Protein Kinase G May Exert Pro-Degradation Inhibition on Nitric Oxide Synthase

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Summary: Nitric oxide synthase (NOS) is regulated by protein-protein interactions. We had earlier shown that PKG inhibits activated NOS in endothelial cells and speculated that PKG phosphorylation of NOS terminates its activity. The present work examines if PKG activation increases breakdown of NOS. Diamino-fluorescein fluorescence spectrometry of real time NO production was used to establish that isolated ovine lung microvascular endothelial cells responded to PKG modulation as previously reported. Fluorescence activated cell sorter (FACS) analysis was used to establish that 8-Br-cGMP, a PKG activator, caused carboxy terminal deletion on NOS, a sign of degradation. Western blot analysis was used to investigate NOS fragments in control and 5 min 8-Br-cGMP treated cells. PKG activator 8-Br-cGMP, at 20 nM, 200 nM, and 2 μM, decreased nitric oxide production in a dose dependent manner (p<0.05 in all cases). PKG inhibitors: 100 μM Rp-8-Br-PET-cGMPS, 50 nM Rp-8-pCPT-cGMPS, or 4 μM Rp-8-Br-cGMPS Na significantly increased NO production (p<0.05) showing that PKG normally inhibits basal NO production. 8-Br-cGMP (100 nM) abrogated the elevation in NO production produced by the PKG inhibitors. FACS analysis revealed that PKG decreased NOS carboxy terminal labeling. Western blot analysis revealed that 8-Br-cGMP increased N-terminal serine-116 phosphorylated NOS fragments of molecular weights of about 60, 50 and 35 kDa. PKG may be a post-activation inhibitor of NOS, possibly important for the degradation of the spent enzyme.

Keywords*:* Protein kinase G, Nitric oxide synthase

INTRODUCTION

Nitric oxide synthase (NOS) is regulated by protein-protein interactions. Recently, we used real time measurement of basal NOS function by DAF assay in live endothelial cells and showed that PKG inhibits NOS. We speculated that PKG phosphorylation of NOS terminates its activity (John *et al*., 2008; John, 2010; John and Raj, 2010). Using FACS analysis, we also showed that PKG caused molecular and phosphorylation changes on the NOS molecule under basal conditions (John and Raj, 2010). In unpublished work, we have observed a differential subcellular colocalization of NOS with caveolin-1 versus PKG under basal conditions. NOS co-localizes with caveolin-1 at plasmallemal and golgi sites and with PKG in the cytosol, ER network, and nuclear sites, especially in vesicular formation, under basal conditions. This may be an indication that the two inhibitors have different effects on NOS function. Caveolin-1 appears to be a pre-activation inhibitor of nascent NOS while PKG appears to be a post-

activation inhibitor of spent NOS. Our present hypothesis is that PKG deactivates active NOS and subjects the spent enzyme to degradation. In this study, western blot analysis was used to observe remnant NOS N-terminal fractions obtained from live endothelial cells in control and cGMP treated cells.

The present report is a snippet of evidence that PKG phosphorylation of NOS precedes NOS degradation. More understanding of the endogenous regulation of basal NOS activity and the metabolic cycle of the NOS enzyme can help us develop even more precise therapeutic strategies for regulating NOS in disease conditions.

MATERIALS AND METHODS

Animals
This work was reviewed and approved by the Animal Care and Use Review Committee of Los Angeles Biomedical Research Institute. Newborn lambs aged <2 d were obtained from Nebeker Ranch (Lancaster, CA). Lung microvascular endothelial cell cultures

were isolated from the lambs and characterized as recently published (John *et al*., 2008; John, 2010).

