

## Cardiotonic steroids stabilize RGS2 protein levels

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*Non-standard abbreviations and acronyms:*

CTS – Cardiotoxic steroids

RAAS – Renin-angiotensin-aldosterone system

GPCR – G protein-coupled receptor

RGS – Regulator of G protein Signaling

GAP – GTPase activating protein.

VSMC – Vascular smooth muscle cells

PL - ProLabel

## Abstract

Regulator of G protein signaling 2 (RGS2), a Gq-specific GTPase activator protein (GAP), is strongly implicated in cardiovascular function. RGS2<sup>-/-</sup> mice are hypertensive and prone to heart failure and several rare human mutations that speed RGS2 degradation have been identified in hypertensive patients. Consequently, pharmacological up-regulation of RGS2 protein levels could be beneficial. We utilized a  $\beta$ -galactosidase complementation method to screen several thousand compounds with known pharmacological function for those that increase RGS2 protein levels. Several cardiotonic steroids (CTS), including ouabain and digoxin increase RGS2 but not RGS4 protein levels. CTS increase RGS2 protein levels through a posttranscriptional mechanism by slowing protein degradation. RGS2 mRNA levels in primary vascular smooth muscle cells are unaffected by CTS treatment while protein levels are increased 2-3 fold. Na/K-ATPase is required for the increase in RGS2 protein levels as the effect is lost in Na/K-ATPase knock-down cells. Furthermore we demonstrate that CTS-induced increases in RGS2 are functional, reducing receptor-stimulated Gq-dependent ERK phosphorylation. Finally, we show that in vivo treatment with digoxin leads to increased RGS2 protein levels in heart and kidney. CTS-induced increases in RGS2 protein levels and function should modify several deleterious mechanisms in hypertension and heart failure. This novel CTS mechanism could contribute to the beneficial actions of low dose digoxin in heart failure. Additionally, our results support the concept of small-molecule modulation of RGS2 protein levels as a new strategy for cardiovascular therapeutics.

## Introduction

Regulators of G protein signaling (RGS) proteins are GTPase accelerating proteins (GAPs) that deactivate GTP-bound G $\alpha$  subunits, thereby reducing the amplitude and duration of G protein-coupled receptor (GPCR) signaling (Ross and Wilkie, 2000; Sjögren et al., 2010). They also represent novel potential drug targets in numerous diseases, including hypertension, heart failure, Parkinson's disease and schizophrenia (Blazer and Neubig, 2009; Blundell et al., 2008; Chowdari et al., 2002; Heximer et al., 2003). Modulation of RGS proteins would alter GPCR signaling in a pathway- and tissue-specific manner (Blazer and Neubig, 2009). While RGS proteins are considered to be difficult drug targets (Tesmer et al., 1997), we and others have made significant progress in developing RGS protein inhibitors (Blazer et al., 2010; Blazer et al., 2011; Roman et al., 2007; Turner et al., 2012). In other cases, however, increasing RGS protein function may be therapeutically beneficial.

Heart failure and hypertension are major clinical problems and treatment is only partially effective (Shah et al., 2011). Inhibition of neurohumoral signaling by catecholamines and the renin-angiotensin-aldosterone system (RAAS) is widely used but mortality rates from heart failure remain high (Shah et al., 2011). Consequently, improved understanding of heart failure mechanisms and new treatments are needed.

RGS2 plays key roles in hypertension and heart failure. RGS2<sup>-/-</sup> mice are hypertensive (Heximer et al., 2003), show enhanced catecholamine-induced cardiomyocyte hypertrophy (Nunn et al., 2010), and have worsened heart failure responses to pressure overload *in vivo* (Takimoto et al., 2009). RGS2 is a relatively selective GAP for Gq-family proteins (Heximer et al., 1997) which mediate phospholipase C and Ca<sup>2+</sup> signaling, including that of many vasoconstrictor agents such as norepinephrine, angiotensin II and endothelin-1. Consequently, hypertension in RGS2<sup>-/-</sup> mice (Heximer et al., 2003; Takimoto et al., 2009), and in humans with low RGS2 expression (Bodenstein et al., 2007; Semplicini et al., 2010; Yang et al., 2005), could be due to increased GPCR-mediated vasoconstriction. On the other hand, RGS2 expression is

increased in patients with Bartter's/Gitelman's syndrome, a disorder characterized by low blood pressure (Calo et al., 2004).

RGS2 protein levels are selectively reduced in ventricular cardiomyocytes early in pressure-overload hypertrophy in mice and RNAi-mediated knock-down of RGS2 leads to increased phenylephrine- and endothelin-induced myocyte hypertrophy (Zhang et al., 2006). Over-expression of RGS2 inhibits  $G_{\alpha_{q/11}}$ -mediated cardiac myocyte hypertrophy (Zou et al., 2006). Endogenous RGS2 also restrains angiotensin-induced cardiac fibroblast signaling, collagen synthesis, and proliferation (Zhang et al., 2011). Furthermore, RGS2 is required for the vasodilation (Sun et al., 2005) and cardiac antihypertrophic effects (Takimoto et al., 2009) of nitric oxide and cGMP.

