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Differential effects of Gqα, G14α and G15α on vascular smooth muscle cell survival and gene expression profiles

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Running title: Distinct Gα signaling pathways

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d) Abbreviations: DMEM, Dulbecco's modified Eagle medium; 2-APB, 2-amino ethoxydiphenyl borate; BIS-I, bisindolylmaleimide; U73122, a PLCβ inhibitor, VSMC, vascular smooth muscle cells, NFAT, nuclear factor of activated T-cells; MAP Kinase, mitogen activated protein kinase; RhoGEF, Rho guanine nucleotide exchange factor; GRK2, G protein receptor kinase 2; EE, glutamate-glutamate epitope tag; TBST, tris buffered saline with 0.1% tween; GCOS, GeneChip Operating Software; RMA, Robust Multichip Analysis; DEVD, substrate amino acid sequence recognized by caspase-3 enzyme; PI3 kinase, phosphatidylinositol 3-kinase; NFκB, transcription factor, nuclear factor κ B; JNK, c-Jun N-terminal kinase; PIP_2, phosphatidylinositol (4,5) bisphosphate; PIP_3, phosphatidylinositol (3,4,5)trisphosphate.

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ABSTRACT

Gqα family members (Gqα, G11α, G14α and G15/16α) stimulate phospholipase Cβ (PLCβ) and inositol lipid signaling, but differ markedly in amino acid sequence and tissue distribution predicting unappreciated functional diversity. To examine functional differences, we compared the signaling properties of Gqα, G14α and G15α and their cellular responses in vascular smooth muscle cells (VSMC). Constitutively active forms of Gqα, G14α or G15α elicit markedly different responses when introduced to VSMC. Whereas each Gα stimulated PLCβ to similar extents when expressed at equal protein levels, Gqα and G14α, but not G15α initiated profound cell death within 48 hours. This response was due to activation of apoptotic pathways since Gqα and G14α, but not G15α, stimulated caspase-3 activation and did not alter phospho-Akt, a regulator of cell survival pathways. Gqα and G14α stimulate NFAT activation in VSMC, but Gα-induced cell death appears independent of PKC, $InsP_3/Ca^{++}$, and NFAT because pharmacological inhibitors of these pathways did not block cell death. Gene expression analysis indicates that Gqα, G14α and G15α each elicit markedly different profiles of altered gene sets in VSMC after 24 hrs. Whereas all three Gα stimulated changes ($\geq$2-fold) in 50 shared mRNA, Gqα and G14α (but not G15α) stimulated changes in 221 shared mRNA of which many are reported to be pro-apoptotic and/or involved with TNF-α signaling. Surprisingly, each Gα also stimulated changes in non-overlapping Gα-specific gene sets. These findings demonstrate that Gqα family members activate both overlapping and distinct signaling pathways, and are more functionally diverse than previously thought.

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INTRODUCTION

Heterotrimeric G proteins (Gαβγ) couple cell surface receptors to intracellular effector and second messenger systems, and are essential for a broad range of cellular activities and patho/physiological processes. Receptor activation stimulates G proteins to dissociate into Gα-GTP and Gβγ complex which act alone or together to regulate target effector protein activity. Gα subunits are GTPases which act as molecular switches, and the lifetime of Gα-GTP dictates the lifetime of the signaling event (Bourne, 1997; Hamm, 1998). The Gα subunits comprise a large, diverse family with 21 isoforms identified. Members of the Gqα class (Gqα, G11α, G14α, G15/16α) activate phospholipase Cβ isoforms (PLCβ). Activation of PLCβ stimulates phosphoinositide (PI) hydrolysis to generate the second messengers inositol triphosphate ($InsP_3$), which releases calcium from intracellular stores, and diacylglycerol (DAG), which activates protein kinase C (PKC).

While Gqα class members share a capacity to activate PLCβ , they also differ markedly in their biochemical properties and tissue distribution. Gq/11α, G14α, and G15/16α exhibit limited amino acid sequence identity with only 57% overall (compared to ~90% for Gs and 85% for Gi), and only 12% within the first 40 amino acids. Gqα and G11α are the most similar of this class and are expressed fairly ubiquitously (Strathmann and Simon, 1990). G14α has a more limited expression pattern (kidney, liver, lung, testis), whereas G15/16α is limited to hematopoietic tissue (Amatruda et al., 1991; Wilkie et al., 1991).

Despite these differences, established models suggest that Gqα family members are functionally redundant and that cellular responses due to activation of linked receptors are due to PLCβ and downstream calcium/PKC pathways. However, growing evidence indicates that many receptors and Gα-mediated effects do not involve inositol lipid signaling. For example,

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several Gq/11α-linked receptors have been shown to stimulate MAP kinase pathways independent of activation of inositol lipid signaling, including m1 muscarinic, thrombin, α1A-adrenergic, and metabotropic glutamate 5 receptors (Heasley et al., 1996a; Hung et al., 1992; Peavy et al., 2001; Qian et al., 1994; Williams et al., 1998). Gβγ subunits released from Gα-GTP also regulate a growing list of signaling proteins (Gutkind, 1998). Furthermore, introduction of constitutively active Gqα family members elicit diverse responses in cell growth, survival and differentiation depending on the identity of the Gα and the cell type involved. Together, these findings indicate receptors and linked G proteins activate multiple parallel signaling pathways.

Consistent with this diversity of cellular responses, additional binding partners have been reported for Gqα distinct from PLCβ including RGS proteins, RhoGEF, GRK2 and Bruton's tyrosine kinase (Bence et al., 1997; Carman et al., 1999; Heximer et al., 1997; Sagi et al., 2001). Whereas RGS proteins clearly modulate the signaling capacity of target Gα, they and other binding partners may also serve as novel Gα effectors to activate parallel downstream signaling proteins and pathways. Together, these findings predict that Gqα family members activate both overlapping and distinct signaling pathways resulting in diverse cellular responses. However, the relative contribution of PLCβ versus distinct pathways to signaling by Gqα family members is unknown.

To examine signaling diversity among Gqα family members, we compared the signaling properties of Gqα, G14α and G15α and their cellular responses in vascular smooth muscle cells (VSMC). We report that Gqα, G14α and G15α elicited markedly different global cellular responses when expressed in VSMC. Whereas each Gα stimulated PLCβ to nearly identical extents when expressed at equal protein levels, Gqα and G14α, but not G15α initiated cell death.

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This cell death was due to activation of apoptotic pathways because Gqα and G14α, but not G15α, stimulated caspase-3 activation. Gqα and G14α also activated NFAT, but Gα-induced cell death is independent of PKC and $InsP_3/Ca^{++}$ and NFAT because pharmacological inhibitors of these pathways did not block cell death. Gene expression studies indicate that Gqα, G14α and G15α each elicit surprisingly different profiles of altered gene sets consisting of both overlapping and distinct sets of mRNA that increase or decrease. Gqα and G14α, but not G15α stimulated changes in 221 shared genes, many with reported roles in apoptosis. Taken together, these findings suggest that Gqα family members activate both overlapping and distinct signaling pathways in VSMC, independent of PLCβ activation, to elicit unique cellular responses.

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MATERIALS and METHODS

Materials: The Luciferase Assay System and the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit to measure lactate dehydrogenase (LDH) were purchased from Promega (Madison, WI). *Myo*-[^{3}H]-inositol was purchased from Perkin Elmer (Boston, MA). Fura-2 AM, Hoechst 33342 dye and rhodamine 110, bis-L-aspartic acid amide fluorescent caspase substrate were purchased from Molecular Probes (Eugene, OR). The caspase-3, Akt and phospho-specific Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). The monoclonal Glu-Glu ("EE") antibody was purchased from Covance Research Products (Princeton, NJ). Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from BioRad (Hercules, CA). Horseradish peroxidase-conjugated goat anti-mouse IgG was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Restriction enzymes and modifying enzymes were purchased from Stratagene (La Jolla, CA) and New England Biolabs (Beverly, MA). 2-APB and BIS I were purchased from Calbiochem (San Diego, CA). Cyclosporin A was a gift from the laboratory of Grace K. Pavlath (Emory University). Recombinant human PDGF-AB was purchased from Invitrogen Life Technologies (Carlsbad, CA). Media and supplements were purchased from Invitrogen Life Technologies, Mediatech (Herndon, VA) and Atlanta Biologicals (Norcross, GA). Other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Fair Lawn, NJ).

Cell culture. A continuous line of rat vascular smooth muscle cells (VSMC) obtained from R.W. Alexander (Emory University) were maintained in DMEM with Pen-Strep (100 U/mL penicillin and 100 μM streptomycin) and 10% fetal bovine serum (FBS) in a 37ºC humidified incubator

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with 5% CO_2. Phoenix retroviral producer cells (no. SD3443, American Type Culture Collection, Rockville, MD) were also grown in DMEM supplemented with 10% FBS and Pen-Strep.

Plasmid constructions. The retroviral expression vectors pTJ66 and pCL1 have been described previously(Murphy et al., 2002). The first set of constructs were created by removing the human Gqα(Q209L), G14α(Q205L) and G15α(Q212L) inserts from GNA0Q0EIC0, GNA140EIC0 and GNA150EIC0 obtained from Guthrie cDNA Resource Center (Sayre, PA). Each construct contained an internal Glu-Glu (EE) epitope tag. Amino acids 171-176 within Gqα corresponding to AYLPTQ were mutated to EYMPTE, and the corresponding amino acids within G14α and G15α were mutated similarly to generate the internal EE epitope tag. The Gqα(Q209L), G14α(Q205L) and G15α(Q212L) inserts were removed by sequential digests with *Xho*I, and then *Pme*I, and inserted into pTJ66, which had been opened with *Bcl*I , blunted with Klenow, then cut again with *Xho*I to yield compatible sites. The second set of constructs were created by removing the Gqα(Q209L) and G15α(Q212L) inserts from GNA0Q0EIC0 and GNA150EIC0 with *Hind*III and *Pme*I digestion, and by removing the G14α(Q205L) insert from GNA140EIC0 with *Bgl*II, followed by a partial cut by *Hind*III. The recovered Gqα(Q209L), G14α(Q205L) and G15α(Q212L) inserts were further subcloned into retroviral vector pCL1, which had been cut open with *Mlu*I, blunt ended with Klenow, and then then cut with *Hind*III.

Retroviral Production and VSMC Infection. Protocols for the transient, helper-virus free production of nonreplicating recombinant retroviruses and VSMC infection have been described previously (Murphy et al., 2002), except that the Phoenix-Ampho producer cell line was used for the work described in this report. Infectious retroviral supernatants were prepared from Phoenix

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producer cells transfected with retroviral plasmids encoding for Gqα(Q/L), G14α(Q/L), G15α(Q/L), or empty vector alone for controls and used fresh or stored at –80^{o}C for future use.

LDH measurements. VSMC in 24-well plates were infected with retroviral expression vectors containing Gqα (Q/L), G14α (Q/L) and G15α (Q/L) or vector only. Immediately after completing the infection protocol, viral supernatant medium was replaced with phenol red-free DMEM supplemented with 10% FBS and Pen-Strep (500 µL/well). Appropriate concentrations of inhibitors or antagonists or vehicle were added to the VSMC and 50µL samples were removed from each well and transferred to 96-well plates and stored at -20ºC. At 24-hour intervals, additional 50µL samples were removed and stored over a 5-day period. At each sampling, 50 µL aliquots with inhibitor or antagonist or vehicle were added to the wells to replenish the volume of medium. At the end of the experiments, the samples were measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) using a Molecular Devices Thermomax microplate reader (Molecular Devices Corp., Sunnyvale, CA).

[^{3}H]-Inositol phosphate production. VSMC were infected with retroviruses encoding Gqα(Q/L), G14α(Q/L), G15α(Q/L), or empty virus as control, and then incubated overnight in DMEM/10% FBS and Pen-Strep with 4 µCi/ml [^{3}H]-inositol. Cells were placed in HEPES-buffered (pH 7.4) DMEM supplemented with 10 mM LiCl at 37ºC and [^{3}H]-inositol phosphates were isolated by ion exchange chromatography as previously described (Heximer et al., 1997).

Calcium mobilization in VSMC. VSMC grown on coverslips were infected with Gqα(Q/L), G14α (Q/L), G15α (Q/L), or empty virus only, and then incubated with 5 µM Fura2-AM for 30

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min at room temperature. Cells were then placed on a microscope stage for imaging of intracellular calcium concentration. External solution contained 150 mM NaCl, 10 mM HEPES, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5.5 mM glucose, pH 7.3, and 325 mOsm. Intensity images of 510 nm wavelength were taken at 340 nm and 380 nm excitation wavelengths, and the two resulting images were taken from individual cells for ratio calculations. Axon Imaging Workbench version 2.2.1 was used for acquisition of intensity images and conversion to ratios.