Demonstration of role of PKG in regulation of NOS activity

To demonstrate the immediate effect of PKG activation on basal NOS activity of the cells, a cell permeable cGMP analogue, 8-Br-cGMP (Fluka Biochemika, Buchs, Switzerland), in concentrations of 20 nM, 200 nM, or 2 μM, was added to confluent microcultures with DAF fluorophores in a reaction medium comprised of PBS plus 5% phenol-red free DMEM, and 1% Gibco® antibiotic/antimycotic mixture. To demonstrate the effect of inhibition of PKG on basal NOS activity, cells were incubated with guanosine-3'-5'-cyclic monophosphoro thionate-8-Br-Rp isomer Na salt (Calbiochem EMD, San Diego, CA) in concentrations of 25 nM, 75 nM and 125 nM 30 min before the DAF combination was added. Experiments were completed as previously reported (John, 2010; John and Raj, 2010).

Demonstration of the effects of PKG inhibitors on PKG

Cells were preincubated with PKG inhibitors, 100 μM Rp-8-Br-PET-cGMPS (Biolog, Hayward, CA), or 50 nM Rp-8-pCPT-cGMPS (Biomol, Plymouth Meeting, PA), or 4 μM Rp-8-Br-cGMPS Na (Calbiochem EMD, San Diego, CA), 30 min before the DAF combination was added and with or without PKG activator, 100 μM 8-Br-cGMP, which was added 5 min before DAF was added. NO produced was measured after 5 min.

Fluorescence of NO produced by cells was calculated as the difference above fluorescence detected in the presence of NOS inhibitor, 200 μM L-NAME. Studies were done thrice. Representative data from single experiments (N=6) are presented.

Fluorescence activated cell sorter demonstration of the effect of 8-Br-cGMP on NOS C-terminal expression.

LMVECs were trypsinized and washed with PBS. Cell suspensions in PBS were split equally into control and 5 μM 8-Br-cGMP (5 minutes) treatment groups and immunolabled for detection of the C-terminal of the NOS molecule by FACS as earlier described (John and Raj, 2010) using a monoclonal antibody (1: 100) for specific probing. The mouse anti NOS antibody used was developed against the immunogen bovine eNOS c-terminal amino acids 1185-1205 (Sigma, St Louis MO). The FACS analysis system comprised of a BD FACS Calibur and a MacIntosh Q3 FACS workstation. The fluorescence intensities of sample populations were determined by Cell Quest software.

Detemination of the effect of 8-Br-cGMP on the immunoblot expression of N-terminal serine 116 phosphorylated NOS in endothelial cells.

To test the effect of 8-Br-cGMP on NOS N-terminal expression, serine 116-NOS immunolabeling in endothelial cells with western blot analysis was used. LMVECs from a single suspension were equally seeded in 100mm diameter dishes. Five-day-confluent triplicate monolayers were used for controls and identical triplicate monolayers of sibling cells were used as tests treated with 300 nM 8-Br-cGMP (Calbiochem, CA) for 15 min before routine immunoblot analysis. After electrophoresis and transfer of proteins to a membrane, the membrane was blocked with 5% non-fat milk and then incubated with a primary antibody against phosphorylated serine-116 region of NOS (1: 200 concentration, Sigma, St. Louis) at 4C for 24h with gentle rocking. The experiment was completed as reported (John *et al*., 2008). Protein bands were visualized by horseradish peroxidase conjugated secondary antibodies (Amersham, Buckinghamshire, UK) using Super Signal® West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) to prepare the developing solution. Membranes were exposed to Blue Lite Autorad Films (ISC BioExpress, Kaysville, UT), and the films were processed in a HOPE developer.

RESULTS

Protein Kinase G inhibits basal NOS activity in neonatal ovine lung microvascular endothelial cells.