An important mechanism of control of RGS proteins is through ubiquitin-mediated proteasomal degradation (Bodenstein et al., 2007; Lee et al., 2005; Lee et al., 2011). RGS4 is degraded through the N-end rule pathway which detects specific destabilizing amino-terminal residues (Bodenstein et al., 2007; Lee et al., 2005). RGS2 is also degraded through the proteasome in transfected HEK-293T cells (Bodenstein et al., 2007) but the specific pathway has not yet been elucidated. This rapid degradation of RGS2 and other RGS proteins by proteasomal mechanisms provides a useful control point at which to pharmacologically modulate RGS levels and function (Sjogren and Neubig, 2010). Given the regulatory role for RGS2 in the control of cardiovascular function and the correlation between low RGS2 protein levels and hypertension and heart failure, pharmacologically increasing protein expression of RGS2 could be a novel approach to treating cardiovascular disease.

In this study, we tested a collection of known drugs to identify small molecules that increase RGS2 expression. Surprisingly, ouabain, digoxin, and other cardiotonic steroids (CTS) act at nanomolar concentrations to increase RGS2 protein levels *in vitro*. We also demonstrate enhanced function of RGS2 in suppressing  $G_q$ -mediated downstream signaling. Digitalis glycosides, including digoxin, are the oldest established therapies for heart failure. The precise

mechanism by which CTS increase contractility and enhance cardiac function is not known. There has, however, been increasing interest (Ahmed and Waagstein, 2009; Gheorghiade and Braunwald, 2009) in the utility of low dose digoxin in improving clinical outcomes in heart failure. The novel effects of digoxin to increase RGS2 protein levels, elucidated here, may contribute to an improved understanding of CTS function and a new cardiovascular therapeutic strategy.

## Materials and Methods

### Materials

MG-132 was from Calbiochem (Quincy, MA). Ouabain was from Tocris (Ellisville, MS). digoxin, lactacystin and cycloheximide were from Sigma-Aldrich (St Louis, MO). If not otherwise indicated all chemicals were from Sigma-Aldrich and all tissue culture supplies were from Invitrogen/Gibco (Grand Island, NY).

### Mammalian expression constructs

A 4-kDa fragment of  $\beta$ -galactosidase (ProLabel™) was added to the C-terminus of human RGS2 and RGS4 (RGS2-PL and RGS4-PL). Human RGS2 and RGS4 were amplified by PCR from the pcDNA3.1 RGS2-HA and RGS4-HA vectors previously described (Bodenstein et al., 2007). Full-length RGS open-reading frames, without the HA tag, were cloned into the pCMV-ProLabel™-C3 (DiscoverRx, Freemont, CA) vector in frame with the C-terminal ProLabel tag. Restriction sites for XhoI (5') and BamHI (3') were added to the RGS2 and RGS4 PCR primers to facilitate insertion into the pCMV-ProLabel™-C3 vector. The primers for amplification of RGS2 were 5'- CGCTCGAGATGCAAAGTGCTATGTTCTTGGC-3' (sense) and 5'- CCGCTCGAGATGCAAAGTGCTATGTTCTTGGC-3' (antisense). The primers for amplification of RGS4 were 5'- CCGCTCGAGATGTGC AAAGGGCTTGCAGGTCTGCC -3' (sense) and 5'- CGCGGATCCGGCACACTGAGGGACCAGGG-3' (antisense).

### Cell Culture and Transfections

Human embryonic kidney HEK-293 or HEK-293T cells were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub> and grown to 95% confluence in Dulbecco's modified Eagle's medium (DMEM; GIBCO, 11995), containing 4.5 g/L glucose, 2 mM L-glutamine, 25 mM HEPES with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were transfected using Lipofectamine 2000 (Invitrogen) at 5 µl per microgram of plasmid

DNA, according to the manufacturer's recommended protocol. All transfections were performed under serum free conditions in Opti-MEM (GIBCO, 31985). Transfections were allowed to proceed for 4-5 h before the media was changed to DMEM with 10% FBS. For transient transfections, experiments were run 48 h after transfection.

To develop HEK-293 stable cell lines expressing RGS2-ProLabel or RGS4-ProLabel, 400 µg/ml G-418 (Gibco) was added to the cells 48h after transfection. The cells were cultured until non-transfected cells had died. To select individual clones, cells were sorted by flow cytometry into 96-well plates at 1 cell/well and cells were allowed to grow in selection medium until colonies formed. Individual clones were expanded to 12-well plates and tested for response to MG-132 using the PathHunter™ ProLabel assay.

Porcine proximal tubular epithelial (LLC-PK) cells with normal (AAC-19) or severely reduced (PY-17; Na/K-ATPase null) expression of the Na/K-ATPase ( $\alpha$ 1) have been previously described (Liang et al., 2006). Cells were maintained in DMEM, 10% FBS and allowed to grow to 95% confluency prior to assay. Puromycin (1 µg/ml) was maintained in the culture medium to maintain shRNA expression.

