SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Materials - Antibodies were from the following sources: ERK-pT^{202}/Y^{204}, JNK-pT$^{183/}$Y^{185}, AKT-pT^{308}, AKT-pS^{473}, Shc-pYY$^{239/240}$, GSK3α/β-pS^{21}/S^{9}, and Bcl-2 (Cell Signaling Technologies); PKCε and Caveolin-3 (BD Transduction Laboratories); PKCδ and GSK3 (Santa Cruz Biotechnology); AKT, FOXO3a-pT^{32}, and EGFR (Upstate Biotechnology); p27Kip and BIM (BD Pharmingen); p66Shc-pS^{36} (Alexis Biochemicals); MnSOD (Assay Designs); PCNA (Abcam). PMA, and PDGF were from Sigma–(St. Louis, MO). 1, 2-dioctanoyl-sn-glycerol (diC8) was from Avanti Polar Lipids, Inc. All other chemicals were reagent grade.

Cardiomyocyte culture **-** Cardiomyocytes were isolated from the hearts of 2-day-old Wistar rats by a trypsin dispersion procedure using a differential attachment protocol to enrich for cardiomyocytes followed by irradiation as described previously [1-3]. The yield of cardiomyocytes typically is 2.5-3 x 10^6 cells per neonatal ventricle. Cells were plated on protamine sulfate-coated culture dishes at a density of 5 x 10^6 cells/100-mm dish. Experiments were performed on cultures grown for 5 days in MEM (Gibco BRL) supplemented with 10% fetal calf serum and then serum-deprived for the subsequent 24 hr.

Adenoviral infections **-** Infections with adenoviral constructs that drive expression of WT-PKCδ, KD-PKCδ, WT-PKCε or β-galactosidase (β-gal) as a control. were performed according to methods described previously [1]. Briefly, infections were performed on cultures that had been grown for 5 days in MEM supplemented with 10% fetal calf serum, with protein extracts prepared 48 hr following infections. Infections also were performed with an adenovirus that drives expression of a 19-mer sequence

corresponding to bases 45–63 in the p66Shc unique N-terminal CH2 domain (Adp66shcRNAi, generously provided by Dr. Irani, University of Pittsburgh Medical Center). In this case, infections were initiated on culture day 1. Studies showing that this Ad-p66ShcRNAi rapidly (within 24 hr) and persistently (up to 5 days) downregulates p66Shc without influencing p46Shc/p52Shc expression in HeLa and endothelial cells have been published [4;5].

Immunoprecipitation and immunoblot analysis. Immunoprecipitation and immunoblotting was performed according to methods described previously or manufacturer's instructions. In each figure, each panel represents the results from a single gel (exposed for a uniform duration); detection was with enhanced chemiluminescence with results quantified by scanning densitometry.

Preparation of caveolae membranes. Fractions enriched in the muscle-specific caveolin-3 isoform were prepared according to a detergent-free purification scheme described previously [2;3]. All steps were carried out at 4°C. Briefly, cells from five 100-mm diameter dishes were washed twice with ice-cold phosphate-buffered saline and then scraped into 0.5 M sodium carbonate, pH 11.0 (0.5 ml/dish). Cells from five dishes were combined (total volume, 2.5 ml) for each preparation. The extract was sequentially disrupted by homogenization with a loose-fitting Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts), and a tip sonicator (three 20-s bursts). The homogenate was then adjusted to 40% sucrose by adding an equal volume of 80% sucrose prepared in MES-buffered saline (25 mM MES, pH 6.5, and 0.15 M NaCl), placed on the bottom of an ultracentrifuge tube, overlaid with a 5 to 30% discontinuous sucrose gradient (3 ml of 5% sucrose and 4 ml of 35% sucrose, both in MES-buffered

