MODULATION OF NITRIC OXIDE RELEASE IN CULTURED HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS BY MYRISTOYLATED-PKC EPSILON ACTIVATOR/INHIBITOR PEPTIDES

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INTRODUCTION

Protein kinase C epsilon (PKCε) is known to increase endothelial nitric oxide synthase (eNOS) activity by binding to a specific receptor for activated C kinase (RACK-1), facilitating its translocation from the cytosol to the cell membrane for phosphorylation of eNOS at serine-1177 to augment activity [1,2]. Previous *in vitro* and *in vivo* animal studies have demonstrated that PKCε activation stimulates eNOS activity, increasing nitric oxide (NO) release; whereas a peptide inhibitor that disrupts PKCε interaction with RACK-1 results in decreased NO release [3,4,5,6,7].

However, the modulation of PKC-mediated eNOS activity (Figure 1) is not well known in human endothelial cells. Moreover, elucidating the role of PKCε in regulating eNOS activity would be essential in the clinical setting of ischemic heart disease. Re-establishing blood flow after thrombus removal results in endothelial dysfunction that is characterized by limited NO bioavailability and excess generation of reactive oxygen species (ROS) by eNOS during reperfusion (Figure 2).

Therefore, we aim to demonstrate enhancement and attenuation of NO release in cultured HUVECs using cell-permeable, myristic-acid conjugated PKCε activator (Myr-PKCε+), Myr-HDAPIGYD (MW = 1097 g/mol), and inhibitor (Myr-PKCε-), N-Myr-EAVSLKPT (MW = 1054 g/mol), in the absence and presence of acetylcholine (Ach). Ach is a well-established positive control to activate eNOS via calcium calmodulin (CAM) and promote NO release from vascular endothelial cells [7, 8]. Determining NO release using selective cell permeable PKCε activator (Myr-PKCε+) and inhibitor peptides (Myr-PKCε-) in cultured HUVECs under normoxic conditions would provide a foundation to test these peptides under hypoxic-reoxygenation conditions when eNOS would be uncoupled and produce ROS instead of NO release.

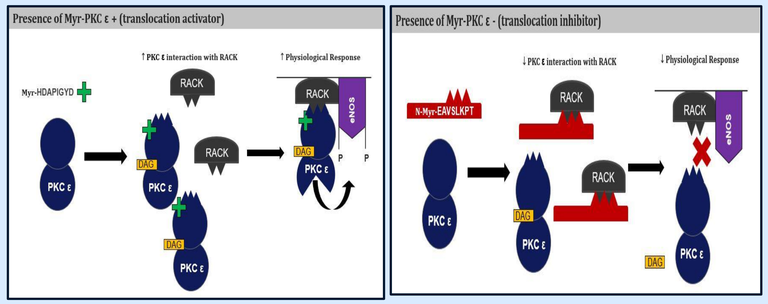

Figure 1. Mechanism of action of Myr-PKCε activator (Myr-HDAPIGYD) and Myr-PKCε inhibitor (N-Myr-EAVSLKPT), adapted from [1]. When activated by diacylglycerol (DG), PKCε binds to specific receptor for activated C kinase (RACK) domain which translocates PKCε to interact with substrates (e.g. eNOS) to produce a physiologic response (left panel). Myr-PKCε+ (in green) prolongs the active confirmation of PKCε to facilitate increased interaction with RACK, resulting in increased physiological response. Myr-PKCε- (in red) inhibits PKCε or translocation by binding to its specific RACK to reduce physiologic response (right panel).