All data were corrected for extraneous fluorescence due to DAF autofluorescence as previously reported (John *et al*., 2008; John 2010). L-NAME decreased fluorescence produced by NO in the LMVEC microcultures from the basal value of 3783.14 ± 51.01 to 1933.33 ± 16.15 (N=6, $p<0.02$). The analogue of cGMP, 8-Br-cGMP, which activates PKG decreased basal NO production when given in concentrations of 20 nM, 200 nM and 2 μM 8-Br-cGMP. The fluorescence was decreased from the basal value of 3783.14 ± 51.01 to 2780.00 ± 21.79, 2439.25 ± 16.09, and 1831.25 ± 15.45 respectively in 5 min. The significance values were $p<0.05$, $p<0.004$, and $p<0.004$ respectively. The results were converted to percentage change in basal NO and plotted against drug concentrations (Figure 1, second to fourth bars, N=6).
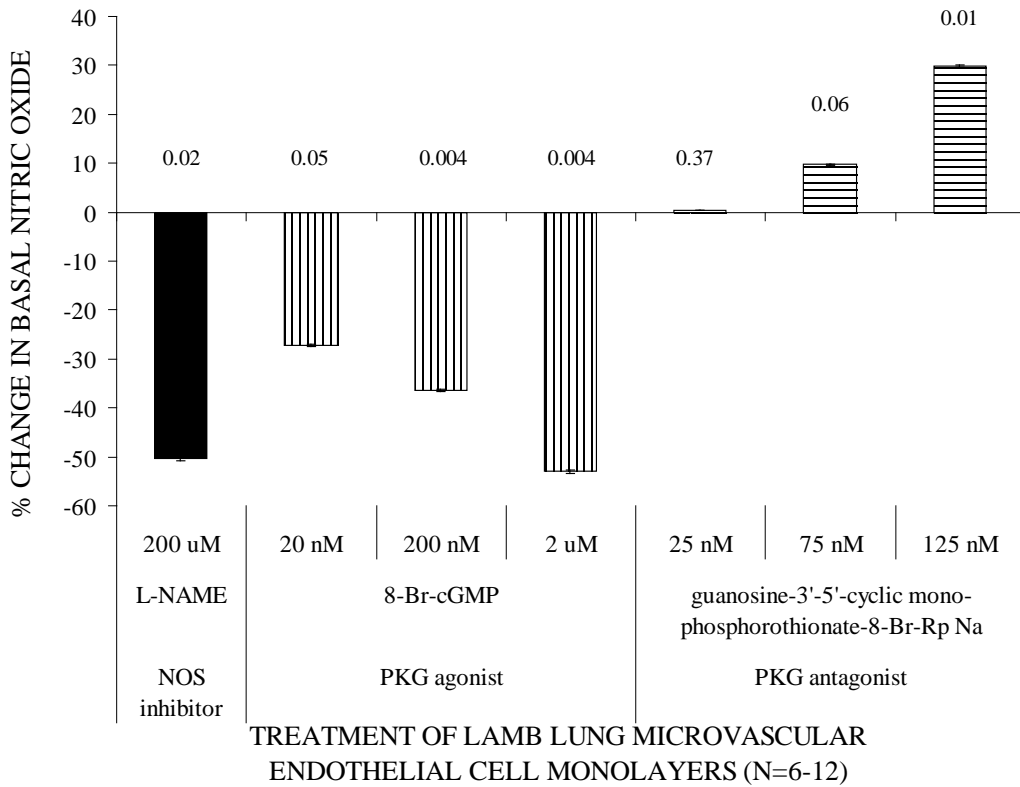
Figure 1.
PKG activation and inhibition have opposite effects on basal NO production in microvascular endothelial cells. Using paired t-tests, the inhibitory responses to PKG activation were all significant at p<0.05 (values shown). The enhancement of NO production by the PKG antagonist was significant at 125 nM (p<0.01). Studies were done at least thrice with similar results. Figure shows means ± SE of representative data with significance levels of differences from basal values indicated for each test.
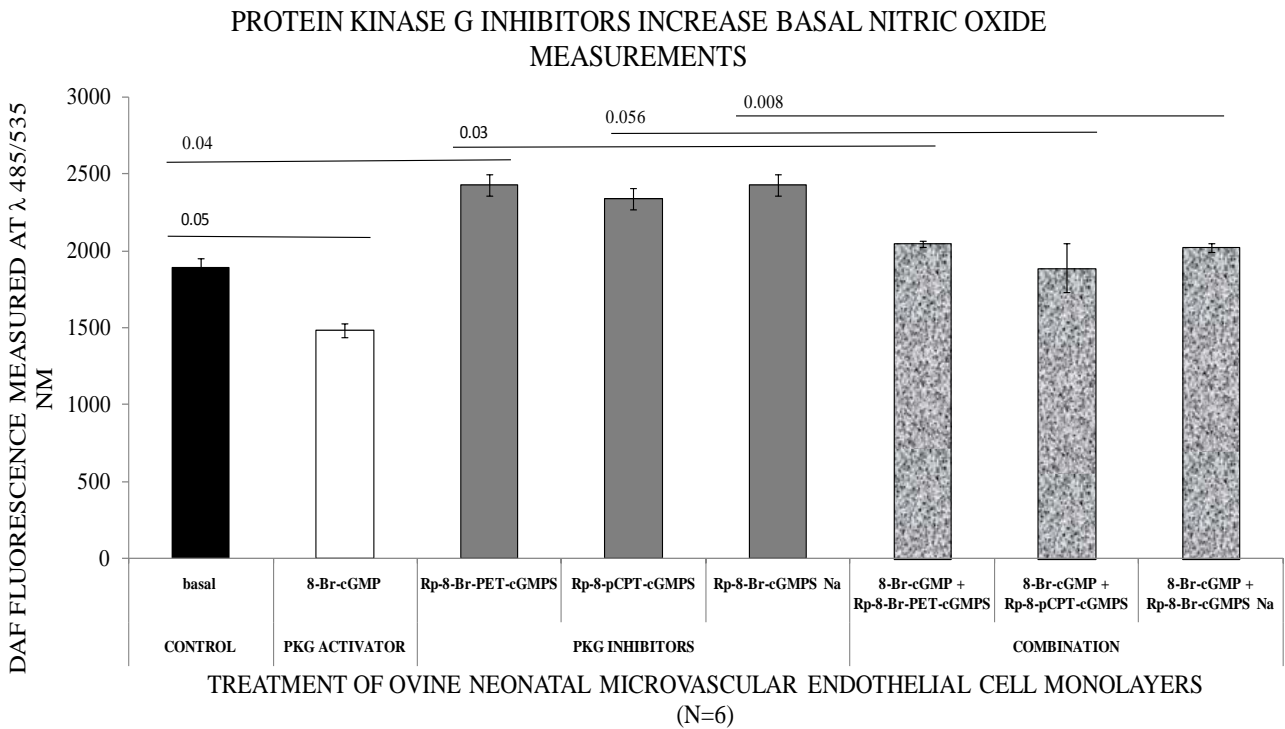

