Proposal to Evaluate the Use of Cyclic GMP Accumulation as a Marker of Nitric Oxide Synthase Activity

Brandon S. Russell & Ryan C. Perkins
Department of Chemistry and Biochemistry
Univeristy of Mississippi
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Specific Aims

The overall goal of this research is to test the validity of the use of 3',5'-cyclic guanosine monophosphate (cGMP) accumulation as a measure of nitric oxide (NO) production and eNOS activity, and to compare the accuracy and efficiency of this method with the standard Griess assay. In order to accomplish this overall goal, two specific issues must be addressed. First we must determine the dose-response relationship between NO and soluble guanylate cyclase (sGC), testing the hypothesis that the relationship is linear over a sufficiently large range of concentrations to make cGMP a useful marker. Next we must measure both the intra- and extra-cellular accumulation of NO in vanadate-treated epithelial cells by the Griess assay and compare these values to the values obtained in the cGMP accumulation assay, testing the hypothesis that the level of NO measured by the cGMP assay will be proportional only to the level of extra-cellular NO measured by the Griess assay.

Background & Significance

Nitric oxide (NO) is an essential biological molecule that is a key component in cellular signaling in the neurons, immune system, and endothelial cells. NO is generated on demand by three difference nitric oxide synthases (NOSes) that are classified according to their location and function: inducible NOS in the immune system creates NO as a non-specific cytotoxic agent, neuronal NOS in the neurons creates NO as a neurotransmitter, and endothelial NOS in the endothelial cells creates NO as a second messenger in vasodilation [1]. These diverse functions make it clear that fully understanding the mechanism of NO action could have far-reaching implications, both in basic biochemical and therapeutically targeted research.

One of the major technical issues in studying NO is its short half-like (approximately a few seconds). While a number of highly sensitive probes that are capable of generating real time

data on NO concentration are commercially available, the invasive and often bulky nature of these probes makes them impractical for *in vivo* studies. The standard protocol for determining NO concentration *in vivo* is the Griess assay, which relies on the fact that the final oxidation product of NO in the presence of oxygen is the stable nitrite anion (NO_2^-). Treatment of a nitrite-containing sample with a sulphanilic acid followed by an azo dye results in the formation of a pink color that is proportional to nitrite concentration and can easily be measured by ultraviolet-visible spectrophotometry [2].

Recent literature [3] has employed a "reporter cell" method for measuring NO concentration, relying on the reaction of NO with sGC found in smooth muscle cells to produce cGMP, and using cGMP accumulation as a measure of NO production. While the discovery of a complimentary assay is an exciting prospect, there are a number of complicating factors involved in this method that have not yet been fully addressed.

The first factor is the question of the dose-response relationship between NO and sGC/cGMP. Self-regulation of a signaling pathway based on accumulation of some downstream species is a common biological feature, and it is possible that relatively long-term or high-concentration exposure to NO as is often seen in experiments is activating such a process in sGC. If this were the case, the relationship between NO concentration and cGMP accumulation would not be linear over a sufficiently large range to compare differences in NO activity across a variety of conditions because many of these conditions could be sufficient to trigger self-regulation of sGC, thus creating a "leveling effect" on the concentration of NO inferred. A related possibility is that long-term or high-concentration exposure to NO activates the phosphodiesterase pathway that degrades cGMP, which would have a similar leveling effect.

The second factor is the issue of intra- versus extra-cellular NO levels. Because the reporter cell assay relies on smooth muscles cells being grown in a co-culture with the cells being studied, the assay is only capable of measuring extra-cellular NO levels. Without also knowing intra-cellular NO levels, we are limited in the number of valid conclusions we can make regarding stimulation of NOS. For example, if we observed that a certain activator showed a five-fold increase in cGMP accumulation compared to another activator, that increase could either be caused by an increase in total NO production or an increase only in extra-cellular NO production. Furthermore, because sGC is not localized to the cell membranes, extra-cellular NO must diffuse into the reporter cells a sufficiently large distance in order to encounter sGC. This diffusion brings into play the variable solubility of NO in aqueous and lipid solutions, and further complicates the dose-response relationship between NO and cGMP.

The standard Griess assay avoids all of these issues. Because it relies on the spontaneous and inorganic oxidation of NO to nitrite rather than on a biochemical signally pathway, there is no possibility of regulation, making the relationship between NO and nitrite linear over an infinite range of NO concentration. Because nitrite is stable in aqueous culture medium as well as the inter-membrane and lipid spaces, the Griess assay can be used to determine both intra- and extra-cellular NO production, giving a more complete understanding of NO production. Given these issues, it is clear that more research is necessary to determine the true usefulness of the reporter cell assay.

Research Design & Methods

To address the first aim we must perform one experiment to determine the dose-response relationship between NO and sGC activity / cGMP accumulation. Rat aortic smooth muscle

cells (RASMC) will be exposed to NO and $O_2/CO_2/Ar$ using a previously described membrane-diffusion gas delivery system [4]. O_2 and CO_2 concentrations will be calibrated to correspond to *in vivo* levels, and total NO dosages will be varied from 0.005μM to 1.000 μM (concentrations represent minimum eNOS activity and ten-times maximum eNOS activity) [5]. Control cells will be exposed only to the same $O_2/CO_2/Ar$ mixture. Following exposure, cells will be collected and cGMP accumulation will be measured by the previously described protocol [3]. Exposures will be conducted in triplicate and dose-response curves will be generated for NO and sGC/cGMP. If the dose-response relationship is linear over a sufficiently large range of concentrations, then this would confirm our hypothesis and validate the ability of the reporter cell assay to measure NO concentrations across a wide variety of conditions. If the dose-response relationship is not linear over a significant range of concentrations, this would disprove our hypothesis and reveal a significant limitation of the reporter cell assay.

To address the second aim we must perform one experiment to compare intra- and extra-cellular NO levels measured by the Griess assay with the NO levels measured by the reporter cell assay. To measure extra-cellular NO levels, bovine lung microvascular cells (BLMVEC) will be cultured and treated with vanadate according to the previously described protocol [3]. Following treatment, cell culture medium will be collected and subjected to the Griess assay: treatment with 2% sulphanilamide /5% phosphoric acid, addition of 0.2% naphthylenediamine dihydrochloride, and absorbance measurement at 540-550nm. To measure intra-cellular NO levels, the same culture and treatment protocol will be followed. After treatment, cell culture medium will be aspirated and the cells collected, sonicated, and centrifuged. Following centrifugation, the supernatant will be subjected to the Griess assay. To measure NO levels by the reporter cell assay, BLMVEC and RASMC will be grown in co-cultures and exposed to vanadate according

to the previously reported protocol [3], and cGMP levels will be determined as described above. Values obtained for NO concentration from the reporter cell assay will then be compared to both intra- and extra-cellular NO concentrations measured by the Griess assay. If the NO concentrations indicated by the reporter cell assay are equal or proportional to the extra-cellular NO concentrations indicated by the Griess assay, then this would confirm our hypothesis and would validate the reporter cell assay for measuring extra-cellular NO. If the NO concentrations indicated by the reporter cell assay are proportional to the intra-cellular NO concentrations indicated by the Griess assay, this would disprove our hypothesis but would indicate a direct link between intra- and extra-cellular NO production, and would validate the reporter cell assay as a general assay for NO production.

Works Cited

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