saline containing 0.25 M sodium carbonate), and centrifuged at 38,000 rpm for 16 to 18 h in a SW40 rotor (Beckman Coulter, Palo Alto, CA). After centrifugation, thirteen 1-ml fractions were collected. A pooled caveolae fraction (fractions 4-5, containing all of the buoyant caveolin-3 immunoreactivity and 0.5-1 % total starting cell protein), a pooled fraction 8-13 (F8-13, which contains the bulk of the cellular material including the cytosol and most of the particulate membrane fraction), and the insoluble pellet (P, which is solubilized in SDS-PAGE sample buffer) were subjected to SDS-PAGE and immunoblotting. The caveolin-3-enriched membrane fraction isolated according to this method is biochemically distinct from the surrounding phospholipid bilayer and is operationally defined as caveolae in this study. We recognize that this buoyant membrane fraction undoubtedly contains both true caveolae (specialized lipid raft membranes that contain caveolin and form invaginations at - or vesicles close to - the surface membrane) and morphologically featureless lipid rafts (that coexist and may even associate with caveolae [6]); biochemical methods to separate these distinct membrane subdomains and experiments to resolve their discrete cellular functions are beyond the scope of this study.

Measurements of intracellular ROS – Cardiomyocytes were loaded with dihydroethidium (10 μM) in the presence of vehicle or stimuli (10 μM NE or 50 μM H_2O_2); stimulation was in the presence of 10 mM Tiron in some experiments. Images were captured every 3-5 min using a Zeiss LSM 510 NLO confocal microscope (excitation 470–490 nm, emission 510–550 nm).

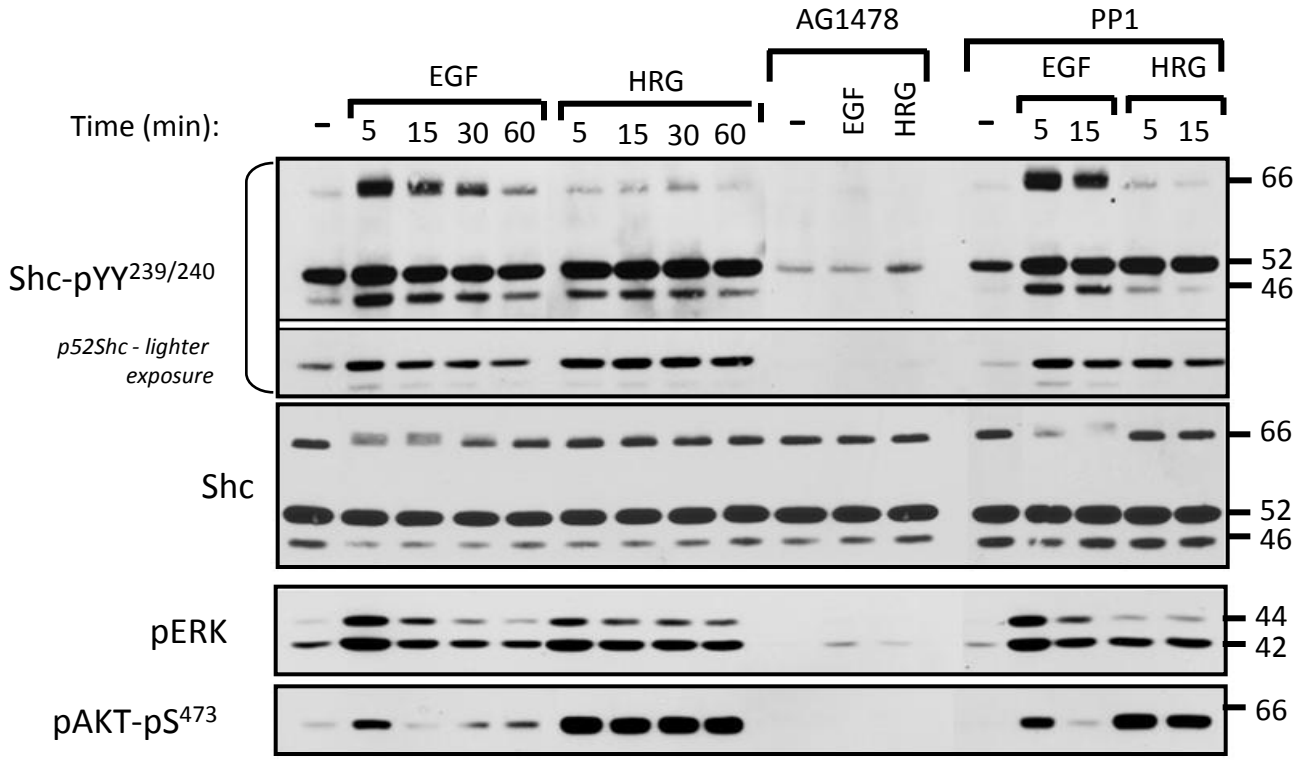
Measurements of Cardiomyocyte Growth – Cell surface area was measured by digitized image analysis as described previously [8]. Briefly, cell were treated with agonists for 72