Figure 2.
Enhanced nitric oxide production by PKG inhibitors is reversed by 8-Br-cGMP. Figure is data, mean ± SE, from a representative experiment with indication of significance levels for differences between pairs.
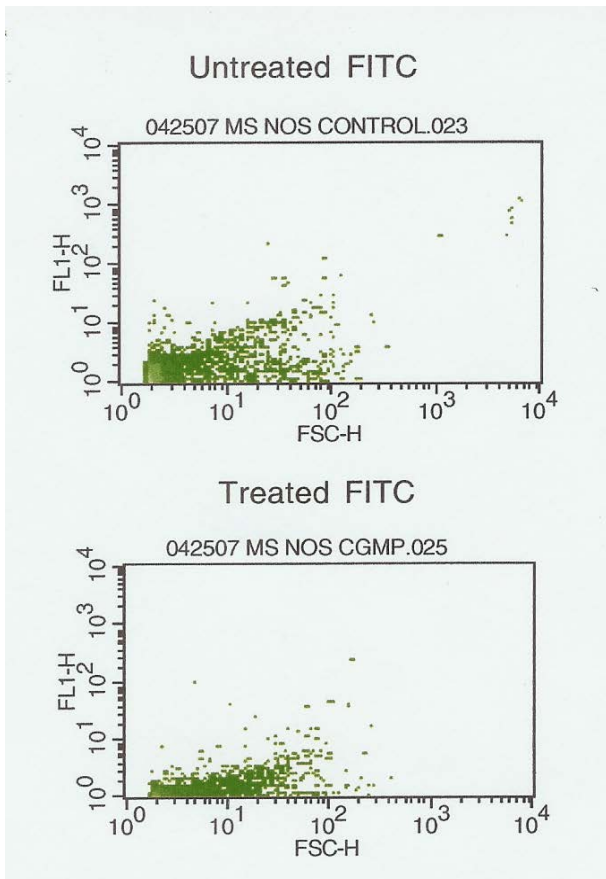
Fig. 3
FACS analysis shows that PKG activation decreases NOS carboxy terminal antibody labeling. In the analysis of fluorescein tagged cells, 10,000 events were taken per sample. Upper channels show control cells and lower channels show PKG activator 5 µM 8-Br-cGMP treated cells. The fluorescence intensity range (y-axis) was decreased by PKG activation. The mean fluorescence value for cells gated at 10^1 fluorescence units (FU) was 98.48 ± 2.13 for controls and 38.62 ± 1.18 for 8-Br-cGMP treated cells, a 60.8 % decrease by PKG activation. Figure is a representative of two studies.

