a stable state as $t \to \infty$. Under certain conditions and parameters sets the system becomes inherently unstable, and as such may be subject to bifurcation, potentially analogous to the symptoms of Type II diabetes.

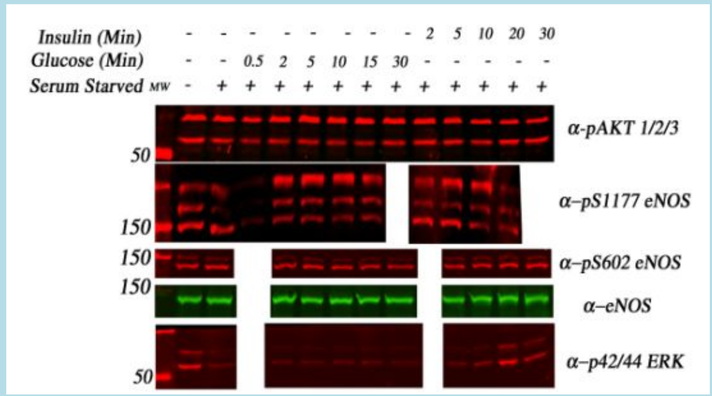


Figure 6: BAEC cells at confluence were serum starved in glucose free media for 3.5 hours. The cells were treated with glucose (20mM) or insulin (100 units/ml) for the indicated times. 10 cm plates were harvested in 0.2 mls lysis buffer and ~10% of the sample were analyzed by western analysis. Changes in phosphorylation status are rapid and oscillate, particularly for pS1177.

$$\partial a1/\partial t = -Ka1\cdot a1/an + a1 - f2d8\cdot Ka2\cdot a1/am + a1 + Ka3\cdot a2/ao + a2 + f2d9\cdot Ka4\cdot a2/ap + a2$$

$$\partial c1/\partial t = -Kc1\cdot c1/cn + c1 - a2d1\cdot Kc2\cdot c1/cm + c1 + Kc3\cdot c2/co + c2$$

$$\partial e1/\partial t = -Ke1\cdot e1/en + e1 - c2d4\cdot Ke2\cdot e1/em + e1 + Ke3\cdot e2/eo + e2$$

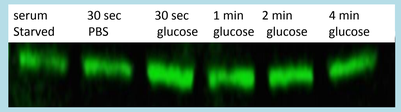
$$\partial b1/\partial t = -Kb1\cdot b1/bn + b1 - e2d7\cdot Kb2\cdot b1/bm + b1 + Kb3\cdot b2/bo + b2$$

$$\partial d1/\partial t = -Kd1\cdot d1/dn + d1 - b2d2\cdot Kd2\cdot d1/dm + d1 + Kd3\cdot d2/do + d2$$

$$\partial f1/\partial t = -Kf1\cdot f1/fn + f1 - d2d6\cdot Kf2\cdot f1/fm + f1 + Kf3\cdot f2/fo + f2$$

$$\partial g1/\partial t = -Kg1\cdot g1/gn + g1 - e2d11\cdot Kg2\cdot g1/gm + g1 + Kg3\cdot g2/go + g2 + f2d10\cdot Kg4\cdot g2/gp + g2$$

Figure 7: System of differential equations used to simulate the behavior of the model shown in Figure 4. The variables used are analogous to those seen in Figure 5. The equations were solved numerically using a fourth order Runge-Kutta program including the option of discrete delays.



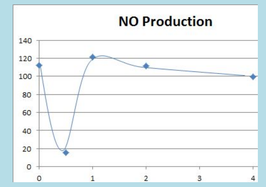
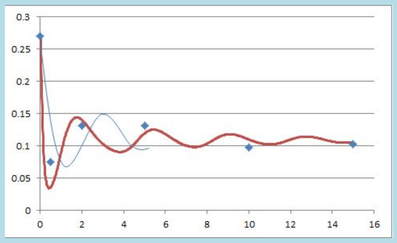
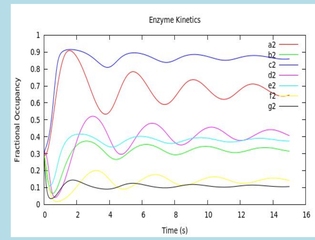


Figure 8: Above: Oscillation of eNOS phosphorylation at T495 by PKC after addition of a bolus of glucose to cultured endothelial cells; T496 phosphoryation has a frequency similar to that of S1177 phosphorylation. Right: output of NO during oscillations.





Figures 10 (Left): Figure 10 shows the plot obtained from the reporter term (g2) in the program (red) against the densitometry measurements obtained from the phosphorylation of Ser1179 (blue dots) along with the original proposed best fit line. **Figure 11(Right):** Figure 11 shows the plot of all the intermediates a2-g2 running the program using the same parameters used to obtain the plot seen in Figure 10.

Conclusions

- When treated with glucose, phosphorylation of the eNOS sites Ser1179 and Thr495 occurs, followed by oscillation
- The oscillations observed at the various phosphorylation sites on eNOS occur via a feedback loop involving a complex system of intermediates including kinases (AKT and PKC) and phosphatases
- The system is stable only under certain conditions, and when those conditions are not met may be subject to bifurcation
- The program constructed allows for accurate modeling of the observed phosphorylated states, and has applications in data confirmation, experimental design, intermediate identification, and can serve as a foundation for future systems that are prone to oscillatory behavior
- Future research into the conditions that create instability may have applications in the diagnosis and treatment of various physiological conditions, such as Type II diabetes

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