### **Preparation of rat aortic vascular smooth muscle cell cultures**

All animal studies were reviewed and approved by the University of Michigan Committee on the Use and Care of Animals. Primary rat aortic vascular smooth muscle cells (VSMC) were prepared as previously described (Atkins et al., 2009). Cells were used from passages 3 to 8.

### **PathHunter™ ProLabel $\beta$ -galactosidase complementation assay**

The 4 kDa PL tag on the C-terminus of RGS2 and RGS4 permitted rapid and quantitative assessment of protein expression. HEK-293 cells expressing RGS2-PL or RGS4-PL were trypsinized and re-suspended in DMEM without phenol red (GIBCO, 21063) containing 4.5 g/L glucose, 2 mM L-glutamine, 25 mM HEPES supplemented with 0.1% bovine serum albumin (BSA) and counted using the Countess automatic cell counter (Invitrogen). Cells were



diluted to 500,000 cells/ml and plated in a white 384-well plate (Corning #3570) in 30  $\mu$ l (15,000 cells/well) DMEM without phenol red plus 0.1% BSA. Cells were allowed to attach for at least 3 h prior to treatment with compounds (10  $\mu$ M for the screen or as indicated). At the end of treatment, the medium was removed using an ELx406 plate washer (BioTek, Winooski, VT) and 5  $\mu$ l/well of Celltiter Fluor viability reagent was added (Promega, Madison, WI). The plate was shaken at 400 rpm for 2 min and incubated at 37°C for 30 min before reading fluorescence (Ex 390 nm/Em 505 nm) on a Pherastar plate reader (BMG, Cary, NC).

The PathHunter™ ProLabel protein expression assay (DiscoverRx) was performed immediately following the viability assay following the manufacturer's general protocol. CL/Lys reagent was prepared by combining 1 part Galacton Star, 5 parts Emerald II, 19 parts CL substrate and 25 parts lysis buffer. 10  $\mu$ l/well CL/Lys reagent was added to each well and the plate was shaken at 400 rpm, for 2 min. Plates were incubated at room temperature for an additional 5 min to allow complete cell lysis. 5  $\mu$ l Enzyme Acceptor (incomplete  $\beta$ -galactosidase) was then added and the plate was shaken at 400 rpm, for 2 min and then incubated in the dark at room temperature for 3 h. Chemiluminescence corresponding to relative RGS2 or RGS4 protein expression was detected on a Pherastar plate reader.

### **Preparation of cell lysates**

HEK-293T cells transiently transfected with C-terminally HA-tagged RGS2-HA (Bodenstein et al., 2007) were plated in 12-well plates in DMEM with 10% FBS and allowed to grow to 95% confluence prior to compound treatment in DMEM with 0.5% FBS. Cells were harvested and lysed on ice by removing medium and adding 100  $\mu$ l RIPA buffer containing protease inhibitors (20 mM Tris-HCl, pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM  $\beta$ -glycerophosphate, 1% Triton X-100, 0.1% SDS, Complete protease inhibitor cocktail (Roche; Pleasanton, CA)). For studies of agonist-induced p42/44-ERK phosphorylation 2 mM sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ) was added to the lysis buffer to inhibit phosphatases. The plate was

shaken for 5 min at 4°C and lysates transferred to plastic centrifuge tubes. Samples were sonicated for 10 min at 4°C in a bath sonicator, centrifuged at 500 x *g* for 3 min and the supernatant used for SDS-PAGE and immunoblot.

For detection of endogenous RGS2 protein expression in VSMC, AAC-19 or PY-17, cells were plated in 6-well plates in DMEM, 0.5% FBS and allowed to grow to 95% confluence prior to treatment with indicated compounds. Cells were trypsinized and harvested in 500 µl PBS on ice. Cells were pelleted by centrifugation (500 x *g* for 5 min) and the pellets were lysed by adding 80 µl RIPA buffer. All samples for Western blot were sonicated for 10 min at 4°C in a bath sonicator, centrifuged at 500 x *g* for 3 min and the supernatant used for SDS-PAGE and immunoblot.

### **SDS-PAGE and Immunoblot**

Protein concentration in the cell lysates was determined using the BCA assay (Pierce; Rockford, IL) and adjusted with an appropriate volume of Laemmli buffer (BioRad; Hercules, CA). Equal amounts of protein in each lane were resolved on a 12% SDS-PAGE gel for 1 h at 160 V. Samples were transferred to an Immobilon-P membrane (Millipore, Billerica, MA) for 1 h at 100 V, 400 mA on ice and subjected to Western immunoblot analysis.

The membrane was blocked with Tris-buffered saline (10 mM Tris, pH 8.0, 150 mM NaCl), 0.1% Tween-20 (TBS-T), 5% (w/v) nonfat dry milk for 30 min at room temperature on an orbital shaker. The membrane was probed overnight at 4°C with primary antibody diluted in TBS-T with 5% (w/v) nonfat dry milk. Rat anti-HA antibody was from Roche (11867423001; 1:1000); rabbit RGS2 antibody was a gift from Dr. David Siderovski (1:2000), rabbit RGS4 antibody was a kind gift from Susan Mumby (1:5000; (Krumins et al., 2004)), p42/44-ERK1/2 antibody was from Cell Signaling (Beverly, MA; 9102; 1:1000) and total ERK1/2 was from Santa Cruz (Santa Cruz, CA; sc-153; 1:1000). Rabbit GAPDH antibody was from Cell Signaling (2118; 1:5000).