hours and then imaged, with 8-10 frames per dish captured at 150x magnification; 30 to 50 cells were analyzed for each treatment. Measurements of protein content were performed in triplicate on cardiomyocytes cultured in serum-free medium with vehicle or the indicated agonists for 48 hr. Cells were rinsed with PBS and scraped into 0.2N perchloric acid, collected by centrifuge at 10,000 g for 10 min, and redissolved in 250 µl of 0.3 N KOH (for 20 min at 60°C). Triplicate aliquots from each sample were assayed according to standard methods for protein by the Lowry method (with bovine serum albumin as a standard).and for DNA using 33258 Hoechst dye (with calf thymus DNA as a standard [7]).

REFERENCES

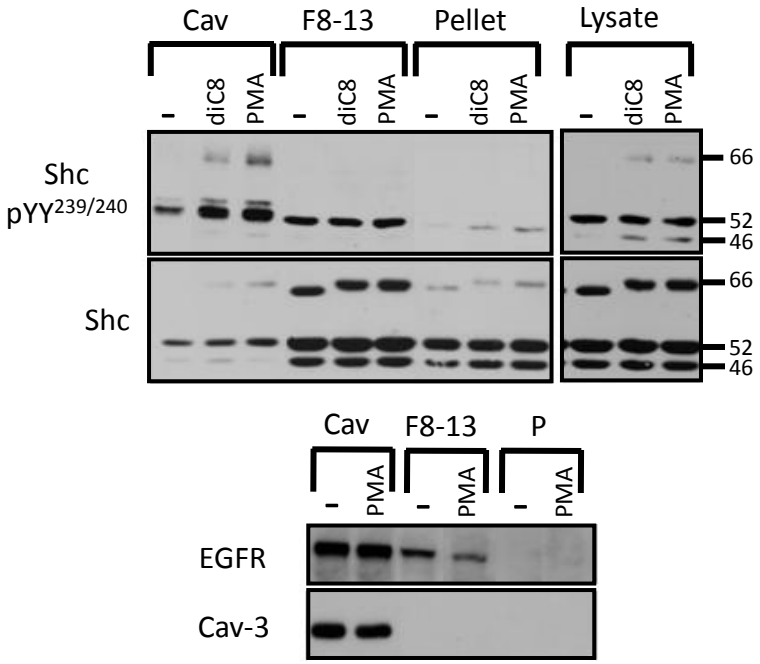
1. Rybin VO, Guo J, Gertsberg Z, Elouardighi H, Steinberg SF. Protein kinase Cε and Src control PKCδ activation loop phosphorylation in cardiomyocytes. *J Biol Chem*. 2007;282:23631-23638.
2. Rybin VO, Xu X, Steinberg SF. Activated protein kinase C isoforms target to cardiomyocyte caveolae. *Circ Res*. 1999;84:980-988.
3. Rybin VO, Xu X, Lisanti MP, Steinberg SF. Differential targeting of β-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae: A mechanism to functionally regulate the cAMP signaling pathway. *J Biol Chem*. 2000;275:41447-41457.
4. Kisielow M, Kleiner S, Nagasawa M, Faisal A, Nagamine Y. Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA. *Biochem J*. 2002;363:1-5.