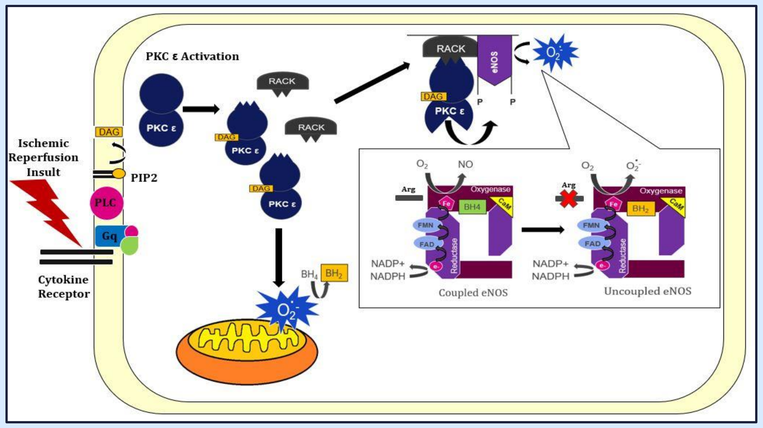

Figure 2. Schematic representation of PKC regulation of eNOS and mitochondrial-derived superoxide (O_2^-) release in myocardial I/R, adapted from [5]. I/R insult activates PKC and opening of mitochondrial potassium ATP channels resulting in uncoupled eNOS activity by PKCε.

HYPOTHESIS

We hypothesize that Myr-PKCε+ would increase NO release, whereas Myr-PKCε- would decrease NO release from cultured HUVECs in the absence and presence of Ach.

METHODS

Measurement of NO release from cultured HUVECs: Single-donor HUVECs (Lonza, Walkersville, MD) at passages 3-4 were grown to confluence in 6-well (10^6 cells/well) plates. NO release was measured in real time using a calibrated NO electrode following the administration of 10 μM Myr-PKCε+ or 10 μM Myr-PKCε- treatments in the absence or presence of 10 μM Ach stimulation of NO release. All treatments were prepared in endothelial growth media (EGM). Basal NO release was determined by measuring the difference between wells with and without cells.

Statistical Analysis: All data in the figures are presented as means ± S.E.M. ANOVA analysis using Student-Neuman-Keuls was used to assess statistical difference in NO release between basal levels vs Myr-PKCε+ treated or Myr-PKCε- treated cultured HUVECs in the absence and presence of Ach stimulation (Ach or EGM); p<0.05 were considered statistically significant.

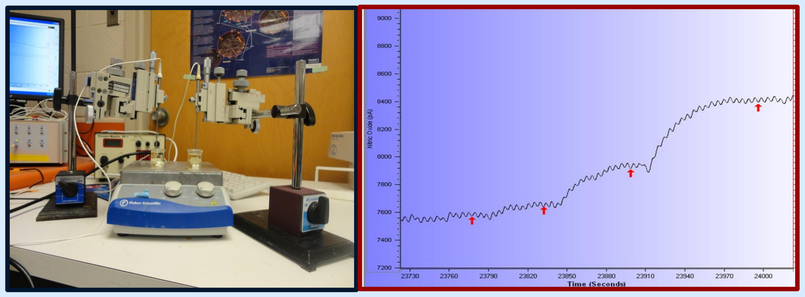

Figure 3. Illustration of NO electrode calibration setup (left), calibration curve (top right) and HUVEC NO measurement in 6-well plates (bottom). The NO electrode was calibrated based on the following reaction: $2KNO_2 + 2KI + 2H_2SO_4 \rightarrow 2NO + I + 2H_2O + 2K_2SO_4$ (e.g. y = 0.9346x + 3.4828; R^2 = 0.997) that corresponded to a 25 nM to 200 nM range (right). Dose responses are indicated by red arrows.