The membrane was washed with TBS-T four times, and probed for 1 h at room temperature with horseradish peroxidase-conjugated (HRP) secondary antibody diluted in TBS-T, 5% (w/v) nonfat dry milk. Rabbit anti-rat (A5795; 1:10,000) and goat anti-rabbit (A0545; 1:10,000) antibodies were from Sigma-Aldrich and the goat anti-mouse antibody was from Santa Cruz (sc-2060; 1:20,000). HRP-conjugated anti-actin (Santa Cruz; sc-1615; 1:10,000) antibody was used as loading control. After four washes in TBS-T the protein bands were visualized on autoradiography film using the Super Signal West Pico chemiluminescent substrate (Pierce), and images were scanned and quantified using the ImageJ software (NIH, Bethesda, MD).

### **Quantitative real-time PCR**

RNA was extracted from VSMC using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Following treatment with DNase, 1  $\mu$ g of total RNA was reverse transcribed into cDNA with random hexamers using the cDNA reverse transcription kit (TaqMan; Applied Biosystems, Branchburg, NJ). Quantitative real-time PCR was performed in 20- $\mu$ l reactions containing 1  $\mu$ l of the cDNA sample, and 0.3  $\mu$ M forward and reverse primers with the RT<sup>2</sup> SYBR Green qPCR Master Mix (SABiosciences, Frederick, MD). Primers for RGS2 (Doupnik et al., 2001) and  $\beta$ -actin (Schoenfeld et al., 1998) were described previously. Reaction mixtures were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s. No-template controls and no-RT controls were run during each experiment to detect RNA and/or DNA contamination. Quantification of relative RGS2 mRNA expression levels was determined by the  $\Delta$ Ct method (Livak and Schmittgen, 2001) using  $\beta$ -actin as endogenous control.

### ***In vivo* treatment with digoxin and RGS2 expression in heart.**

All animal protocols are consistent with guidelines from the NIH and the University of Michigan Committee on the Use and Care of Animals which approved all procedures. Male

C57BL/6 (8-13 weeks old) were housed in cages under specific pathogen-free conditions, maintained in a temperature-controlled room with a 12:12 h light/dark cycle and provided with standard chow and water *ad libitum*.

Mini-osmotic pumps (Model 2002, Alzet, Cupertino, CA, USA) with 0.9% saline containing DMSO (0.04%; control) or digoxin (2 µg/kg/day) were incubated overnight in sterile saline at 37°C prior to implantation. Animals were anaesthetized with a mixture of ketamine (100 mg/kg) and xylazine (7.5 mg/kg) and afterwards underwent a small incision midline at the base of the scapula. The skin was retracted, and a small subcutaneous pocket was made for the osmotic mini-pump placement. All pumps were inserted with the flow moderator pointed posteriorly away from the surgical site. After seven days treatment, the animals were euthanized by intra-peritoneal injection of Pentobarbital (100 mg/kg) and tissues removed.

### **Data analysis and statistics**

All data were analyzed using GraphPad Prism 5.0 (GraphPad; LaJolla, CA). Dose response curves were fit using non-linear regression. Datasets with three or more groups were analyzed with one-way ANOVA with Bonferroni's post hoc test for multiple comparisons and datasets with two groups were analyzed using Student's t-test. Data are presented as mean±S.D. and a *p*-value less than 0.05 was considered significant.

## Results

### **RGS2 and RGS4 are degraded through the proteasome**

Pharmacologically increasing RGS2 protein expression (and thereby function) could represent a novel strategy in the treatment of hypertension and heart failure. To identify small molecules that selectively increase RGS2 protein expression, we utilized the PathHunter™ ProLabel  $\beta$ -galactosidase complementation assay. HEK-293 cell clones stably expressing human RGS2 or RGS4 C-terminally tagged with a 4 kDa part of  $\beta$ -galactosidase (hereafter termed RGS2-PL and RGS4-PL) were developed. Since both RGS2 and RGS4 have previously been shown to be degraded through the proteasome (Bodenstein et al., 2007), the cell lines were validated using the proteasome inhibitors MG-132 (Fig. 1A) and lactacystin (Fig. 1B). Both compounds increased the chemiluminescent signal corresponding to RGS2 and RGS4 protein in a concentration-dependent (Fig. 1) and time-dependent (Supplemental Fig. 1) manner. The increased level of RGS2 protein expression after proteasome inhibitor treatment was confirmed using HEK-293T cells transiently transfected with HA-tagged human RGS2 (Fig. 1C). Furthermore, we demonstrate for the first time that endogenous RGS2 protein in VSMC is increased by MG-132 and lactacystin (Fig. 1D).