Detection of caspase-3 activity and chromatin condensation. Twenty-four hours after infection with the retroviral expression vectors, VSMC were washed twice with buffer (150 mM NaCl, 10 mM HEPES, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5.5 mM glucose, pH 7.3), then incubated with the fluorescent caspase substrate, rhodamine 110, bis-L-aspartic acid amide (1.5 μg/mL), for 30 minutes. The VSMC were washed again with buffer and observed under an Olympus IX51 inverted microscope. Images were captured using Image-Pro Plus software (Version 4.5.1, Media Cybernetics, Silver Spring, MD) from the microscope equipped with an Olympus Q Color 3 camera through a Chroma 41001 (FITC/EGFP/Bodipy/Fluo 3/DiO) fluorescent filter set (Chroma Technology Corp., Brattleboro, VT). For images of chromatin condensation, the infected VSMC were washed twice with buffer, then incubated with Hoechst 33342 dye (10 μg/mL) for 15 minutes. The VSMC were washed again with buffer and images were captured as described above through a Chroma 31000 (DAPI/Hoechst/AMCA) filter set.

Transcription factor luciferase reporter. The retroviral NFAT-specific luciferase reporter vector has been described (Murphy et al., 2002). VSMC stably expressing the NFAT luciferase reporter were grown in 24-well plates and infected with one round of Gqα(Q/L), G14α(Q/L),

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G15α(Q/L), or empty vector alone for controls, as described above. Twenty-four hours after infection, VSMC were serum-starved overnight. VSMC were lysed and luciferase activity monitored on a Turner Designs luminometer (Sunnyvale, CA) utilizing Luciferase Assay System kit (Promega, Madison, WI).

SDS-polyacrylamide gel elecrorphoresis and immunoblot analysis. Whole cell extracts of VSMC were prepared by lysing the cells in a buffer (50mM Tris-HCl, 50mM NaCl, 5mM EDTA, 10mM EGTA, 1mM Na3VO4, 2mM Na4P2O7·10H2O, 10μg/mL leupeptin, 2μg/mL aprotinin, and 1% Triton X-100) collecting, adding Laemmli sample buffer, sonicating briefly and boiling for 5 minutes. Samples were loaded and resolved on SDS-polyacrylamide gels and transferred to nitrocellulose. For detection of the Glu-Glu (EE) epitope tag, the membranes were blocked for 1 hour at room temperature with 5% nonfat milk in TBST, and probed with the monoclonal anti-Glu-Glu (EE) antibody (1:2000 in 5% nonfat milk-TBST) overnight at 4ºC. Membranes were washed with TBST and incubated for 1 hour in horseradish peroxidase-conjugated goat anti-mouse IgG (1:20,000 in TBST). Membranes were again washed in TBST and immunoreactive proteins detected by chemiluminescence. All other immunoblots were performed using an identical protocol except for the indicated specific antisera. For detection of caspase-3, membranes were probed with the polyclonal caspase-3 antibody (1:1000 in 5% nonfat milk-TBST) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 in 5% nonfat milk-TBST). For detection of Akt, membranes were probed with the polyclonal Akt antibody (1:1000 in 5% nonfat milk-TBST) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 in 5% nonfat milk-TBST). For detection of phospho-Akt, membranes

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were probed with the polyclonal phospho-Akt antibody (1:1000 in TBST) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 in 5% nonfat milk-TBST).

Statistical treatment of data. Significant differences among treated and control samples were determined using one-way ANOVA with Tukey's and Dunnett's post tests. Significant differences between conditions ($p < 0.05$) are indicated in each figure and legend, where appropriate.

Total RNA isolation and gene microarray studies: VSM cells (confluent 6 wells, 35 mm dishes) were infected with empty retrovirus (control) or retroviruses encoding Gqα(Q/L), G14α(Q/L) or G15α(Q/L) (experimental). After 24 hr, total RNA was extracted from cells in some wells using Trizol according to manufacturer's protocol (Invitrogen). Remaining sister cells continued to grow and were either observed for an additional 48 hr (72 hr total) to confirm cell death or were harvested and immunoblotted (anti-EE) to confirm expression of Gα-EE. The quality and amount of RNA were confirmed by ethidium bromide staining of an agarose gel and by spectrophotometry. Total RNA samples from three separate identical experiments were then sent to the UCLA/NHLBI Shared Microarray Facility (Dr. Stanley Nelson, Director) for processing and analysis. The quality of RNA samples were again assessed at the Microarray Facility to determine suitability for analysis. Samples (cDNA) were generated and hybridized with a GeneChip array (Affymetrix High Density Rat Genome 230-2.0) containing 31,042 oligonucleotides representative of the entire rat genome. For each gene sequence, 11 different complimentary oligo pairs were present on the array to normalize differences in transcriptional levels (as detected by intensity values) of each gene (www.affymetrix.com). Microarrays were

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processed using the Affymetrix recommended protocol for hybridization, washing and staining, and results were quantitated using the GeneChip Scanner 3000 High Resolution Scanner and GeneChip Operating Software (GCOS) from Affymetrix. Resulting microarry data sets (CEL files) were analyzed at the Emory Biomolecular and Computing Resource using the R-Bioconducting package (www.bioconductor.org) analysis for Affymetrix arrays (Gautier et al., 2004). Data sets were loaded into the R-Bioconductor package and resulting RMA (Robust Multichip Analysis) values (Bolstad et al., 2003; Irizarry et al., 2003) were generated using the following parameters: RMA background correction method, quantile normalization method, PM (perfect match) only values, and the median polish summary method for signal calculation (Irizarry et al., 2003). RMA values were imported into GeneSpring v6.2 (Silicon Genetics, Redwood City, CA) and expression values for each gene were normalized across chips to the median value of each gene. In each experiment, fold change values (experimental vs. control) were determined, and a cutoff value of 2.0 was arbitrarily applied to ascertain genes that were differentially expressed across experiments and between the conditions within each experiment (Gqα, G14α or G15α versus control). Gene ID, annotation (where known), fold change for each condition, average fold change, and range were reported for each condition. Lists of affected genes were sorted by an increase or decrease in fold change (vs. control) for each condition. Gene lists generated were compared to determine which gene(s) overlapped in each replicate experiment, and instances where genes changed less that 2-fold in one or more experiments are not reported. Venn diagrams were used to sort differential expression in one or multiple experimental conditions (overlapping and non-overlapping).

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RESULTS

Gqα, G14α and G15α have differential effects on vascular smooth muscle cell survival.

To study signaling characteristics of the Gqα family of G proteins, we used a retroviral infection method to introduce constitutively active (GTPase-deficient, Q/L) mutants of Gqα, G14α and G15α into rat vascular smooth muscle cells (VSMC). Differences in cell morphology and viability were evident within 24 hours after infection in cells expressing Gqα(Q/L) and G14α(Q/L), but not those expressing G15α(Q/L) or control retroviral vector only (Figure 1A). These changes in morphology became more profound over time, and nearly all Gqα- and G14α-infected cells were dead after 120 hours. Cells infected with wild type (not constitutively active) Gqα, G14α and G15α grew normally and were not different from control, and C2C12 mouse myoblasts infected with Gqα (Q/L), G14α(Q/L) or G15α(Q/L) did not die (data not shown). We measured cytosolic lactate dehydrogenase (LDH) released into the culture medium as a method for the quantification of cell death (Figure 1B). At 72 hours increases in LDH levels were 2- to 3-fold greater for cells infected with Gqα(Q/L) and G14α(Q/L) than G15α(Q/L) or control retroviral vector only. We propose that distinct and divergent signaling pathways from Gqα, G14α and G15α mediate the selective cell death seen in VSMC.

Gqα(Q/L), G14α(Q/L) and G15α(Q/L) stimulate inositol lipid signaling to similar extents (Figure 2). Gqα family members link cell surface receptors to phosphoinositide hydrolysis and are reported to activate phospholipase Cβ (PLCβ) equally in cell-free recombinant systems (Hepler et al., 1993) and intact cells (Lee et al., 1992). We measured the capacity of Gqα(Q/L), G14α(Q/L) and G15α(Q/L) to stimulate phosphoinositide hydrolysis in rat VSMC by the accumulation of radioactive labeled inositol phosphates in the presence of lithium chloride. Cells

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infected 24 hours earlier with Gqα(Q/L), G14α(Q/L), or G15α(Q/L) produced similar levels of PI hydrolysis (Figure 2A). We confirmed the expression of each mutant by immunoblotting with an antibody to detect an internal glutamate-glutamate (EE) tag introduced into Gqα(Q/L), G14α(Q/L) and G15α(Q/L) constructs. Equal samples of lysates from cells infected at the same time as those used for the PI hydrolysis experiments showed similar levels of expression of each Gα protein (Figure 2A).

Since Ca^{++} is the second messenger downstream of InsP production, we measured cytosolic Ca^{++} levels at the point of early onset of cell death to determine if expression of Gα resulted in measurable differences in cytosolic Ca^{++}. Twenty four hours after infection, we measured intracellular calcium levels in infected cells with the fluorophore Fura-2 (Molecular Probes, Eugene, OR) and found that resting cytosolic calcium concentrations were similar for Gqα(Q/L), G14α(Q/L) and G15α(Q/L), (Ratio $F_{340/380}$: 0.223±0.060, 0.252±0.049, 0.253±0.040, 0.234±0.057, respectively; n=3, mean±S.E.M.) and not different from vector only control. Our finding that Gα-expressing cells generate inositol phosphates after 24 hrs without a parallel rise in cystolic [Ca^{++}] suggested that cellular Ca^{++} stores were depleted and/or a desensitization of Ca^{++} signaling. This idea was supported by our finding that activation of endogenous Gq/11-linked purinergic receptors with ATP resulted in agonist responses (amplitude and time-to-maximal response) for cytosolic Ca^{++} that were severely blunted and similar in cells expressing Gqα(Q/L), G14α(Q/L or G15α(Q/L) when compared to cells expressing empty virus (data not show). A time course experiment measuring accumulation of inositol phosphates also demonstrated no differences in the kinetics of PI hydrolysis among Gqα(Q/L), G14α(Q/L) and G15α(Q/L) (Figure 2B). Because Gqα(Q/L), G14α(Q/L) and G15α(Q/L) all stimulate inositol lipid/Ca^{++} signaling pathway similarly but induce cell death differentially, we propose that

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signaling pathways leading to cell death in rat VSMC infected with Gqα(Q/L) and G14α(Q/L) are independent of inositol lipid signaling.

Gqα(Q/L) and G14α(Q/L) activate caspase-3 (Figures 3 and 4). Cell death may result from general cytotoxicity (necrosis), activation of programmed cell death (apoptosis) pathways, or inhibition of cell survival pathways. To test whether cell death in the infected VSMC was apoptotic, we used a fluorogenic substrate (Z-DEVD-Rhodamine 110, Molecular Probes, Eugene, OR) containing the recognition site for caspase-3 (DEVD), a central component of apoptotic cell death pathways. Cleavage of the substrate by activated caspase-3 produces a fluorescent product (Molecular Probes, Eugene, OR). Twenty four hours after infection with Gqα(Q/L), G14α(Q/L), G15α(Q/L), or vector only, VSMC were incubated with the Rhodamine 110 substrate and observed by fluorescent microscopy. Cells infected with Gqα (Q/L) and G14α (Q/L) showed extensive fluorescence, indicative of caspase-3 activation. Cells infected with G15α(Q/L) or vector only showed little or no fluorescence (Figure 3, *middle panel*). We also incubated infected VSMC with the nuclear stain Hoechst 33342 (Molecular Probes, Eugene, OR) to detect nuclear chromatin condensation as another marker for apoptotic cell death. Cells infected with Gqα(Q/L) and G14α(Q/L) exhibited condensed nuclei, but cells infected with G15α(Q/L), or vector alone did not (Figure 3, *bottom panel*). As an additional measure of caspase-3 activation, samples of lysates from infected cells were subjected to immunoblotting with a caspase-3 antibody (Figure 4). Cells infected with Gqα(Q/L) and G14α(Q/L) showed the appearance of a distinct band at 16kDa corresponding to the large fragment of caspase-3, generated by proteolytic cleavage of the full length precursor (Nicholson et al., 1995). Cells infected with G15α(Q/L) or vector only, did not show the appearance of the fragment (Figure 4). Based on these results, we conclude that Gqα(Q/L) and G14α(Q/L), but not G15α(Q/L) mediate

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cell death through an apoptotic pathway involving activation of caspase-3.