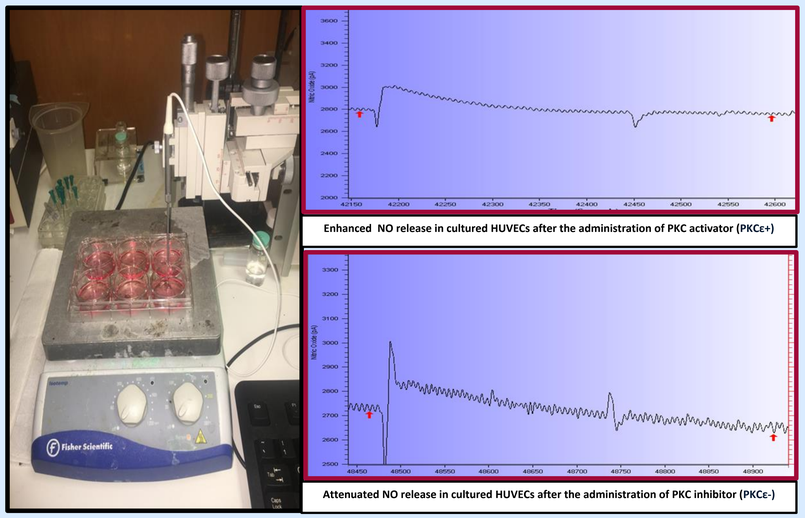

Figure 4. Illustration of real-time NO release measurements in cultured HUVECs using calibrated NO electrode (left). Representative tracings of NO released from cultured HUVECs was measured after administration of the 10 μM PKCε activator (top right) or 10 μM inhibitor (bottom right) in the absence and presence of Ach (10 μM). Basal NO release was determined by measuring the difference between a well containing cells and one well containing only endothelial growth media (EGM) without cells. Picoamp responses (red arrows) were converted to picomoles/10^6 cells based on the calibration curve of the NO electrode.

RESULTS

Basal NO release (83±12 pmol) was determined by measuring the difference between wells with and without HUVECs (n=9, P<0.05). In the absence of Ach stimulation, Myr-PKCε+ treatments significantly enhanced NO release to 136±13 pmol (n=6, p<0.05) and Myr-PKCε- attenuated total NO release to 16±27 pmol (n=7, p<0.05) compared to basal levels.

As a positive control, 10 μM Ach significantly enhanced NO release to 153±11 pmol above basal levels (p<0.05, n=20). In the presence of Ach simulation, Myr-PKCε+ still significantly increased NO release above basal levels to 129±17 pmol (p<0.05, n=8) and Myr-PKCε- decreased NO release to 34±12 pmol, (p<0.05, n=7) compared to basal levels, as shown in Figure 5.

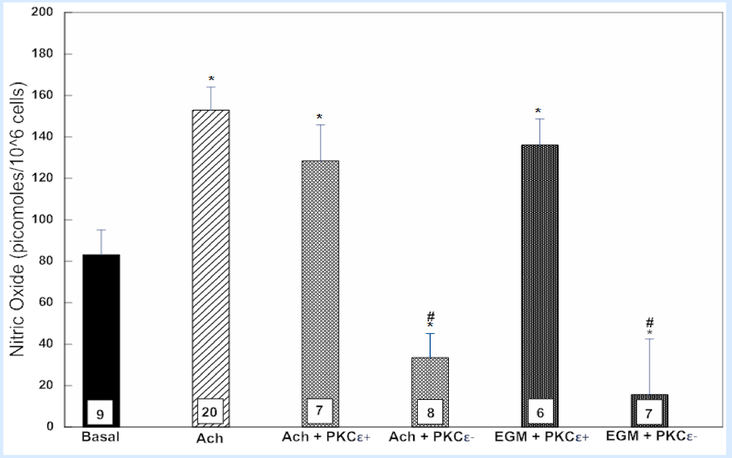

Figure 5. NO release (picomoles) in cultured HUVECs (10^6 cells/well) following Myr-PKCε+ (10 μM) and Myr-PKCε- (10 μM) treatments in the absence and presence of ACh stimulation. EGM represents negative control containing only endothelial growth media without Ach; *p<0.05 vs. Basal values; #p<0.05 vs. Myr-PKCε+ treatments.

CONCLUSION

These results suggest that in cultured HUVECs, Myr-PKCε+ presumably increases NO release via activation of eNOS, whereas Myr-PKCε- attenuates eNOS activity by inhibiting its phosphorylation; thus these peptides demonstrate comparable effects across species in regulating NO release. The understanding of the role of eNOS modulation is important for developing therapeutic interventions in the context of human organ-ischemia reperfusion injury, in which uncoupled eNOS produces ROS instead of NO.

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