Protein Kinase G inhibitors enhance NO production in untreated neonatal ovine lung microvascular endothelial cells.
PKG inhibitor, guanosine-3'-5'-cyclic monophosphoro thionate-8-Br-Rp isomer Na salt in concentrations of 25 nM, 75 nM and 125 nM increased basal NO production from 3539.00 ± 28.41 to 3554 ± 30.46, 3881.83 ± 36.47, and 4598.33 ± 50.24 respectively (N= 6 per group). The PKG inhibitor at a concentration of 125 nM significantly increased basal NO (p<0.01). The results were converted to percentage change in basal NO production and plotted against drug concentrations (Figure 1, last 3 bars).

In another study, the effect of PKG inhibitors without and with cGMP were compared using fluorescence of NO produced by cells calculated as the difference above fluorescence detected in the

presence of NOS inhibitor 200 uM L-NAME. The PKG activator 100 µM 8-Br-cGMP decreased NO production from the basal value of 1856.7 ± 62.6 to 1451.5 ± 34.1 (N=6, p<0.05) (Figure 2, second bar).
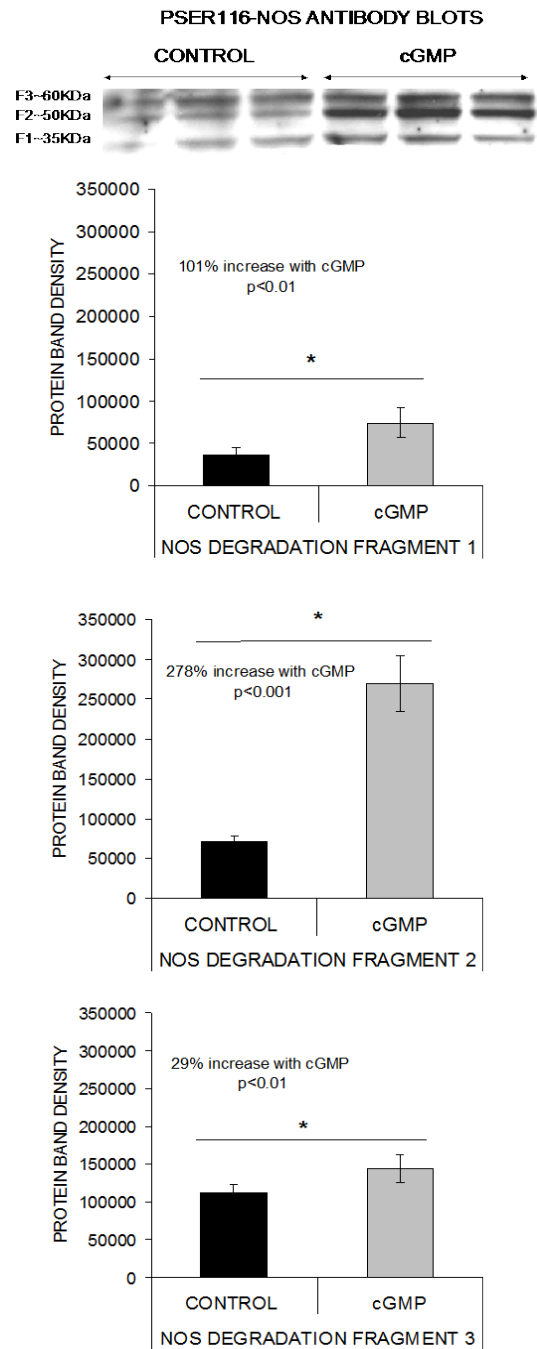