Gqα(Q/L) and G14α(Q/L) do not alter Akt activity in VSMC (Figure 5). Based on reports that the constitutively active mutant Gqα(Q209L) inhibits the Akt cell survival pathways in cardiomyocytes (Howes et al., 2003), we sought to determine whether Gqα(Q/L), G14α(Q/L) and G15α(Q/L) affected the Akt activity in VSMC. After retroviral infection with Gqα(Q/L), G14α(Q/L), G15α(Q/L), or vector only, we lysed VSMC 12 or 24 hours later and subjected samples to immunoblotting with a phospho-specific antibody to detect activated Akt. We detected no changes in the phosphorylation of Akt in cells infected with Gqα(Q/L), G14α(Q/L), G15α(Q/L), or vector only (Figure 5). We also detected no changes in Akt activation in infected cells stimulated with PDGF, which has been shown to stimulate Akt through stimulation of PI3 kinase (Ballou et al., 2003). Based on these results, we conclude that Gqα(Q/L)- and G14α(Q/L)-mediated cell death in VSMC is not dependent on inhibition of Akt activity.

Inhibition of the PLCβ-PKC-calcium pathways does not prevent Gα-directed VSMC death (Figure 6). To determine whether Gqα(Q/L)- and G14α(Q/L)-mediated cell death is dependent on activation of PLCβ, we treated infected VSMC with inhibitors of the PLCβ-PKC-calcium pathway. Because inhibition of PLCβ with the aminosteroid U73122 was cytotoxic in controls and all treatments, we used inhibitors of the second messengers in the pathway, PKC and IP_3. After VSMC were infected with Gqα(Q/L), G14α(Q/L) and G15α(Q/L), or vector only, we supplemented growth medium with the PKC inhibitor, bisindolylmaleimide I (BIS), or with the IP_3 receptor antagonist, 2-APB. Samples of growth medium were taken at 24-hour intervals to measure cytosolic LDH release as a measure of cell death. Neither BIS nor 2-APB prevented cell death induced by infection with Gqα(Q/L) or G14α(Q/L) (Figure 6). This result is consistent

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with the idea that Gqα(Q/L)- and G14α(Q/L)-mediated cell death in VSMC is not dependent on PLCβ or its second messengers.

Inhibition of Gqα(Q/L) and G14α(Q/L) activation of NFAT does not prevent VSMC death (Figure 7). In considering possible downstream signals mediating cell death through alteration of gene expression, we examined changes in activity of transcription factors often associated with stimulation of G protein coupled receptors linked to inositol lipid and Ca++/PKC pathways. The transcription factor NFAT has been reported to be a component of Gqα-mediated anti-apoptotic pathways in cardiomyocytes (Pu et al., 2003). NFAT activation is initiated through calcium release and activation of calmodulin and subsequent activation of calcineurin. Calcineurin binding to NFAT dephosphorylates and causes activated NFAT to translocate to the cell nucleus where it begins transcription of target genes. We used a VSMC line stably infected with an NFAT-specific luciferase reporter to determine whether differences existed in the ability of Gqα(Q/L), G14α(Q/L) and G15α(Q/L) to stimulate NFAT (Boss et al., 1998). We infected the NFAT-VSMC with Gqα(Q/L), G14α(Q/L), G15α(Q/L), or vector only. After 24 hours, cells were serum starved and luciferase activity was measured 24 hours later. Gqα(Q/L) and G14α(Q/L) increased NFAT-specific luciferase activity significantly more than vector only, but G15α(Q/L) did not increase NFAT activity significantly different than vector only (Figure 7A). We also tested whether cyclosporine A (CsA), a calcineurin inhibitor, blocked NFAT activation in these cells. Supplementing the growth medium of VSMC with CsA did not alter the cell death induced by infection with Gqα(Q/L) or G14α(Q/L). The capacity of Gqα(Q/L) and G14α(Q/L) to activate NFAT is greater than G15α(Q/L) in VSMC, but activation of NFAT does not appear to alter the outcome of cell death (Figure 7B).

Gqα(Q/L) and G14α(Q/L) and G15α(Q/L) stimulate changes in both overlapping and

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distinct expressed genes sets in VSMC (Figure 8 and Tables 1-5). Our findings suggest that Gqα family members activate both overlapping (e.g. PLCβ-dependent) and distinct (e.g. PLCβ-independent) signaling pathways. To investigate this further, we examined mRNA expression profiles in VSMC following infection with either control virus or virus encoding Gqα(Q/L), G14α(Q/L) or G15α(Q/L). We examined mRNA levels in cells 24 hr post-infection when they were entering early stages of apoptosis but before cell death was prevalent. Cells were lysed and total RNA isolated. Expressed mRNA was examined in recovered samples where virally infected sister cells were observed to: 1) express Gqα(Q/L), G14α(Q/L) or G15α(Q/L) by anti-EE immunoblot, and 2) proceed to cell death after 48-72 hrs in the case of Gqα(Q/L) and G14α(Q/L) (data not shown). Total RNA was extracted from these samples, and mRNA isolated. Samples were then subjected to cDNA microarray analysis as described in Methods. Sample mRNA was hybridized to a rat DNA microarray chip (Affimetrix RAE-2.0) spotted with samples from genes representing the entire rat genome. Samples were normalized (see Methods) and examined for genes that changed (increase or decrease) 2-fold or greater in each experiment relative to control cells infected with empty retrovirus. Instances where genes changed less than 2-fold in one or more experiments are not reported.

Results of these cDNA microarray analysis are summarized in Figure 8 and Tables 1-5. Of 31,042 genes examined, a total of 422 mRNA changed (2-fold or greater increase or decrease) in each experiment with VSMC expressing Gqα(Q/L) after 24 hr. In parallel samples, 341 mRNA and 74 mRNA changed in cells expressing G14α(Q/L) or G15α(Q/L). Figure 8 illustrates expressed gene sets that changed and were either shared (overlapping) or distinct (non-overlapping) among the three Gα. All three Gα shared 50 genes that changed (23 increase, 27 decrease), and those genes with known identities and their reported functions are

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listed in Table 1. The genes that changed most robustly and were shared by all three Gα are Cyclooxygenase 2 (COX-2), and a muscle cytoskeletal protein (Krp1/sarcosin); mRNA for the interleukin 1 receptor antagonist (Il1rn) decreased markedly. Surprisingly, each Gα also stimulated changes in distinct gene sets. Gqα(Q/L) stimulated changes in 150 unique genes (74 increased, 76 decreased) that did not change with G14α(Q/L) or G15α(Q/L). Of these, only 37 are defined and are listed with their reported functions in Table 2 (the remainder are undefined expressed sequence tags, or ESTs). The mRNA most robustly expressed (22-fold) in cells expressing Gqα(Q/L) encodes Gqα, though we cannot rule out the possibility that this reflects cross-reactivity between the probes and retrovirally expressed recombinant Gqα. G14α(Q/L) stimulated changes in 70 genes (45 increased and 25 decreased) not changed by Gqα(Q/L) or G15α(Q/L). G15α(Q/L) stimulated changes in 18 genes (16 increased and 2 decreased) not changed by Gqα(Q/L) or G14α(Q/L). Identified genes sets that changed uniquely in response to either G14α(Q/L) or G15α(Q/L) and their reported functions are listed in Table 3.

Gqα(Q/L) and G14α(Q/L) stimulate changes in a shared set of genes, some with established roles in apoptosis and TNF-α signaling (Figure 8; Tables 4 and 5). Most germane to the present study, 221 genes changed (137 increased, 84 decreased) in response to Gqα(Q/L) and G14α(Q/L), but not G15α(Q/L) (Figure 8, Tables 4 and 5). When comparing the samples using a three-way cluster analysis, changes in gene sets were most similar for Gqα(Q/L) and G14α(Q/L) and more divergent for G15α(Q/L) (data not shown). Of the genes that changed, only a subset are defined with annotations (38 that increased, 11 that decreased), the rest being undefined ESTs. Of note, several of these gene products are reported to be pro-apoptotic in various cell lines, and are bolded (Table 4). The individual gene which increased most robustly

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(12-fold) in response to Gqα(Q/L) and G14α(Q/L) is TNF-α stimulated gene 6 (TSG-6), which (as its name implies) is strongly upregulated by TNF-α and binds to the extracellular matrix sugar hyaluronan. Other genes reported to be strongly upregulated by TNF-α that are also upregulated by Gqα(Q/L) and G14α(Q/L) include the enzyme which produces hyaluronan, hyaluronan synthase (Has2), the multi-drug resistance gene (Mdr1a), which is induced during cell stress, and the Urokinase/Plasminogen receptor (uPAR-1). Gene products that are robustly upregulated and pro-apoptotic, and also either stimulate TNF-α synthesis or mediate TNF-α signaling include activating transcription factor 3 (Atf3/LRF1), a CREB-like nuclear transcription factor, the TGF-β-like cytokine bone morphogenic protein 2 (BMP2), MAPKinase kinase kinase 8 (Map3K8;Tpl2;Cot), the nuclear transcription factor NGFI-B/Nurr77, the oxidized LDL-receptor 1 (LOX-1) and connexin 37. The gene most robustly down-regulated by Gqα(Q/L) and G14α(Q/L) is the receptor for angiotensin II (AT1A-R) (Table 5).

TNF-α alone or in combination with calcium ionophore does not stimulate VSMC death. Since Gqα(Q/L) and G14α(Q/L) stimulated an increase in numerous genes reported to be pro-apoptotic and/or involved with TNF-α signaling, we tested if TNF-α, either alone or in the presence of increased intracellular calcium, stimulates VSMC death. Cells treated with increasing concentrations of TNF-α alone (0-100 ng/ml) exhibited no cell death, even at concentrations (30-100 ng/ml) reported to stimulate cell death in other sensitive cells (data not shown). In separate experiments, we tested if TNF-α acted synergistically with calcium to stimulate VSM cell death. As expected, ionomycin alone was cytotoxic at high concentrations (0.1-1 μM), whereas intermediate and low concentrations of ionomycin were not (data not shown). Addition of high concentrations (100 ng/ml) of TNF-α did not alter the potency of ionomycin-induced cell death (data not shown), suggesting TNF-α does not act synergistically

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with ionomycin to shift the sensitivity of VSMC to cell death at low or modest intracellular [Ca^{++}].

DISCUSSION

Gqα family exhibit striking differences in sequence homology and tissue distribution which predicts unappreciated differences in cellular functions. Consistent with this idea, we found that Gqα, G14α and G15α exert different effects on VSMC survival and gene expression patterns. Constitutively activated forms of Gqα and G14α, but not G15α or control, stimulate caspase-3 activation and apoptotic cell death when expressed in VSMC. These effects are specific to the cell type involved and the activation state of Gα, because VSMC expressing inactive Gα or myoblasts expressing active Gqα, G14α and G15α do not undergo cell death. Each Gα elicits a markedly different profile of altered gene sets in VSMC. Whereas all three Gα stimulated changes in a shared set of mRNA, Gqα and G14α (but not G15α) stimulated changes in a much larger shared set of mRNA of which several are reported to be pro-apoptotic and involved with TNF-α signaling. Surprisingly, each Gα also stimulated changes in non-overlapping Gα-specific gene sets. These findings demonstrate that Gqα family members are functionally diverse and activate both overlapping and distinct signaling pathways.

Established models indicate that Gqα family members exert their cellular actions by activating PLCβ. We found that constitutively active Gqα, G14α and G15α stimulate accumulation of inositol phosphates equally in both the kinetics and magnitude of response. PLC activity is sustained until cell death is complete with Gqα(Q/L) and G14α(Q/L), and for viable cells expressing G15α(Q/L) over the same period (data not shown). Despite the accumulation of inositol phosphates, intracellular calcium levels were suppressed and no different for any of the Gα-expressing or control cells after 24 hrs, suggesting possible depletion

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of internal calcium stores. Consistent with this idea, we found that activation of endogenous Gq/11α-coupled purinergic receptors in Gα-expressing cells resulted in markedly reduced calcium responses (data not shown). Similar desensitization of calcium signaling was reported in other cells (SLCC, Swiss-3T3 and different VSMC) following G16α(Q/L) stimulated phosphoinositide hydrolysis (Heasley et al., 1996b; Higashita et al., 1997; Lobaugh et al., 1996; Qian et al., 1994). Even though the three Gα stimulated $InsP_3/Ca^{++}$ similarly, their effects on VSMC survival differed suggesting that Gqα- and G14α-initiated cell death is not mediated by components of the PKC/IP_3-Ca^{++} signal pathway alone. In support of this, inhibition of PKC activity, IP_3 binding to its receptor, or Ca^{++} stimulated activation of calcineurin/NFAT failed to block Gα-induced apoptosis.