### **Identification of small molecule enhancers of RGS2 protein expression through high-throughput screening**

The stable HEK-293 cell lines expressing RGS2-PL and RGS4-PL were tested in a small molecule screen utilizing the  $\beta$ -galactosidase complementation assay. In order to assess and correct for toxicity of the compounds, the assay was multiplexed with a fluorescent viability assay (Celltiter Fluor; Promega) in the same well. MG-132 was used as a positive control for RGS up-regulation and two compound libraries, the Microsource Spectrum 2000 and the Biofocus NCC (NIH collection of FDA approved drugs), were screened at a concentration of 10

$\mu$ M (total ~2,900 compounds). Both contain compounds with previously defined pharmacological properties.

Hits in the screen were defined as compounds that increased RGS protein expression >3 S.D. above baseline (approximately 20% or greater). Only one of the 2,900 compounds (thimerosal) affected expression of both RGS2 and RGS4, demonstrating distinct mechanisms of regulation and strong specificity of the assay. Compounds shown to increase the chemiluminescence signal corresponding to RGS2 protein levels in the primary screen were analyzed and divided into groups based on their known pharmacology (Supplemental Table 1). One group of compounds identified was the CTS (i.e. digitalis glycosides). They are known for their ability to inhibit pump function of the Na/K-ATPase and are commonly used in the treatment of atrial fibrillation and congestive heart failure. Given the known role for RGS2 in the cardiovascular system these compounds were chosen for further analysis.

### **The CTS ouabain and digoxin selectively increase RGS2 protein expression**

Two CTS, ouabain and digoxin were chosen for follow-up studies and we initially ascertained the selectivity of these compounds using the PathHunter™ ProLabel assay. Both ouabain and digoxin increase RGS2 protein chemiluminescence in a concentration-dependent manner (Fig. 2). The EC<sub>50</sub> values for increasing RGS2 protein were 32±8 nM and 83±36 nM for ouabain and digoxin, respectively. Neither ouabain nor digoxin increased RGS4 expression (Fig. 2A, B). Their effects on RGS2 protein expression were confirmed by Western blot in HEK-293T cells transiently transfected with RGS2-HA (Fig. 2C). They also increased the level of endogenous RGS2 protein in rat VSMC (Fig. 2D).

In contrast to proteasome inhibitors, ouabain and digoxin did not increase RGS2 protein levels with short term treatment. The CTS-induced effect on RGS2 protein levels was only detected after overnight treatment (Supplemental Figure 2), indicating an indirect mechanism of these compounds on RGS2 protein expression.

### **Posttranscriptional Action of CTS on RGS2**

Based on previous literature (Bodenstein et al., 2007) and our own data (Fig. 1), we hypothesized that ouabain and digoxin increase RGS2 protein expression through a posttranslational mechanism. To examine this model, we considered whether increased transcription might be a factor. It is unlikely that these compounds activate the promoter for RGS2 transcription since RGS4 protein expression was not enhanced in our HEK-293 cell lines where it is under regulation of the same CMV promoter as RGS2. In VSMC, where the endogenous RGS2 promoter drives RGS2 expression, there was no effect on RGS2 mRNA levels by either ouabain or digoxin (both 100 nM) as demonstrated by real-time PCR (Table 1) despite the significant increase in protein level (Fig. 2D). Interestingly, the effects on RGS2 mRNA levels by the two proteasome inhibitors, MG-132 and lactacystin, were divergent. Lactacystin (10  $\mu$ M), which is more specific as a proteasome inhibitor (Fenteany et al., 1995), did not significantly increase RGS2 mRNA levels whereas overnight treatment with MG-132 (10  $\mu$ M) resulted in a 10-fold increase in RGS2 mRNA (Table 1). This increase could explain the difference in the magnitude by which the two proteasome inhibitors increase RGS2 protein levels (MG-132 20-fold vs. lactacystin 10-fold; Fig. 1). Overall, these data support a posttranscriptional mechanism for CTS-induced upregulation of RGS2 protein.

### **RGS2 protein is stabilized by CTS**

To further elucidate the means by which ouabain and digoxin increase RGS2 protein levels, we investigated the effect of these compounds on the cellular half-life of RGS2 protein. RGS2-PL cells were exposed to the protein translation inhibitor cycloheximide with or without pre-treatment with ouabain, digoxin or MG-132 and RGS2 levels were measured at different time points. Under control conditions, RGS2 is rapidly degraded ( $t_{1/2}$  17 $\pm$ 6 min). MG-132 (10  $\mu$ M) completely blocks the degradation of RGS2 (Fig. 4A) consistent with the known

proteasomal pathway for RGS2 degradation (Bodenstein et al., 2007) (Fig. 1). Ouabain and digoxin treatment (both 100 nM) significantly increased the half-life of RGS2 ( $t_{1/2}$  78±43 and 54±26 min, respectively) by a magnitude similar to that of the increase in protein expression in both HEK-293 cells and VSMC (Fig. 4A, B). The increase in RGS2 protein half-life taken together with the lack of effect on RGS2 mRNA levels supports a model whereby CTS increase RGS2 expression through posttranslational protein stabilization.