5. Yamamori T, White AR, Mattagajasingh I, Khanday FA, Haile A, Qi B, Jeon BH, Bugayenko A, Kasuno K, Berkowitz DE, Irani K. p66shc regulates endothelial NO production and endothelium-dependent vasorelaxation: implications for age-associated vascular dysfunction. *J Mol Cell Cardiol*. 2005;39:992-995.
6. Oh P, Schnitzer JE. Segregation of heterotrimeric G proteins in cell surface microdomains. G_q binds caveolin to concentrate in caveolae, whereas G_i and G_s target lipid rafts by default. *Mol Biol Cell*. 2001;12:685-698.
7. Vijayan K, Szotek EL, Martin JL and Samarel AL. Protein kinase C-α-induced hypertrophy of neonatal rat ventricular myocytes. *Am J Physiol Heart Circ Physiol*.2004; 287: H2777-H2789.
8. Sabri A, Muske G, Zhang H, Pak E, Darrow A, Andrade-Gordon P, Steinberg SF. Signaling properties and functions of two distinct cardiomyocyte protease-activated receptors. *Circ Res*. 2000; 86:1054-1061.

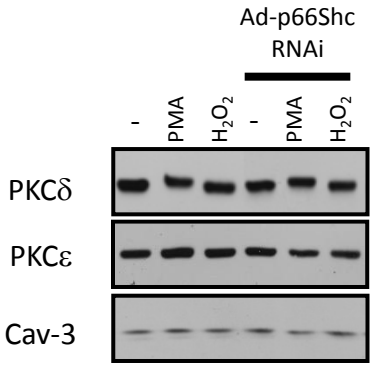


Online Figure I. EGFR activation of p66Shc-YY$^{239/240}$ phosphorylation.

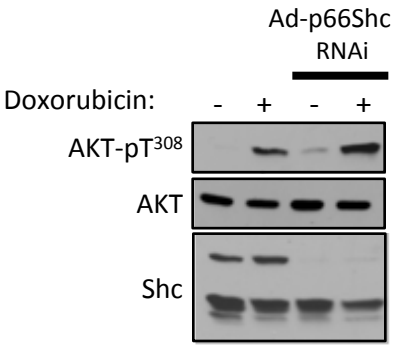
Immunoblotting on cell lysates from cardiomyocytes pretreated for 30 min with vehicle, PP1 (10 μM), or AG1478 (2 μM) followed by stimulations with 100 nM EGF or 100 nM heregulin (HRG). Stimulations were for 5 min, unless indicated otherwise. All results were replicated in three separate culture preparations.