Our report adds to others showing differences among the Gqα family members in their binding partners and cellular responses. For example, G15α and G16α are "promiscuous" in coupling to various GPCR compared to the selective Gqα and G11α (Offermanns and Simon, 1995), and G16α does not interact with GRK2 like other Gqα family members (Day et al., 2003). Many reports demonstrate that Gqα family members activate distinct downstream signaling events leading to different cell fates. Constitutively active G16α is a more potent stimulator of NFκB than Gqα in HeLa cells (Yang et al., 2001), and inhibits growth of SCL carcinoma cells and Swiss 3T3 cells (Heasley et al., 1996b; Qian et al., 1994). Constitutively active Gqα and G16α induce cell differentiation and activate JNKs in PC-12 cells (Heasley et al., 1996a). Gqα family members also have been linked to activation of caspases and apoptotic pathways in various cell types. In COS-7 and CHO cells, constitutively active Gqα causes apoptosis independent of PKC (Althoefer et al., 1997). In cardiomyocytes, constitutively active

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Gqα causes apoptosis through cytochrome *c* release from mitochondria, and caspase activation (Adams et al., 2000). This Gqα-induced apoptosis is mediated through inhibition of the PI3K/Akt cell survival pathway characterized by depletion of PIP_2 availability, generation of PIP_3 and dephosphorylation of Akt (Howes et al., 2003). In contrast, we find no differences in resting or stimulated Akt phosphorylation in VSMC expressing constitutively active Gα subunits. However, in Rat-1 fibroblasts, active Gqα inhibits PI3K activity upstream from Akt, suggesting an interaction between Gqα and PI3K (Ballou et al., 2003). A subsequent report demonstrated that constitutively active Gqα stimulates GSK-3β in HEK cells through inhibition of PI3K and activation of Csk tyrosine kinase, but independent of PLCβ and apparently not involving Akt (Fan et al., 2003). Activation of Gqα family members also results in cell fates other than death. Constitutively active Gqα stimulates cell transformation of NIH-3T3 cells and differentiation of PC12 cells (De Vivo et al., 1992; Heasley et al., 1996a). We find that myoblasts expressing Gqα(Q/L), G14α(Q/L) and G15α(Q/L) do not undergo cell death. Taken together with our experiments comparing G14α, these findings suggest that Gqα family members recruit overlapping and distinct signaling pathways leading to distinct cell-specific effects on cell fate.

To examine differences in cellular responses to Gqα family members, we analyzed expressed gene sets in VSMC. While all 31,042 genes of the rat genome were screened, less than half of the genes that changed in response to Gα are defined (the rest being undefined expressed sequence tags, ESTs) which provides only a partial picture of the expression profiles. Nevertheless, important (albeit incomplete) information was obtained. During the first 24 hrs, Gqα, G14α and G15α each elicit changes in both overlapping (shared) and distinct (i.e. Gα-specific) gene sets but exhibit surprisingly different gene profiles. The overlapping sets of genes

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that changed included those shared by all three Gα and by two of the three Gα. Of the latter, Gqα and G14α stimulated changes in a large set of shared genes (221) whereas G15α exhibited little or no overlap with either Gqα (1 shared) or G14α (5 shared). This suggests Gqα and G14α are functionally related and is consistent with our findings that Gqα and G14α but not G15α, stimulated VSMC death. Most of the shared genes robustly upregulated (5-fold or more) by both Gqα and G14α have reported roles in apoptosis and/or TNFα signaling. In particular transcription factors Atf3/LRF1 and NGFI-B/Nurr77, the cytokine bone morphogenic protein 2 (Bmp2), the Ser/Thr kinase Map3K8, and the membrane channel protein connexin 37 are each reported to be pro-apoptotic (Hartman et al., 2004; Kume and Kita, 2004; Patriotis et al., 2001; Seul et al., 2004; Watanabe et al., 2001; Zhang et al., 2001) and upregulated by TNFα or to mediate its actions (Dumitru et al., 2000; Gruber et al., 2003; Hofnagel et al., 2004; Inoue et al., 2004; Mashima et al., 2001; van Rijen et al., 1998). Other robustly upregulated genes including the hyaluronan binding protein, TSG-6, and the enzyme that makes hyaluronan, hyaluronan synthase 2 (Has2), the urokinase/tPA receptor (uPAR-1), and the p-glycoprotein multi-drug resistance gene (Mdr1a), while not directly linked to apoptosis, are reported to either mediate TNF-α actions or to be upregulated by TNF-α (Ijuin et al., 2001; Lee et al., 1993; Piguet et al., 1999; Theron et al., 2003). A shared link between many of the remaining genes and apoptotic pathways or TNFα signaling is not readily apparent.

Our findings suggest that the downstream actions of Gqα and G14α may converge on signaling pathways utilized by TNF-α, or that they may stimulate VSMC to synthesize or release TNFα to exert autocrine actions. Indeed, TNF-α has been shown to stimulate apoptosis in other VSMC by direct and autocrine mechanisms (Boyle et al., 2003). However, we found that activation of TNF-α receptors alone with high concentration of cytokine was not sufficient to

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stimulate death of our aortic smooth muscle cells, nor did TNF-α act synergistically with ionophore to sensitize VSM cell death to lower levels of intracellular calcium (data not shown). While these findings do not exclude the possibility that Gqα and G14α converge on TNF-α signaling pathways, they do suggest additional mechanisms are involved in cell death. In this regard, previous studies have shown that Gq/11-mediated activation of RhoA contributes to Gα-directed death of HeLa cells (Ueda et al., 2004). It will be of interest to know if RhoA has similar cell-death promoting activity in VSMC or whether the RhoA pathway might sensitize VSMC to agents such as TNF-α. This can be one focus of further studies to identify mechanisms underlying Gqα- and G14α-directed cell death.

All three Gα stimulated changes in shared gene sets indicating functional overlap, perhaps linked to activation of inositol lipid signaling. Consistent with this hypothesis, the gene most robustly upregulated by all three Gα is COX-2, which has been shown to be strongly upregulated (mRNA) in VSMC in response to activation of PLCβ-linked receptors and/or Ca^{++} and protein kinase C (Robida et al., 2000). Surprisingly, each Gα stimulated changes in a relatively small number of overlapping genes shared by all three, yet a comparatively large number of non-overlapping Gα-specific genes. This novel finding suggests that, unlike established models, Gqα family members each stimulate unique signaling profiles. Gqα, G14α and G15α exhibit marked differences in overall amino acid sequence and tissue distribution which predicts functional differences, and distinct gene expression profiles for each Gα further supports this hypothesis. G14α and G15α are limited in their tissue expression patterns and not likely expressed in VSMC. Therefore, it will be of interest to compare gene expression profiles

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and functional differences among Gqα family members in cell lines that natively express multiple Gqα family members.

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FIGURE LEGENDS

Figure 1. **Constitutively active Gqα and G14α, but not G15α, cause cell death.** Rat vascular smooth muscle cells (VSMC), infected with retroviruses to express constitutively active mutants of Gqα (Q209L), G14α(Q205L), and G15α(Q212L), or empty virus only, were observed after infection, and samples of growth medium were taken at 24-hour intervals for measurement of released cytosolic lactate dehydrogenase (LDH) as an indicator of cell death. (A) VSMC expressing Gqα(Q/L) and G14α(Q/L) exhibit morphology characteristic of cell death at 24 hours post-infection. (B) LDH activity increases more in cells infected with Gqα(Q/L) and G14α(Q/L) than those infected with G15α(Q/L) or vector. Each point represents the mean ± S.E.M. of three experiments performed in duplicate.

Figure 2. **Constitutively active Gqα, G14α and G15α stimulate phosphoinositide hydrolysis in rat vascular smooth muscle cells.** Rat VSMC infected with retroviruses encoding Gqα(Q209L), G14α(Q205L), G15α(Q212L), or empty virus only, were labeled overnight with ^{3}H-inositol and the accumulation of ^{3}H-inositol phosphates in the presence of lithium chloride measured 24 hours after infection. (A) When expressed in VSMC, Gqα(Q/L), G14α(Q/L), and G15α(Q/L) stimulated phosphoinositide hydrolysis at similar levels over 30 minutes in the presence of lithium chloride. An immunoblot shows the expression of each Gα using an antibody to detect the EE-epitope tag present in each Gα protein. The graph represents the mean ± S.E.M. of three experiments performed in triplicate, expressed as the fold increase of basal (vector only) activity. (B) Gqα(Q/L), G14α(Q/L), and G15α(Q/L) exhibit similar kinetics for the stimulation

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of phosphoinositide hydrolysis. The figure shows a representative time-course experiment performed in duplicate.

Figure 3. **Constitutively active Gqα and G14α, but not G15α, increase caspase-3 activity in intact VSM cells.** Caspase-3 activity in rat VSMC infected with Gqα(Q209L), G14α(Q205L), G15α(Q212L), or empty virus only, was observed 24 hours after infection. (Top panels) Gqα(Q/L)- and G14α(Q/L)-infected cells displayed morphology indicative of cell death (*top panels*). The cell-permeable fluorescent substrate for caspase-3, Rhodamine-110 (Molecular Probes, Eugene, OR) shows elevated activity in VSMC infected with Gqα(Q/L) and G14α(Q/L), but not G15α(Q/L) or vector only (*middle panels*). The cell-permeable fluorescent nuclear stain, Hoechst 33342 (Molecular Probes, Eugene, OR), shows chromatin condensation in nuclei of VSMC infected with Gqα(Q/L) and G14α(Q/L), but not G15α(Q/L) or empty virus only (*bottom panels*).

Figure 4. **Constitutively active Gqα and G14α, but not G15α, increase caspase-3 activity in intact VSM cells.** Caspase-3 activity in rat VSMC infected with Gqα(Q209L), G14α(Q205L), G15α(Q212L), and empty virus only, was observed 24 hours after infection. Immunoblot analysis of cell lysates prepared 24 hours after infection from VSMC. Cells infected with Gqα(Q/L) and G14α(Q/L) show cleavage of caspase-3 (appearance of 16.5 kDa fragment) indicative of elevated activity.

Figure 5. **Akt phosphorylation is unchanged by constitutively activated Gqα, G14α or G15α in rat vascular smooth muscle cells.** Rat VSMC, infected with retroviruses encoding

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Gqα(Q209L), G14α(Q205L), G15α(Q212L), or empty vector, were lysed at 12 and 24 hours after infection, treated for 30 minutes with PDGF or vehicle, then prepared for immunoblotting with a phospho-specific Akt antibody. No changes were observed in basal or stimulated levels of Akt phosphorylation between VSMC infected with Gqα(Q/L), G14αQ/L) or G15α (Q/L) and vector only.

Figure 6. **Blocking protein kinase C and inositol trisphosphate receptors does not inhibit Gqα(Q/L)- and G14α(Q/L)-induced cell death.** Rat VSMC, infected with Gqα(Q209L), G14α (Q205L), and G15α (Q212L), or vector only were incubated in growth medium containing the PKC inhibitor BIS, the IP_3 receptor antagonist, 2-APB, or vehicle. Samples of growth medium were taken at 24-hour intervals for measurement of released cytosolic LDH. At 72 hours, no differences in LDH activity were detected between treated and untreated conditions. The graph represents the mean ± S.E.M. of 3 experiments performed in duplicate.