### **CTS induce increased RGS2 protein expression through actions on the Na/K-ATPase**

Ouabain and digoxin act on the Na/K-ATPase, the major housekeeping ion pump in many cell types (Skou, 1957) but they may have actions on other targets as well (Yeh et al., 2001). To determine the role of Na/K-ATPase in the effects of ouabain and digoxin on RGS2 protein expression, we utilized LLC-PK cells with normal expression of the Na/K-ATPase (AAC-19 line) or with Na/K-ATPase ( $\alpha$ 1) reduced by 90% with stable shRNA (PY-17 line). These cell lines have previously been used to study mechanisms of Na/K-ATPase function (Liang et al., 2006). In AAC-19 cells, both ouabain and digoxin increased endogenous RGS2 protein expression (Fig. 5). Consistent with a mechanism mediated through the Na/K-ATPase, no such increase was observed in the PY-17 cells with reduced Na/K-ATPase expression (Fig. 5). Hence, RGS2 protein expression is stabilized by CTS through a mechanism that is dependent on the Na/K-ATPase.

### **Molecular mechanism of CTS-mediated RGS2 upregulation**

To elucidate whether ouabain and digoxin-mediated stabilization of RGS2 protein is mediated by blockade of the Na/K-ATPase pump function *per se* or if it is related to actions of the Na/K-ATPase to turn on intracellular signaling cascades such as PI-3-kinase, Src, or downstream Ras/Raf/MAPK pathways (Xie and Cai, 2003) we used inhibitors of Src and PI-3-kinase to study effects on RGS2 protein levels. Activation of signal transduction cascades have been shown to



occur at lower (10-100 nM) concentrations of digoxin, similar to those causing RGS2 upregulation. Neither the Src inhibitor PP2 nor the PI-3-kinase inhibitor LY294002 had any effect on basal RGS2 protein levels in HEK-293 cells. Inhibiting either Src or PI-3-kinase had no effect on CTS-induced increase in RGS2 protein levels (Supplemental fig. 3). Blocking effects of CTS on Na/K-ATPase pump function by lowering extracellular K<sup>+</sup> also showed no effects on RGS2 protein upregulation induced by ouabain and digoxin (data not shown). At this point the molecular mechanism of CTS-induced upregulation of RGS2 remains unclear.

### **Increased protein levels of RGS2 have functional effects on GPCR signaling**

To investigate whether digoxin-mediated increases in RGS2 protein expression have functional effects on signaling via GPCRs, HEK-293T cells were transiently transfected with the Gq-coupled M<sub>3</sub> muscarinic receptor alone or in combination with RGS2-HA. Cells were treated with 1 μM of the muscarinic agonist carbachol and agonist-induced ERK1/2 phosphorylation was analyzed by Western blot (Fig. 6). Carbachol stimulation resulted in a strong increase in phosphorylated ERK1/2 which was slightly (but not statistically significant) suppressed in the presence of RGS2. Digoxin treatment (100 nM) had no effect on M<sub>3</sub> receptor signaling in the absence of RGS2. However in the combined presence of RGS2 and digoxin, the carbachol-induced ERK1/2 phosphorylation was significantly reduced, as expected given the increase in RGS2 protein levels (Fig. 6). Hence, increasing RGS2 protein levels by means of pharmacological treatment with digoxin has functional effects on signaling through a Gq-linked GPCR.

### **Digoxin increases RGS2 protein levels in *in vivo*.**

To assess whether pharmacological treatment with digoxin would increase RGS2 protein levels *in vivo*, we treated mice with 2 μg/kg of digoxin daily for 7 days and measured RGS2 protein expression in the heart and other tissues. RGS2 protein levels, compared to the loading

control, were significantly increased in the heart (Fig. 7A). The effect on RGS2 mRNA levels was minimal and not significant (control  $100 \pm 34.5\%$ ; digoxin  $141 \pm 50.9\%$ ), confirming a posttranscriptional mechanism for digoxin-mediated upregulation of RGS2 protein levels. RGS4 protein levels were not significantly changed in heart following digoxin treatment, confirming that CTS selectively stabilize RGS2 over RGS4 (Fig. 7C). Interestingly, RGS2 protein levels were also increased in the kidney (Fig. 7B) where RGS2 has been proposed to exert effects on blood pressure homeostasis (Gurley et al., 2010).

## Discussion

High throughput chemical screens are used to identify new drugs but can also provide mechanistic insights on existing drugs. They can also reveal entirely new therapeutic indications for old drugs in a process termed “repurposing”. In the present study, we use a cell-based high-throughput chemical screen around a new cardiovascular target, the regulator of G protein signaling (RGS) proteins. We tested several thousand known drugs and bioactive molecules for a potential role in enhancing RGS2 and/or RGS4 expression and function and have identified a novel mechanism for digoxin and other cardiotonic steroids. A notable observation in the primary screen was the lack of overlap between the hits at the two RGS proteins screened. Out of almost 3,000 compounds only one increased expression of both RGS2 and RGS4. This was unexpected due to the large number of possible mechanisms that could lead to increased protein expression, including proteasome inhibition and promoter activation. This demonstrates a high degree of specificity in the manner that these two proteins are regulated.

