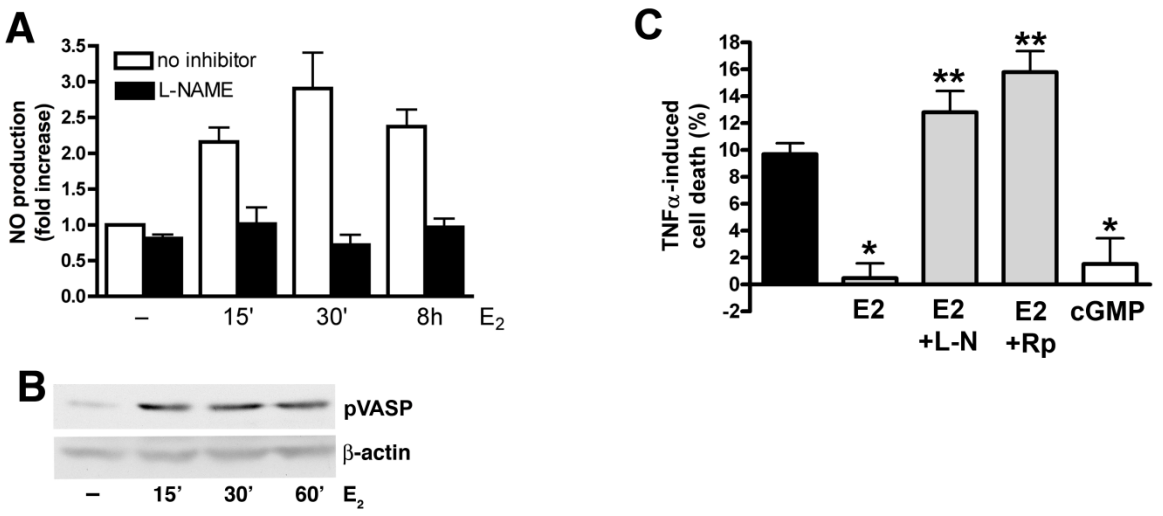
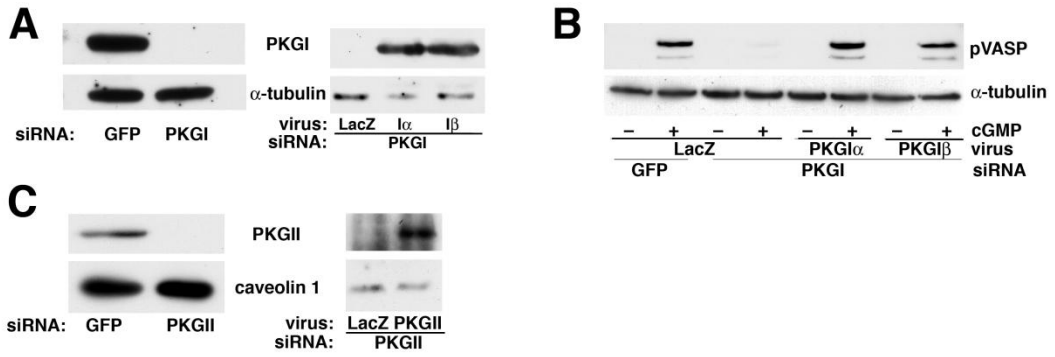
SUPPLEMENTAL FIGURE 1



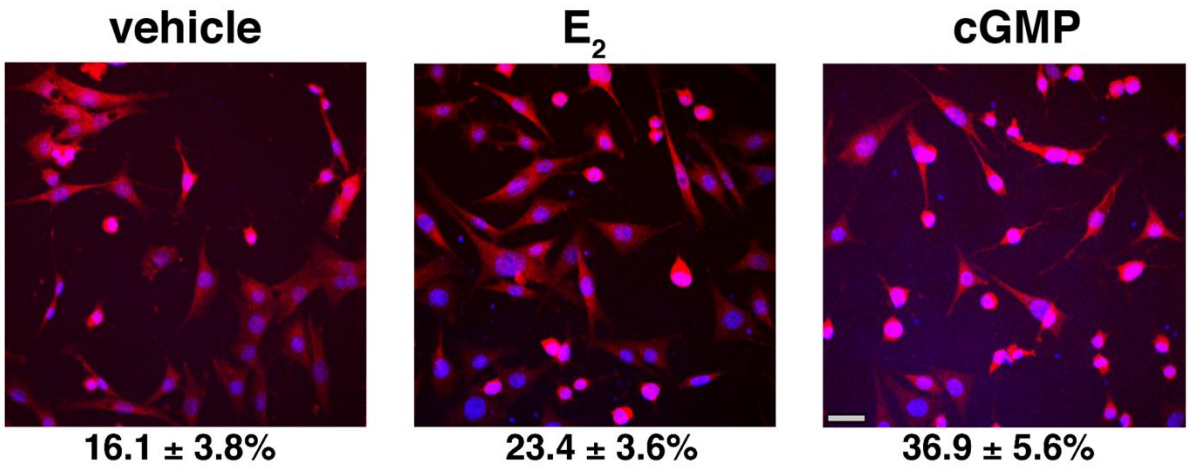
Suppl. Fig. 1: Estradiol increases NO production and VASP phosphorylation in MLO-Y4 osteocytic cells and protects MC3T3 preosteoblast-like cells from TNF-α-induced apoptosis. ***A.*** MLO-Y4 cells were treated with vehicle (white bars) or 4 mM L-NAME (black bars) for 1 h, and then received 100 nM estradiol (E_2) for the indicated times. NO production was measured as described in Experimental Procedures. ***B.*** MLO-Y4 cells were treated with the phosphodiesterase inhibitor vardenafil (1 μM) for 1 h; they then received 100 nM estradiol for the indicated times, and cell lysates were analyzed by Western blotting with an antibody specific for VASP phosphorylation on Ser^{259}, a preferred PKG phosphorylation site. The β-actin blot served as a loading control. ***C.*** MC3T3 cells were transferred to medium containing 1% serum and treated with TNF-α (5ng/ml) plus cycloheximide (10 ng/ml) for 4 h. Some cells received 100 nM estradiol (E2, grey bars) or 100 μM 8-CPT-cGMP (cGMP, white bar) for 30 min prior to the addition of TNF-α plus cycloheximide. Some cells were pre-treated with vehicle, 4 mM L-NAME (L-N) or 100 μM *Rp*-8-CPT-PET-cGMPS (Rp) for 30 min prior to receiving estradiol as indicated. Cell death was measured by trypan blue uptake as described in Experimental Procedures.