Figure 7. **Inhibition of NFAT stimulated by Gqα(Q/L) and G14α (Q/L) in rat vascular smooth muscle cells does not prevent Gα-induced cell death** VSMC lines stably expressing an NFAT-specific luciferase reporter were infected with Gqα(Q209L), G14α(Q205L), G15α(Q212L), or empty virus only. After 24 hours infected cells were serum-starved for an additional 24 hours (48 hrs post-infection) and assayed for NFAT activity. (A) VSMC infected with Gqα (Q/L) and G14α (Q/L), showed elevated NFAT activity significantly different from those infected with vector only. VSMC infected with G15α(Q/L) was not significantly different from those infected with empty virus only. The graph represents the mean ± S.E.M. of six experiments performed in quadruplicate. (*, $p<0.05$) (B) Rat VSMC, infected with retroviruses

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encoding Gqα(Q/L), G14α(Q/L), G15α(Q/L), or empty virus only were incubated in growth medium containing the calcineurin inhibitor, cyclosporin A (CsA), or vehicle. Samples of growth medium were taken at 24-hour intervals for measurement of released cytosolic LDH. At 72 hours, no differences in LDH activity were detected between treated and untreated conditions. The graph represents the mean ± S.E.M. of 3 experiments performed in duplicate.

Figure 8: **Gene expression profiles of VSMC after 24 hr infection with Gqα (Q/L), G14α(Q/L) or G15α (Q/L).** VSMC were infected with Gqα(Q209L), G14α(Q205L), G15α(Q212L), or virus carrying empty vector (control). After 24 hours cells were either harvested and total RNA recovered or sister cells were cultured for an additional 72 hrs and observed. Total RNA derived from cultures where sister samples were observed to both express Gα (by EE-antibody) and to progress to cell death after 72 hr (in the case of Gqa(Q/L) and G14α (Q/L)) were submitted for DNA micrroarray analysis (N=3 for each condition) and data processing as described in Materials and Methods. A) Venn diagrams showing the number of genes that increased 2-fold or more over control for each Gα. in each experiment. B) Venn diagram showing the number of genes that decreased 2-fold or more over control for each Gα in each experiment.

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Table 1: Defined genes up- or down-regulated by Gqα, G14α and G15α in VSMC

Gene/ mRNA	Mean Fold-Change Gqα (Range)	G14α (Range)	G15α (Range)	Cellular Roles
Up-regulated genes*				
Cyclooxygenase 2 (Cox-2)	28.2 (12.2-44.2)	22.5 (10.8-34.2)	7.8 (3.8-11.8)	Prostanoid synthesis; Ca++ reg
Kelch related protein 1 (Krp1; Sarcosin)	7.6 (3.4-11.9)	9.2 (3.3-15.0)	8.4 (3.7-13.0)	Cell motility; cytoskeleton
Tissue plasminogen activator (Plat; tPA; PATISS)	4.6 (3.3-5.9)	2.8 (2.3-3.3)	2.5 (2.3-2.6)	Protease, cleaves plasminogen
Cytokine, small inducible 2 (Cxcl2; Mip-2)	4.4 (3.9-4.9)	3.3 (3.9-4.6)	3.4 (3.3-3.6)	Chemotactic agent for PMNL
Dihydropyrimidinase (Dpys;DHP)	3.3 (3.0-3.7)	4.0 (3.0-5.0)	5.0 (3.7-6.4)	Pyrimidine degradation pathway
Down-regulated genes#				
Interleukin 1 receptor antagonist (Il1rn; Il1Ra)	-9.4 (-9.4,-9.5)	-10.9 (-10.7,-11.2)	-3.2 (-3.1, -3.2)	Natural IL1 receptor antagonist
Selenium binding protein 2 (Selenbp2)	-5.9 (-4.2, -7.7)	-5.2 (-4.2, -6.3)	-3.4 (-2.7, -4.2)	Uncertain (vesicle transport?)
Adrenomedullin, hypertensive peptide (Adm)	-4.8 (-4.3, -5.4)	-5.1 (-4.6, -5.6)	-2.4 (-2.2, -2.5)	Hypotensive peptide (CLCR)
Arginase 1 (Arg1)	-3.2 (-2.6, -3.8)	-3.2 (-2.9, -3.4)	-2.7 (-2.4, -2.9)	Metabolism of L-arginine
Natriuretic peptide clearance receptor	-3.4 (-2.6, -4.2)	-3.9 (-2.8, -4.9)	-2.8 (-2.2,-3.4)	Bind / clear natriuretic peptide
Bone morphogenic protein 4 (Bmp4)	-3.2 (-3.0, -3.4)	-3.1 (-2.8, -3.4)	-2.4 (-2.1, -2.8)	TGF-β family growth factor
Growth arrest and DNA damage-inducible 45alpha (Ddit1, Gadd45a)	-2.4 (-2.2, -2.5)	-2.4 (-2.3, -2.6)	-2.8 (-2.6, -3.0)	p53-responsive stress protein

* List includes all defined annotated genes (5) of 23 total that increased. # List includes all defined annotated genes (7) of 27 total that decreased.

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***Table 2:* Genes that are up- or down-regulated by Gqα, but not G14α or G15α**

Gene/ mRNA	Mean Fold-change Gqα	(Range)	Cellular Roles
Upregulated genes*			
Heterotrimeric G protein alpha subunit, Gqα	22.0	(19.0-25.0)	Link GPCR to activation of PLCβ
Nuclear receptor (Nr4a3)	6.3	(2.2-10.5)	Thyroid/steroid transcription factor
Interleukin 6 (IL6; IFN-β2)	4.7	(4.0-5.4)	Cytokine, linked to cell activation
N-myc downstream regulated gene 2 (Ndrg2)	4.7	(2.2-7.1)	Uncertain, differentiation related gene
cAMP/cGMP phosphodiesterase (PDE10A3)	3.3	(2.2-4.1)	Hydrolyzes cyclic nucleotides
Olfactomedin related ER localization protein (Olfm)	3.2	(2.8-3.6)	Uncertain
Reelin (Reln)	2.8	(2.6-3.0)	Extracellular matrix protein
Solute carrier protein, family 21(Slc21a12)	2.7	(2.1-3.4)	Organic anion transporter
Nuclear hormone receptor (nurr1; NGFI-B)	2.7	(2.6-2.8)	Nuclear receptor, implicated in Parkinson's
ADP-ribosylation factor 2 (Arf2)	2.7	(2.5-2.9)	GTPase, membrane trafficking
Growth response protein, insulin-induced (Insig-1;CL-6)	2.6	(2.2-3.0)	ER membranes protein, lipid metabolism
Hepatic glycogen phosphorylase (Pygl)	2.6	(2.1-3.2)	Glucose catabolism
Insulin-induced growth response protein (CL-6)	2.6	(2.2-3.1)	Regulation of cholesterol concentrations
CPB/P300-interacting transactivator 2 (Mrg1; Cited2)	2.6	(2.3-2.9)	Co-activator of PPAR nuclear receptors
RhoB	2.6	(2.4-2.7)	Monomeric GTPase, antiproliferative
Proteinase-activated receptor 2 (PAR-2)	2.5	(2.2-2.8)	Cell surface GPCR for thrombin
Glycoprotein (CD44)	2.4	(2.1-2.8)	Cell adhesion, promotes growth and invasion
Phospholipid scramblease 1 (Plscr1)	2.4	(2.3-2.6)	Transbilayer movement of membrane phospholipids
Beta-galactoside-alpha 2,6-sialtransferase	2.4	(2.2-2.5)	Increase surface sialic acid
cAMP-response element modulator (Crem)	2.3	(2.3-2.4)	Transcription factor for cAMP-regulated genes
Adenylyate kinase 4 (Ak4)	2.2	(2.1-2.4)	Interconversion of AMP to ADP
Hypoxia induced gene (Hig1)	2.1	(2.1-2.2)	Upregulated by hypoxia, unknown biological activity
Actin, alpha 1 (Acta1)	2.1	(2.0-2.2)	Contractile protein, cellular structure and integrity
Down-regulated genes#			
2'5'-oligoadenylate synthetase	-3.5	(-3.3, -3.7)	Synthesizes oligoadenylates from ATP
Stromal cell-derived growth factor-1 (SDF-1)	-3.1	(-2.1, -4.0)	Chemokine, CXCR4 receptor agonist
Endothelin 1 (Edn1)	-2.7	(-2.5, -2.9)	Endothelium-derived peptide, vasoconstrictor
Amphiregulin (Areg)	-2.6	(-2.2, -3.0)	Transactivated agonist for EGF receptor
Transforming growth factor β2 (TGF-β2)	-2.6	(-2.2, -2.9)	Cytokine involved in cell growth and differentiation
Strathmin-like, neural specific protein 10 (Scg10)	-2.6	(-2.2, -3.0)	Tubulin binding, microtubule destabilizer
Growth arrest specific 6 (Gas6)	-2.5	(-2.1, -2.8)	Ligand for Axl/Sky subfamily of receptor tyrosine kinases
SH3-binding protein 5 (Btk-associated) (Sh3bp5;Sab)	-2.5	(-2.3, -2.6)	Intermediary of Btk- and JNK signaling
NF1-A3	-2.4	(-2.0, -2.7)	Nuclear transcription factor
Neuropilin	-2.3	(-2.2, -2.4)	Cell surface receptor involved in angiogenesis
NGF-induced, early growth response 1 gene (Egr1; Ngf1)	-2.3	(-2.1, -2.5)	Nuclear transcription factor (immediate)
Matrix metalloproteinase 16 (Mmp16)	-2.2	(-2.1, -2.3)	Proteases of extracellular matrix proteins
NF1-B3	-2.2	(-2.1, -2.2)	Nuclear transcription factor
Frizzled gene (Frzzled)	-2.1	(-2.1, -2.1)	GPCR for Wnt, important in development

* List includes all defined annotated genes (23) of 74 total that increased. # List includes all defined annotated genes (14) of 76 total that decreased.

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Table 3: Genes that are up- or down-regulated by either G14α or G15α, but not Gqα

G14α regulated Gene/ mRNA	Mean Fold-change G14α(Range)		Cellular Roles
Up-regulated genes*			
Heparin binding EGF-like growth factor (proHB-EGF)	3.4	(2.5-4.4)	Transmembrane precursor of HB-EGF
Leukemia inhibitory factor (Lif)	2.8	(2.2-3.5)	Neuropoietic cytokine
Ectonucleotide pyrophosphatase/ phosphodiesterase 1 (Enpp1)	2.7	(2.5-2.8)	Membrane protein, conversion of nucleotides
ATP-binding cassette transport protein (Abcb1a; Mdr1)	2.5	(2.1-3.0)	Xenobiotic membrane transporter
A kinase (PRKA) anchor protein (gravin) 12 (Akap12)	2.4	(2.3-2.5)	Kinase scaffolding protein
FMS-like tyrosine kinase (Flt1)	2.4	(2.3-2.5)	Receptor for VEGF-A, placental GF
Potassium chanel K6 (TWIK-2; Kcnk6)	2.3	(2.1-2.5)	Outward rectifying potassium channel
Core promoter element binding protein (Copeb)	2.3	(2.1-2.5)	Transcriptional activator
LIM domain-containing; Enigma homolog (Enh)	2.2	(2.1-2.4)	PKC binding scaffolding protein
Down-regulated genes#			
Xanthine dehydrogenase (Xdh)	-2.8	(-2.2,-3.5)	Converts xanthine to urate
C-CAM4	-2.7	(-2.0,-3.3)	Cell-cell adhesion molecule
Fc receptor, IgG, low affinity III (FcgammaR3a)	-2.6	(-2.1,-3.0)	Immune complex receptors
Leucine arylaminopeptidase (Lap1; Anpep)	-2.6	(-2.6,-2.7)	Aminopeptidase for modification of MHCII molecules

* List includes all defined annotated genes (9) of 45 total that increased. # List includes all defined annotated genes (4) of 25 total that decreased.

G15α regulated Gene/ mRNA	Mean Fold-change G15α(Range)		Cellular Roles
Up-regulated genes*			
Arg/Abl-interacting protein (ArgBP2)	3.5	(2.5-4.4)	Adaptor protein, signaling mediator
NADH/NADPH mitogenic oxidase subunit p65-mox (Nox1)	2.9	(2.4-3.3)	NADPH oxidases, superoxide generating
Ceruloplasmin (Cp; CERP)	2.6	(2.5-2.7)	Copper-binding serum protein
cAMP-reg. guanine nucleotide exchange factor (Epac2)	2.5	(2.4-2.7)	cAMP-binding protein, modulates monomeric GTPases
Exodus/MIP-3 alpha/LARC; CC chemokine ST38	2.4	(2.3-2.5)	Chemokine
CDP-diacylglycerol synthase (Cds1)	2.3	(2.2-2.5)	Involved in phosphatidylinositol metabolism
Lymphotactin; small inducible cytokine, C1 (Scyc1)	2.3	(2.3-2.3)	Chemotactic chemokine
AMP-activated protein kinase (Prkaa2)	2.2	(2.1-2.2)	Rate limiting enzyme in malonyl-CoA synthesiss
Down-regulated genes#			
No annotated genes (of 2 total)			

* List includes all defined annotated genes (8) of 16 total that increased. # List includes all defined annotated genes (0) of 2 total that decreased.