G protein coupled receptors play key roles in the physiology and pathophysiology of cardiovascular disease and heart failure (Brinks and Eckhart, 2010; Hendriks-Balk et al., 2008; Salazar et al., 2007). Consequently, we (Sjögren et al., 2010; Zhong and Neubig, 2001) and others (da Costa Goncalves et al., 2008; Doggrell, 2004; Riddle et al., 2005) have suggested that RGS proteins, including RGS2, could represent novel cardiovascular therapeutic targets. While significant progress has been made in identifying RGS inhibitors (Blazer et al., 2011; Turner et al., 2012), enhancing RGS action is more of a challenge (Sjogren and Neubig, 2010).

Modulating proteasomal degradation of RGS2 and other RGS proteins could be a useful control point at which to enhance RGS2 expression levels and function (Sjogren and Neubig, 2010). The degradation of the closely related RGS4 and RGS5 is mediated by the N-end rule pathway (Bodenstein et al., 2007; Lee et al., 2005) and the specific E1, E2, and E3 ubiquitination enzymes involved in their degradation were recently characterized (Lee et al., 2011). However for RGS2 this pathway is not yet characterized. More detailed information about

the specific enzymes involved in RGS2 protein degradation will be useful in the rational development of drugs that aim to selectively increase RGS2 protein expression. In the current study, we show for the first time in vascular smooth muscle cells that proteasome inhibition as well as ouabain and digoxin markedly enhance endogenous RGS2 protein expression. It is striking that the action of digoxin is selective for RGS2 over RGS4 even *in vivo* which indicates that it is not related to gross inhibition of proteasomal function but that it somehow targets mechanisms related to RGS2 degradation specifically.

The digoxin-induced increase in RGS2 expression is accompanied by an RGS2-dependent reduction in Gq-mediated ERK phosphorylation responses in transfected HEK-293 cells. If this proves to be true also for endogenous RGS2 (i.e VSMC and *in vivo*) it would be expected to suppress signaling by many Gq-coupled receptors such as angiotensin, vasopressin, thromboxane A<sub>2</sub>, thrombin,  $\alpha_1$ -adrenergic, and endothelin. Furthermore, RGS2 has non-G protein-mediated effects such as directly inhibiting cAMP production by the cardiac Type V and VI adenylyl cyclase isoforms (Gu et al., 2008; Salim et al., 2003) and inhibiting protein translation (Nguyen et al., 2009). These could reduce detrimental  $\beta$ -adrenergic receptor signaling, pro-apoptotic, and hypertrophic mechanisms in heart failure. The combination of broad Gq-dependent as well as Gq-independent mechanisms provides several potentially beneficial effects of increased RGS2 protein expression. At this stage we cannot state whether pharmacologically enhanced RGS2 protein expression has functional effects *in vivo*. However, previous studies show that even small changes in RGS2 protein levels (i.e. heterozygous RGS2<sup>+/-</sup> mice) have an impact on endogenous GPCR signaling (Heximer et al., 2003). We therefore predict that digoxin-mediated increased RGS2 protein levels observed in heart and kidney could impact canonical as well as non-canonical RGS2 protein function.

Also, the effects on other components affected by digoxin will need to be investigated. Chronic digoxin treatment, and subsequent increases in RGS2 protein levels, could lead to

altered expression of the Na/K-ATPase and changes in the relative expression and/or function of proteins involved G protein-mediated signaling. These are all possible scenarios that should be investigated but are beyond the scope of our current study.

Our data show that ouabain and digoxin stabilize RGS2 protein through a mechanism that is dependent on the Na/K-ATPase. At this point we cannot say whether the effects are mediated through inhibition of Na/K-ATPase pump function or induction of signaling cascades. Subsequently, at this point it is unclear how these compounds mediate increases in RGS2 protein levels. However, the selectivity over the closely related RGS4 both in vitro and in vivo suggest that it is not related to a global cellular mechanism, but a specific RGS2 related effect, possibly through a decrease in negative regulation of RGS2 protein.

Since adverse effects related to excessive pump inhibition may contribute to the loss of survival benefit at higher plasma concentrations (and doses) of digoxin in the therapy of heart failure (Ahmed and Waagstein, 2009), it will be important to understand the relative structure-activity-relationships (SAR) for blocking ion transport, inducing signaling pathways, and modulating RGS2 expression. A divergence in SAR among those effects could lead to the discovery of less toxic compounds that might have selective actions to induce RGS2 protein increases. Indeed, a CTS analog ouabain inhibitor, rostafuroxin, is in clinical trials for hypertension (Lanzani et al., 2010).