SUPPLEMENTAL FIGURE 2



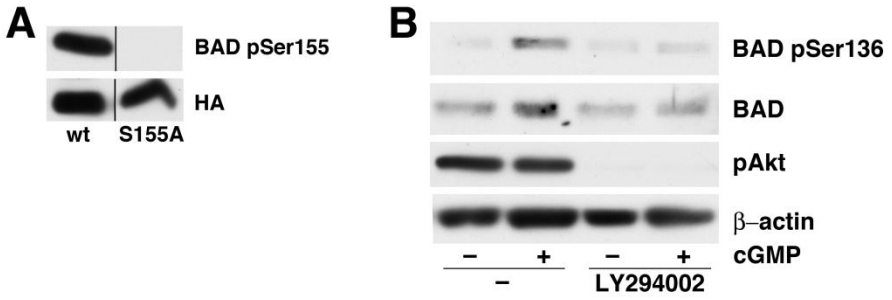
Suppl. Fig. 2: siRNA-mediated depletion and viral reconstitution of PKGI or II in MLO-Y4 cells. ***A.*** Cells were transfected with siRNAs targeting either GFP or the common C-terminus of PKGI, as indicated. The PKGI siRNA-transfected cells in the right panel were additionally infected with adenoviruses encoding LacZ, siRNA-resistant PKGIα, or PKGIβ. Cell homogenates were fractionated by differential centrifugation, and cytosolic fractions were analyzed by Western blotting for endogenous PKGI expression (left panel) and viral PKGIα/β expression (right panel) using an antibody specific for the common C-terminus of PKGIα/β . The α-tubulin blot served as a loading control. ***B.*** MLO-Y4 cells were transfected with GFP- or PKGI-specific siRNAs and were infected with LacZ or PKGIα/Iβ virus as in *A*. Cells were serum-starved, treated for 30 min with 100 μM 8-pCPT-cGMP as indicated, and analyzed by Western blotting with an antibody specific for VASP phosphorylated on Ser^{259}, with α-tubulin serving as a loading control. ***C.*** MLO-Y4 cells were transfected with GFP- or PKGII-specific siRNAs; the PKGII siRNA transfected cells in the right panel were additionally infected with LacZ or PKGII virus. Cells were fractionated as in *A*, and membrane fractions were examined for endogenous PKGII expression (left panels) or viral PKGII expression (right panels). Blots probed with a caveolin-1-specific antibody served as loading controls.

SUPPLEMENTAL FIGURE 3



Suppl. Fig. 3: Effect of estradiol and cGMP on the nuclear localization of ERK. MLO-Y4 cells were treated with vehicle, 100 nM estradiol or 100 μM 8-pCPT-cGMP (cGMP) for 1 h, and analyzed by immunofluorescence staining for ERK (red) and DNA staining with Hoechst 33342 (blue), as described in Experimental Procedures. Three hundred cells in three random fields were evaluated, and the percentage of cells showing nuclear staining for Erk is shown below.

SUPPLEMENTAL FIGURE 4



Suppl. Fig. 4: Specificity of the BAD pSer^{155} antibody, and effect of PI3K inhibition on cGMP-induced BAD phosphorylation on Ser^{136}. ***A.*** Cell lysates from MLO-Y4 cells transfected with HA-tagged wild type (wt) or mutant BAD (S155A) were analyzed by Western blotting using the antibody specific for BAD phosphorylated on Ser^{155} (upper panels) or anti-HA antibody (lower panel), to demonstrate specificity of the antibody. ***B.*** MLO-Y4 cells were transfected with wild type BAD and infected with PKGIα virus as described in Figure 4D. Cells were treated with vehicle or 10μM LY294002 for 1 h, followed by treatment with 100 μM 8-pCPT-cGMP for 1 h, as indicated. At this 1 h time point, cGMP-induced Akt phosphorylation had already decreased back to baseline.