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Table 4: Defined genes upregulated by Gqα and G14α, but not by G15α in VSMC

Upregulated Gene/ mRNA*,#	Mean Fold-change Gqα (Range)	G14α (Range)	Cellular Roles
Surface receptors/ ligands/ signaling proteins			
Urokinase/ Plasminogen Receptor (uPAR-1)	7.2 (6.9-7.5)	8.2 (8.2-8.3)	cell-surface tPA receptor; **TNFα induced**
Bone morphogenic protein 2 (BMP-2)	**6.3 (4.1-8.4)**	**6.0 (3.0-8.9)**	**TGF-β-like peptide; TNFα induced**
Follistatin (Fst; FOL1)	6.1 (5.5-6.7)	4.0 (4.0-4.1)	inhibits FSH release
B-type natriuretic factor (BNF)	5.7 (4.7-6.7)	4.5 (3.7-5.2)	vasoactive peptide
LDL-receptor 1, oxidized (Orl1; LOX-1)	**4.0 (2.9-5.0)**	**3.3 (3.1-3.4)**	**Lectin/lipoprotein receptor; TNFα induced**
Anti-proliferative, B-cell translocation gene 2 (BTG-2; Tis21; PC3)	**3.4 (3.0-3.7)**	**3.5 (2.6-4.3)**	**p53 effector; blocks proliferation**
Vascular endothelial growth factor A 110 (VEGF)	2.9 (2.5-3.3)	2.9 (2.5-3.3)	mitogen, primarily for vascular endothelial cells
Osteoclast inhibitory lectin (Ocil)	2.6 (2.5-2.6)	2.4 (2.4-2.4)	TM protein; binds lectins; killer cells
Transporters/ Channels			
Multi-drug resistance gene (Mdr1a)	7.8 (3.7-11.9)	5.6 (3.7-7.5)	ABC transporter, drug efflux; **TNFα-induced**
Gap junction membrane channel α4 (Connexin37)	**5.5 (4.0-7.0)**	**4.7 (3.5-5.8)**	**component of gap junctions; TNFα-induced**
Monocarboxylate transporter (Mct3)	3.6 (3.0-4.3)	3.2 (2.8-3.6)	PM transporter for pyruvate and lactate
Kinases / phosphatases			
MAP-kinase phosphatase (Cpg21)	6.2 (5.4-7.0)	7.4 (6.8-8.0)	dual specificity phosphatase for Erk1
Serum/glucocorticoid regulated kinase (Sgk)	4.9 (4.8-5.0)	3.7 (3.7-3.9)	dual spec. phosphatase; regulates ion channels
MAPKinase kinase kinase 8 (Map3K8;Tpl2;Cot)	**4.7 (3.5-6.0)**	**2.8 (2.4-3.3)**	**Ser/Thr kinase for JNK; regs TNFα synthesis**
Hexokinase 2 (Hk2)	4.0 (3.2-4.8)	3.8 (3.6-4.0)	phosphorylates glucose to glucose-6-phosphate
Protein tyrosine phosphatase	**3.9 (3.7-4.1)**	**3.0 (2.7-3.3)**	**Dual-specific phosphatase for MAPkinases**
Pyruvate dehydrogenase kinase 1 (Pdk1)	3.4 (3.4-3.5)	2.7 (2.5-2.8)	inactivates PDH; terminates pyruvate decarboxyl.
Sphingosine kinase 1c (Sphk1c)	2.4 (2.2-2.7)	2.4 (2.2-2.5)	phosphorylates sphingosine; proliferation
Transcription factors and modulators			
Activating transcription factor (Atf3/LRF1)	**7.8 (7.4-8.4)**	**5.3 (4.8-5.8)**	**CREB-like transcription factor; TNFα-induced**
NGF-induced transcription factor (NGFI-B;Nurr77)	**5.3 (2.7-7.8)**	**3.3 (2.0-4.6)**	**transcription factor; mediates TNFα actions**
Gonadotropin inducible ovarian transcription factor-1 (Giot1)	4.2 (4.1-4.3)	2.2 (2.2-2.3)	transcriptional repressor, gonadotropin-induced
cAMP responsive element modulator (CREM)	3.0 (2.7-3.3)	2.5 (2.2-2.8)	cAMP regulated transcriptional modulator
Rat nuclear receptor (RNR-1)	3.6 (2.7-4.5)	2.4 (2.0-2.8)	transcription factor
Nuclear factor Nfil3/E4BP4	**2.3 (2.3-2.5)**	**2.3 (2.2-2.4)**	**leucine zipper transcription factor**
Extracellular matrix			
TNF-alpha stimulated gene 6 (TSG-6)	12.1 (6.2-17.9)	12.7 (7.1-18.2)	binds hyaluronan, arthritis; **TNFα-induced**
Hyaluronan Synthase 2 (Has2)	4.8 (3.1-6.4)	5.4 (2.9-7.9)	produces extracellular hyaluronan; **TNFα-induced**
UDP Galactose-4-epimerase (GALE)	2.3 (2.1-2.5)	2.4 (2.4-2.4)	epimerization of UDP-glycoproteins, glycolipids
Disintegrin and metalloprotease (ADAMTS-1)	2.9 (2.8-3.0)	2.4 (2.3-2.5)	cleavage of extracellular proteoglycans
Membrane proteoglycan (NG2)	2.7 (2.4-3.0)	2.2 (2.2-2.3)	proteoglycan
Metabolism and other:			
Calponin 1 (CaP; Cnn)	3.5 (3.3-3.6)	3.1 (2.6-3.6)	Smooth muscle contraction; **TNFα regulated**
Annexin A3 (Anx3; Lipocortin 3)	3.2 (2.8-3.5)	2.6 (2.3-2.8)	membrane trafficking/fusion
Adenoviral interacting protein 3 (Bnip3)	**3.2 (2.8-3.5)**	**2.6 (2.3-2.8)**	**BCL2-binding, pro-apoptotic**
UDP-glucuronosyltransferase	3.2 (2.6-3.8)	3.3 (2.6-4.1)	metabolism of endo- and xenobiotics
T-cell death-associated gene (Tdag)	**2.9 (2.4-3.5)**	**3.1 (2.5-3.7)**	**inhibits protein synthesis; apoptotic**
Alpha-crystallin (Hsp22)	2.8 (2.4-3.1)	2.1 (2.1-2.1)	stress-inducible chaperonin; **induces TNFα**
UDP-glucuronosyltransferase 1A7 (UGT1A7)	2.8 (2.4-3.2)	2.7 (2.2-3.2)	metabolism of endo- and xenobiotics
Antizyme Inhibitor	2.8 (2.8-2.8)	2.1 (2.0-2.2)	inhibits ODC antizyme
Neurominadase 1 (Neu1)	2.2 (2.2-2.2)	2.2 (2.2-2.2)	modifies glycoproteins and glycolipids

***Bold: indicates genes with reported roles in apoptosis and/or TNFα signaling (see text). #Listed are defined annotated genes (38) of 137 total that changed.**

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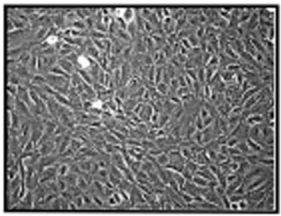
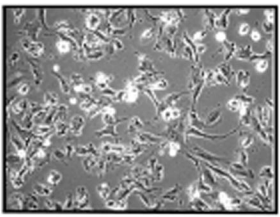
Table 5: Defined genes down-regulated by Gqα and G14α, but not by G15α in VSMC

Downregulated Gene/ mRNA*,#	Mean Fold-change Gqα(Range)		G14α(Range)		Cellular Roles
Surface receptors / ligands/ signaling proteins					
Angiotensin II receptor. Type 1A (AT1A)	-6.0	(-4.9, -7.0)	-5.7	(-5.2, -6.2)	Receptor (GPCR) for angiotensin II
Growth factor receptor binding protein 14 (Grb14)	-3.9	(-3.8, -4.0)	-3.3	(-3.3, -3.3)	Tyr Kinase receptor adaptor protein
Vasopressin receptor 1A (AVPR1a)	-3.6	(-3.3, -3.9)	-3.2	(-3.1, -3.4)	Receptor (GPCR) for vasopressin
Potassium channel, tandem pore (TREK-1, KCNK2)	-2.6	(-2.4, -2.8)	-2.4	(-2.0, -2.7)	"Leak" potassium channel
Fractaline (Cx3cl1)	**-2.7**	**(-2.2,-3.2)**	**-2.2**	**(-2.0, -2.6)**	**Inducible cytokine; reg by TNFα**
Nuclear transcription factors/modulators					
Cardiac adriamycin responsive protein (CARP)	-3.5	(-3.4, -3.6)	-2.7	(-2.0, -3.4)	Nuclear transcription factor
Mesenchyme homeobox 2 protein (Meox2)	-2.8	(-2.1, -3.5)	-2.6	(-2.1, -3.2)	Nuclear homeobox transcription factor
Butyrate response factor (Brf1)	-2.5	(-2.1, -2.8)	-2.3	(-2.1, -2.6)	Immediate early, transcription factor
Signal transducer/activator transcription 1 (Stat1)	**-2.4**	**(-2.4, -2.4)**	**-2.5**	**(-2.0, -3.0)**	**transcription factor; reg by TNFα**
Other					
Guanylate binding protein 2, INF-inducible (Gbp2)	-3.4	(-3.2, -3.5)	-3.1	(-3.1, -3.2)	INF-induced GTPase, undefined
Microsomal glutathione-S transferase (Mgst1)	-3.3	(-2.8, -3.8)	-3.0	(-2.8, -3.3)	Protection from oxidative stress

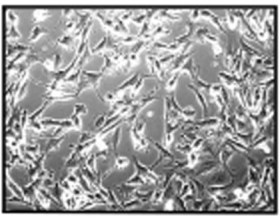
***Bold: indicates genes with reported roles in apoptosis and/or TNFα signaling (see text). #Listed are defined annotated genes (11) of 85 total that changed.**



Vector



Gq (Q209L)



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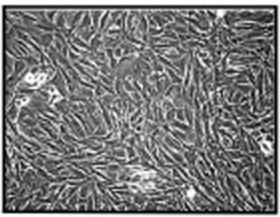