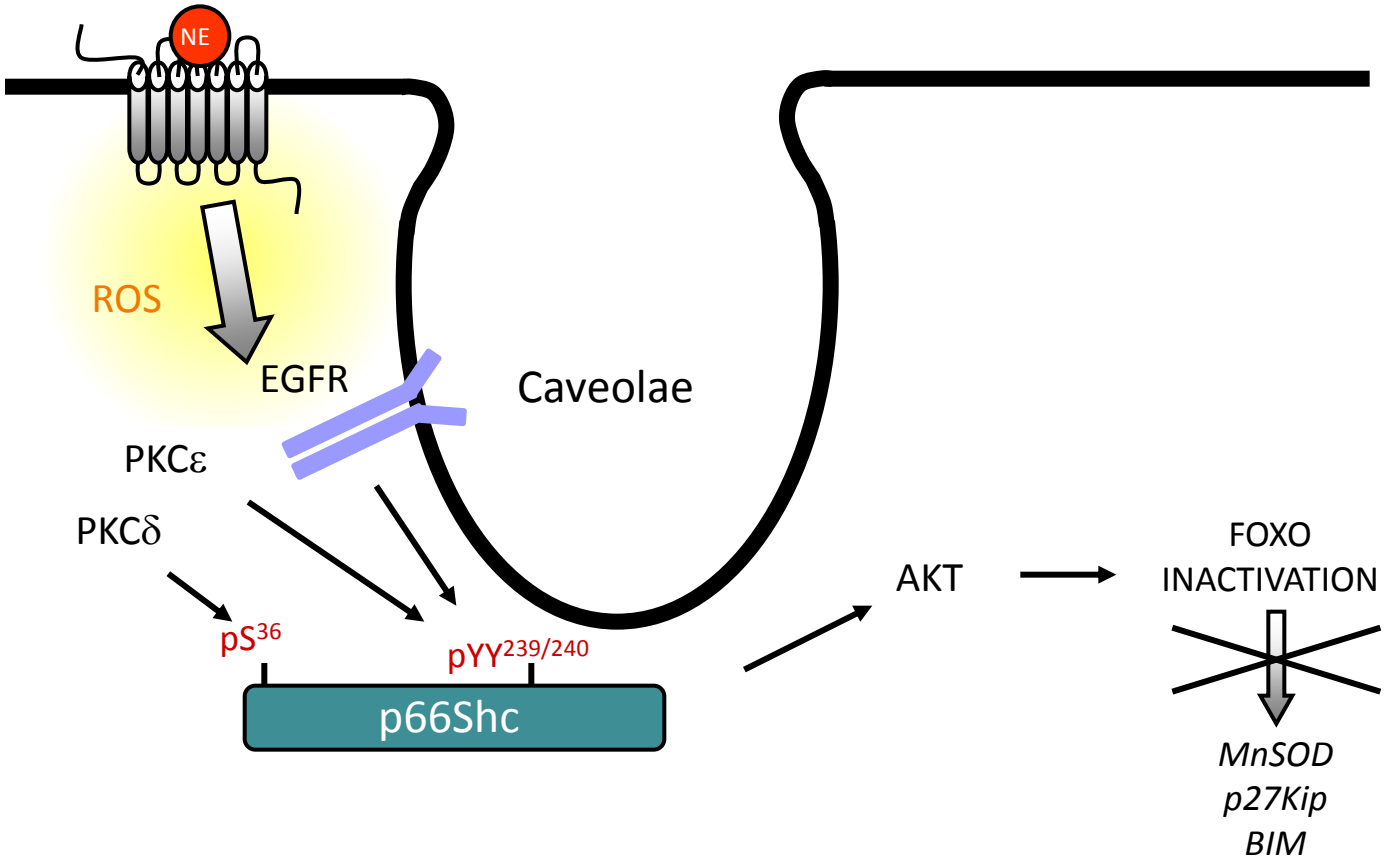
Online Figure II: DiC8 and PMA increase p66Shc-YY$^{239/240}$ phosphorylation in caveolae; EGFR immunoreactivity is enriched in caveolae. Cardiomyocytes were treated with diC8 (58 μM) or 300 nM PMA (each for 20 min) prior to isolation of caveolae and immunoblotting on caveolae (Cav), F8-13, and pellet fractions. Results were replicated in three separate experiments.



Online Figure III: Ad-p66Shc-RNAi does not alter PKCδ, PKCε, or caveolin-3 expression in cardiomyocytes.



Online Figure IV: Ad-p66Shc-RNAi does not prevent doxorubicin-dependent AKT activation. Lysates used for immunoblotting studies in Fig 8G were probed for AKT-T^{308} and AKT protein immunoreactivity. Results for Shc protein expression are reproduced from Fig 8G.



Online Figure V: Schematic of the α_1-adrenergic receptor-EGFR-p66Shc pathway in caveolae that requires ROS and nPKCS and activates the AKT-FOXO3a pathway.
See text.