Fig. 4
PKG activation increases anti-serine116-NOS antibody labeling of NOS N-terminal fractions in normal endothelial cells. The film developed from western blotting showed that serine-116 phosphorylated NOS can be normally found in control cells as enzyme fragments and that these fragments are increased by PKG activation.

In this study, comparing the effects of preincubation with three PKG inhibitors in sibling cells, 100 µM Rp-8-Br-PET-cGMPS, 50 nM Rp-8-pCPT-cGMPS, or 4 µM Rp-8-Br-cGMPS enhanced

basal NO production from 1856.7 ± 62.6 to 2362.7 ± 55.5 (p<0.04), 2316.7 ± 55.13, and 2358 ± 47.4 respectively (N=6, p<0.05 in all cases) (Figure 2, third to fifth bars). The effect of the PKG inhibitors was attenuated by 100 μM 8-Br-cGMP given with DAF to give values of 2001.5 ± 10.7, 1864 ± 123.2, 1981 ± 16.9 (p=0.03, p=0.056, p=0.008 respectively, N=6 in all cases) (Figure 2, last three bars).

PKG activation decreases NOS carboxy terminal whole-cell immunofluorescence expression.

The treatment of cells with 5 μM 8-Br-cGMP caused decrease in NOS detection by the NOS carboxy terminal moiety primary antibody given less than 5 min before fixation. In the FACS analysis of fluorescein tagged cells, 10,000 events were taken per sample. The mean fluorescence value for cells gated at 10^1 fluorescence units (FU) was 98.48 ± 2.13 for controls and 38.62 ± 1.18 for 8-Br-cGMP treated cells, a 60.8 % decrease by PKG activation (Figure 3). A small proportion of 0.8% of the controls and 0.19% of the 8-Br-cGMP treated had greater than 10^1 FU, a 75.25 % decrease by 8-Br-cGMP.

PKG activation increases N-terminal serine116 phosphorylated NOS immunoblot expression.

Control endothelial whole cell lysates showed the presence of serine 116 phosphorylated NOS fragments. These were picked up in bands representing molecular weights of 60, 50 and 35 kDa (Figure 4). Lysates of sibling cells treated with 8-Br-cGMP and expressing similar actin contents showed an increased expression of serine 116 phosphorylated NOS bands. The mean percentage increase in band density for each NOS protein N-terminal fraction is plotted and ranged from 29% - 278%, a significant increase in all cases (Figure 4).