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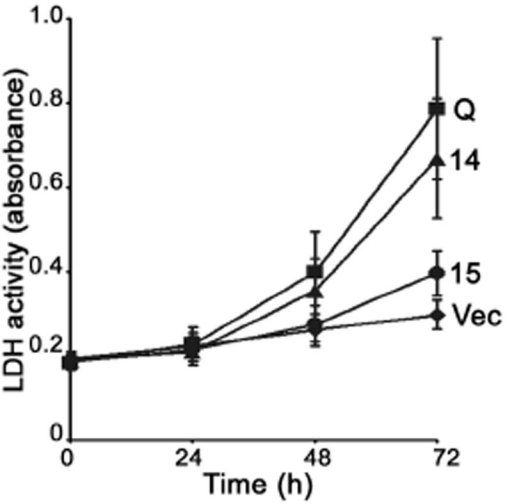
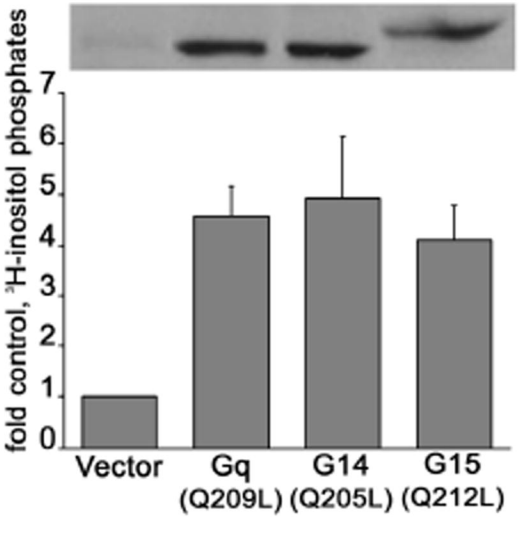
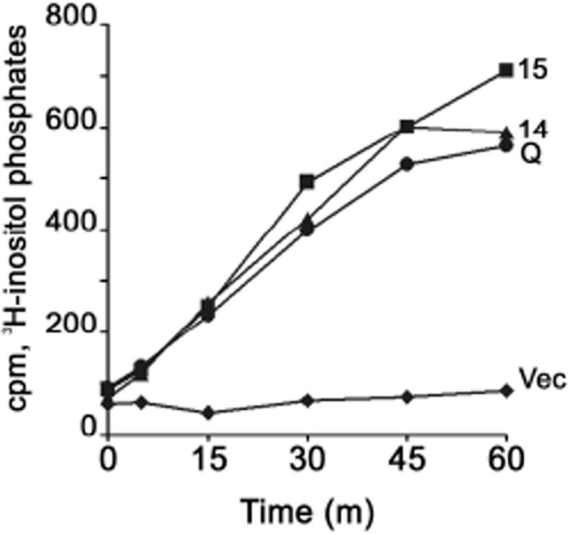
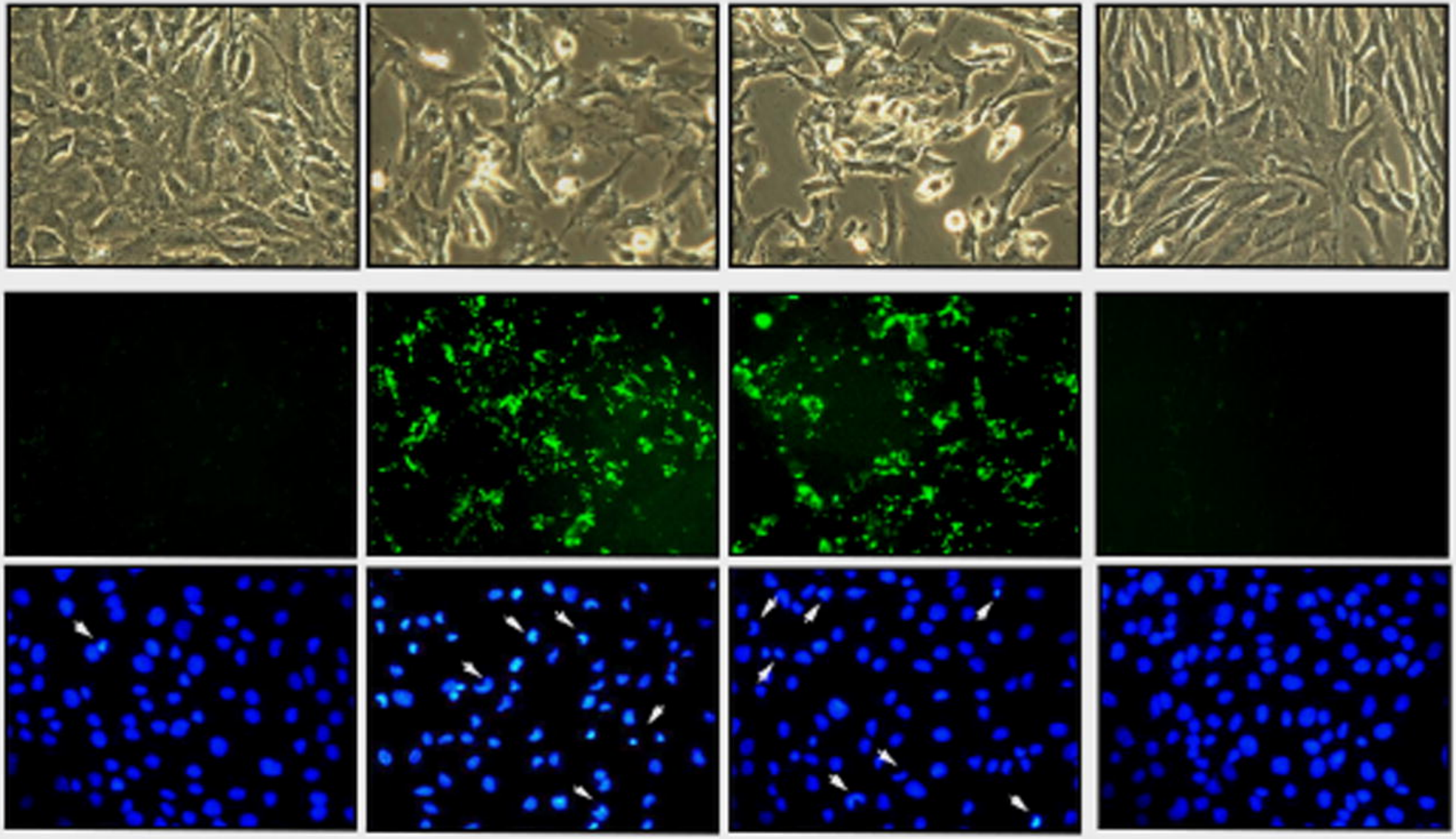



fold control, ^{3}H-inositol phosphates
0
1
2
3
4
5
6
7
Vector
Gq
(Q209L)
G14
(Q205L)
G15
(Q212L)


cpm, ^{3}H-inositol phosphates
0
200
400
600
800
0
15
30
45
60
Time (m)
15
14
Q
Vec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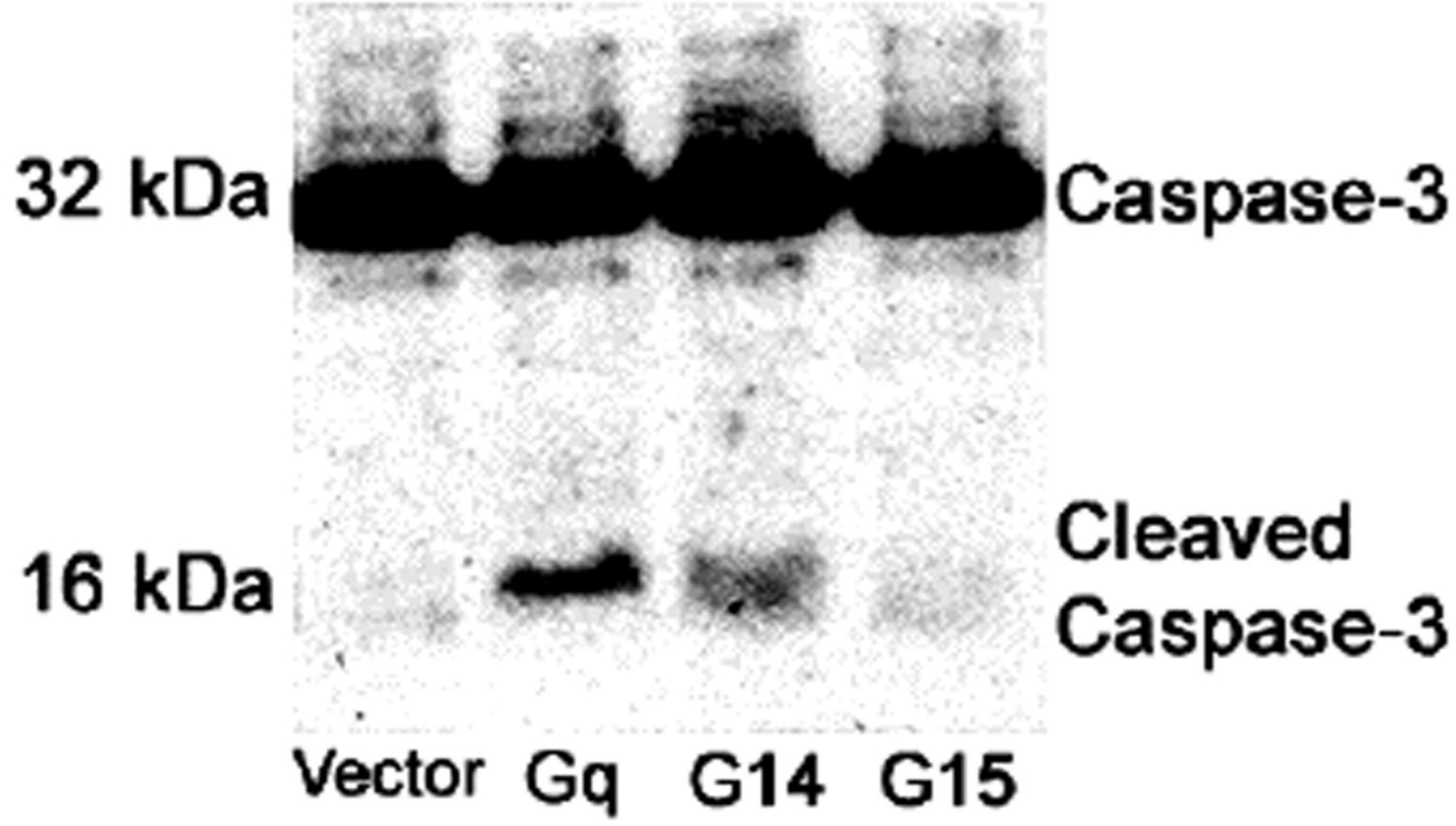
Gq (Q209L)
G14 (Q205L)
G15 (Q212L)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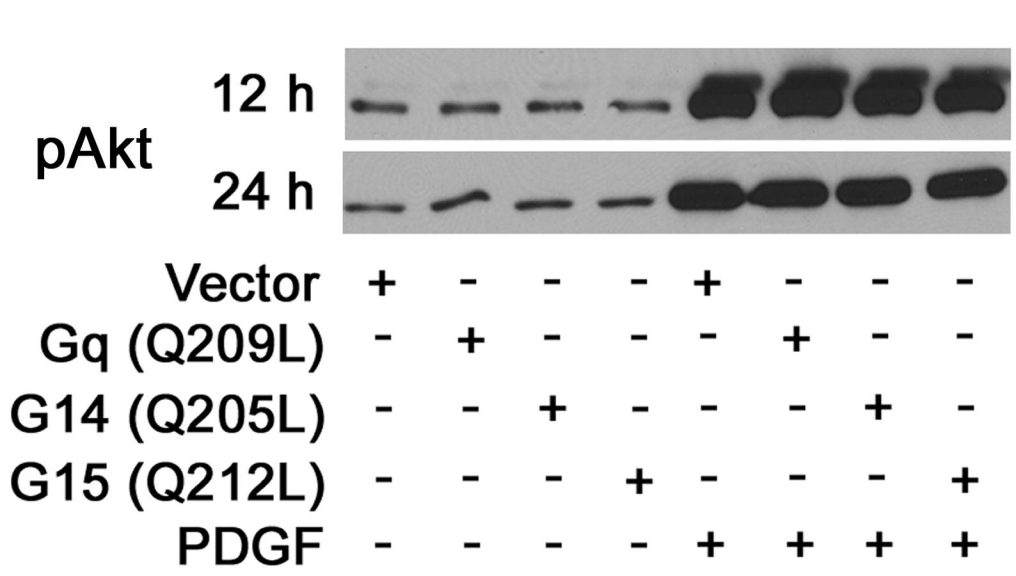
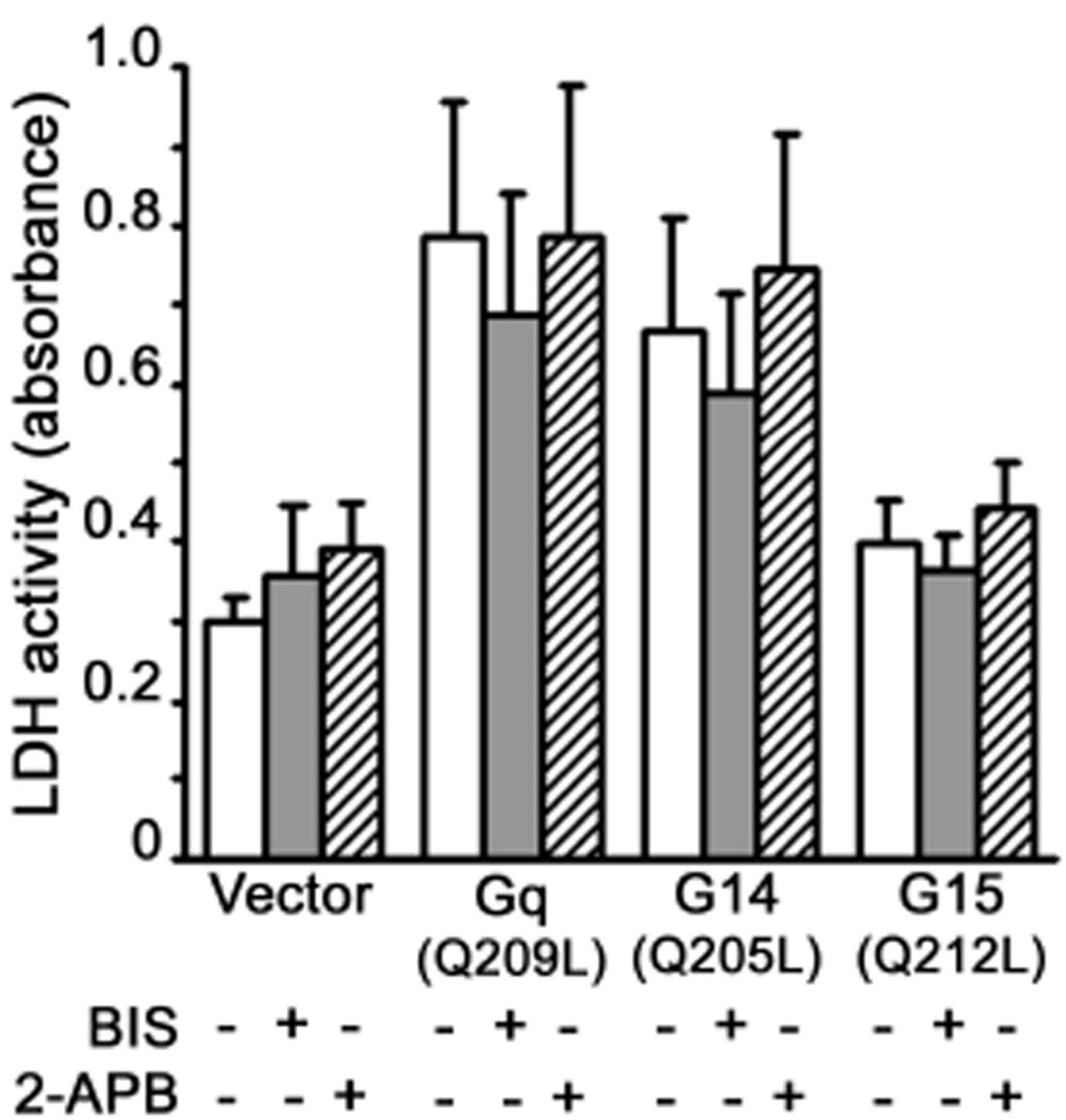
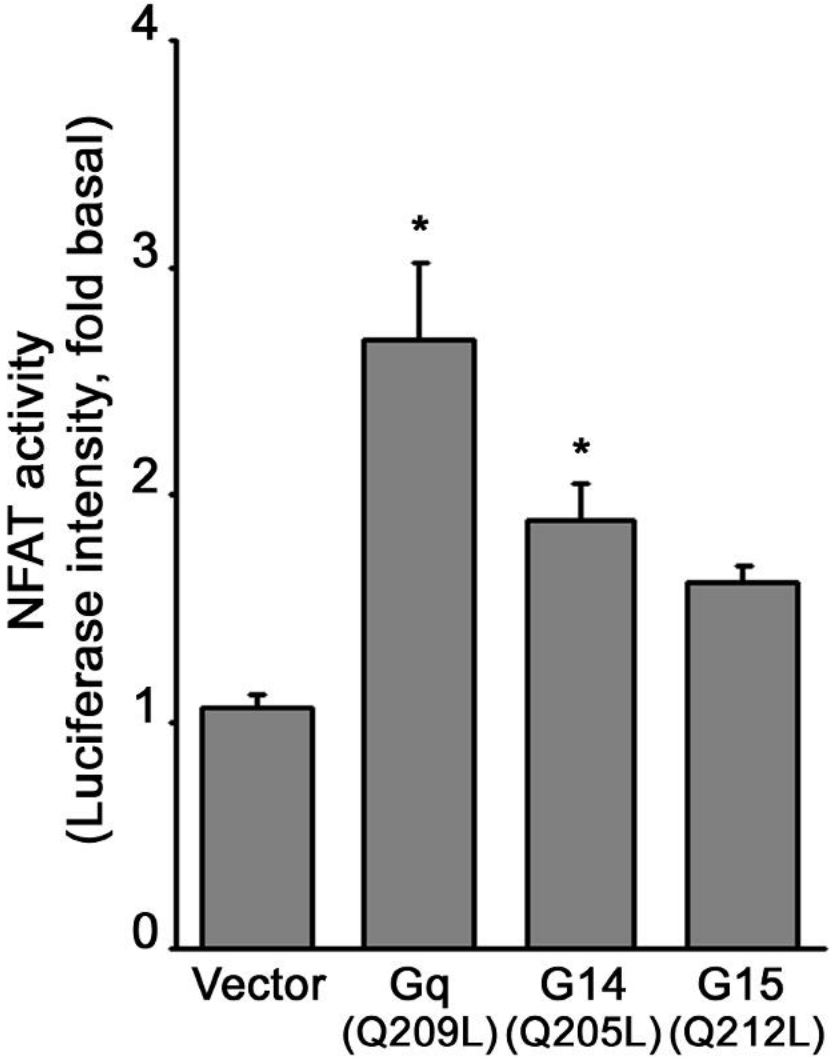
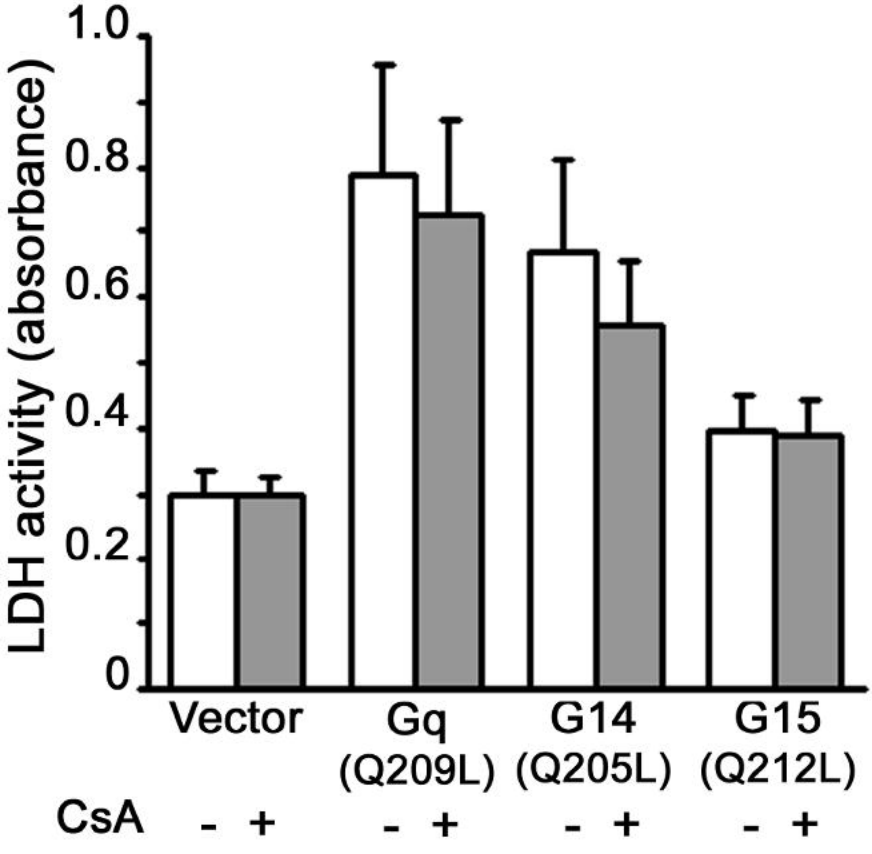
Caspase-3
16 kDa
Cleaved
Caspase-3
Vector
Gq
G14
G15


pAkt
12 h
24 h
Vector + - - - + - - -
Gq (Q209L) - + - - - + - -
G14 (Q205L) - - + - - - + -
G15 (Q212L) - - - + - - - +
PDGF - - - - + + + +


LDH activity (absorbance)
1.0
0.8
0.6
0.4
0.2
0
Vector
Gq
(Q209L)
G14
(Q205L)
G15
(Q212L)
BIS - + - - + - - + - - + -
2-APB - - + - - + - - + - - +


NFAT activity
(Luciferase intensity, fold basal)
4
3
2
1
0
*
*
Vector
Gq
(Q209L)
G14
(Q205L)
G15
(Q212L)


LDH activity (absorbance)
1.0
0.8
0.6
0.4
0.2
0
Vector
Gq
(Q209L)
G14
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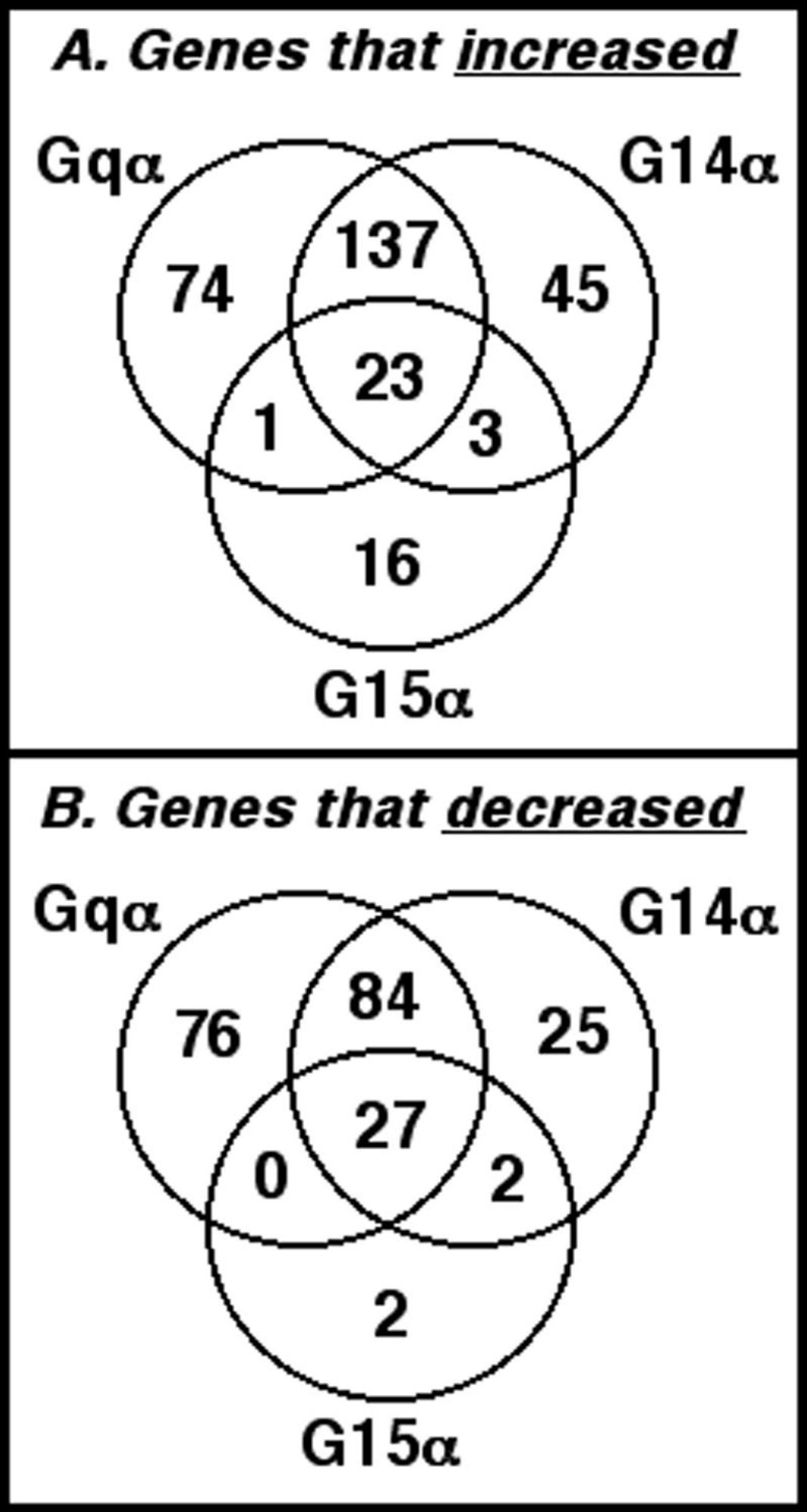
G15
(Q212L)
CsA
- + - + - + - +


A. Genes that increased
Gqα
G14α
74
137
45
23
1
3
16
G15α
B. Genes that decreased
Gqα
G14α
76
84
25
27
0
2
2
G15α