DISCUSSION

The importance of nitric oxide synthase (NOS) which produces nitric oxide (NO) for vasodilatation and other vascular functions (Furchgott and Vanhoutte, 1989; Palmer *et al.*, 1987; Ignarro *et al.* 1987) has made this enzyme a topic for in-depth research in the past 20 years. Inadequate NO leads to endothelial dysfunction. Diseases such as sexual dysfunction, pulmonary hypertension, heart failure, and atherosclerosis are linked to endothelial dysfunction involving nitric oxide synthase (Arnal *et al.*, 1999; Fostermann and Munzel 2006). We have been studying how basal NO production is controlled in endothelial cells (John *et al.*, 2008; John, 2010; John and Raj, 2010). Similar to our earlier publications, we show here, using DAF assays, that cGMP, a PKG activator, decreases NOS function in producing nitric oxide in live endothelial cells (John, 2010; John and Raj, 2010) (Figure 1 and 2). The present and the

published data indicate that PKG is involved in the termination of basal NOS activity. The PKG activator, 8-Br-cGMP, inhibits basal NO production in a dose-dependent manner (Figure 1). Three different PKG inhibitors enhanced basal NO production in cells that received no other drug (Figure 2) and the effect is reversed by 8-Br-cGMP. It appears that the immediate effect of PKG inhibition is that basally active NOS is potentiated and enhanced NO is detected. Similar to earlier publication also (John and Raj, 2010), we show here that in-cell immunolabeling of carboxy-terminal of NOS is decreased by cGMP in FACS analysis (Figure 3), therefore PKG may be causing deletion of carboxy terminal of NOS (possibly a degradative deactivation of the active enzyme).

Figure 4 is a western blot of serine 116 phosphorylated NOS appearing in molecular weight fragments of about 60, 50 and 35 kDa, an indication that the N-terminal remnants of NOS may be normally found in control cells and may be increased by PKG activation. From previous publication, apparently, 8-bromo-cGMP instantaneously causes both increase in phosphorylated serine 116 N-terminal recognition and decrease in phosphorylated serine 1177 carboxy terminal recognition of NOS protein in these quiescent endothelial cells (John and Raj, 2010).

In contrast to decreased carboxy terminal labelling observed, endothelial cells with added cGMP appear to display more N-terminal immunolabelling of NOS than controls (Figure 4). Decreased C-terminal and increased N-terminal detection may be indicating that degradation of NOS begins at the carboxy terminus and the N-terminus may not be subjected to the same process of degradation as the carboxy terminus or its degradation lags behind that of the carboxy terminus. If this were the case, NOS-N-terminal detection by western blotting should be the same for controls and for cGMP treated cells. The NOS N-terminal accumulation observed by western blotting (Figure 4) may indicate that this fragment is actually retained in the cell and normally utilized. Antibodies against serine 116-phosphorylated NOS, (serine 116 being in the N-terminal region) showed punctuate and vesicular immunoflourescence in the ER network and in the nuclear regions of these endothelial cells (John *et al.*, 2008). If the N-terminal fragment is relocated and transferred to the nuclear region it is hypothetical that it may actually act as a transcription factor for the production of new NOS enzyme in normal endothelial cells.

In the present study, the duration of exposure of the endothelial cells to cGMP is only 5 minutes and is probably not enough time to observe a genomic effect. Rather, it appears that the rate of production

of the N-terminal fragment (by NOS degradation) in the endothelial cells with added cGMP (Figure 4) is faster than the basal rate of a hypothetical utilization of the N-fragment (such as translocation of the N-terminal to the nucleus and its utilization to generate fresh enzyme), therefore it accumulates. This may be a snippet of evidence that NOS is replenished by a PKG-dependent feedback process to maintain basal NO production in normal endothelial cells. We need to study this in depth. It is not known how NOS is continually replenished to sustain basal NO production under physiologic conditions, a vital function in the cardiovascular system.

To follow up the speculation of this report, we would need kinetics comparison of the effects of cGMP on the rate and duration of inhibition of NOS enzyme and NO production, the rate of generation of N-terminal fragments and their stability, and the rate of de novo production of NOS enzyme in normal cells and in cells with disturbed vesicular processes. We would also need to study NOS N-terminal permeated cells to determine if this molecular fragment is truly involved in NOS transcription and synthesis and thus could be a drug candidate for cardiovascular diseases.

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