It has long been known that the rodent Na/K-ATPase display far lower sensitivity to CTS than its human counterpart (Detweiler, 1967). In light of this it may be surprising that equal concentrations of ouabain and digoxin produced similar effects of RGS2 protein levels in HEK-293 cells and rat VSMC. Despite this, several studies have demonstrated effects on the rodent Na/K-ATPase at very low concentrations of CTS. Abramowitz et al. showed that 100nM Ouabain induced a significant increase in rat VSMC proliferation (Abramowitz et al., 2003). Furthermore, VSM express both high and low affinity Na/K-ATPase and very low concentrations (<1nM) can modulate aortic VSM contraction (Weiss et al., 1993). It is not clear whether these

effects are a direct result of ouabain inhibiting Na/K-ATPase pump function or by inducing signal transduction cascades. It has however been suggested that acute effects of CTS (5-10 min) require much higher concentrations ( $\mu\text{M}$ ) than the long term effects (hours-days; nM) that are observed in the present study (Quintas et al., 2010).

CTS effects to increase RGS2 expression may influence other aspects of cardiovascular and non-cardiovascular function. RGS2<sup>-/-</sup> mice show enhanced M<sub>3</sub> receptor responses in the atrium and are more susceptible to atrial arrhythmias (Tuomi et al., 2010). Since we found that digoxin causes an RGS2-dependent suppression of M<sub>3</sub> receptor signaling (Fig. 6), this could have relevance in atrial fibrillation, the best accepted clinical indication for digoxin. The story on hypertension is more complex. Since low RGS2 levels in mice causes hypertension (Heximer et al., 2003) and heterozygous destabilizing mutations in RGS2 are found selectively in hypertensive humans (Bodenstein et al., 2007; Yang et al., 2005), one would predict that increased RGS2 protein levels from CTS treatment could ameliorate high blood pressure. However, endogenous ouabain has long been identified as a pro-hypertensive factor (Bagrov et al., 2009), though this is controversial (Nicholls et al., 2009). Also, genetic alterations in Na,K-ATPase have opposite effects on blood pressure in different models (Hou et al., 2009; Rindler et al., 2011). Consequently, the actions of ouabain and other CTS on blood pressure are probably multifactorial with contributions from pump inhibition, effector signaling, and now the increases in RGS2 expression described here. More studies will be needed to fully understand this topic.

Outside of the cardiovascular system, CTS are being considered as novel cancer therapies. RGS2 protein levels are downregulated in androgen-independent prostate cancer cells and in clinical specimens (Cao et al., 2006). Overexpression of RGS2 in LNCaP cells suppressed cell growth, whereas silencing RGS2 had the opposite effect (Cao et al., 2006). In light of our present data, there may be a role for RGS2 in these effects of CTS in prostate cancer models.

In summary, we have developed an approach to identify compounds or treatments that enhance RGS protein expression and function. Furthermore, we report the novel observation that digoxin and other CTS selectively increase RGS2 protein expression *in vitro* and *in vivo*. This may represent an element of the mechanism of beneficial effects of CTS treatment in heart failure. A clear assessment of the relative *in vivo* contribution of RGS2 regulation vs. other actions of CTS will be needed in future work. Furthermore, our data provide proof-of-principle for the concept of increasing RGS2 activity as an approach to the treatment of heart failure and hypertension. Digoxin has been shown to have clinical benefits in heart failure even in the context of ongoing angiotensin converting enzyme inhibitor treatment (Ahmed and Waagstein, 2009); it is plausible that increased RGS2 protein expression could be a factor. More detailed *in vivo* studies will be needed to investigate this possibility.

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## **Authorship contributions:**

Participated in research design: Sjogren, Parra, Heath, Xie, Neubig

Conducted experiments: Sjogren, Parra, Heath

Performed data analysis: Sjogren, Parra, Heath, Xie, Neubig

Contributed new reagents and analytical tools: Atkins, Xie

Wrote or contributed to the writing of the manuscript: Sjogren, Parra, Atkins, Xie, Neubig



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## Footnotes

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## Figure Legends

**Figure 1. RGS2 and RGS4 are degraded by the proteasome.** HEK-293 cells transfected with either RGS2 or RGS4 c-terminally tagged with the 4kDa ProLabel tag were treated with the proteasome inhibitors MG-132 (**A**) or lactacystin (**B**) overnight at 37°C. Both inhibitors increase RGS2 and RGS4 chemiluminescence in a dose-dependent manner. These results were confirmed with western blot in HEK-293 cells transfected with HA-tagged RGS2 (**C**) and endogenously expressed RGS2 protein in vascular smooth muscle cells (**D**). **E.** The specificity of the RGS2 antibody was confirmed with Western blot using heart tissue from wild-type and RGS2<sup>-/-</sup> mice. This antibody has also previously been published (Takimoto et al., 2009). \* $p < 0.05$ ; \*\*\* $p < 0.001$  using one-way ANOVA with Bonferroni's post hoc test for multiple comparisons.

**Figure 2. Ouabain and digoxin selectively increase RGS2 protein expression.** Ouabain (**A**) and digoxin (**B**) increases RGS2 protein expression in stably transfected HEK-293 cells in a concentration-dependent manner as demonstrated by the PathHunter<sup>TM</sup> ProLabel assay. The effect is selective over the closely related RGS4 as neither compound increases RGS4 protein expression using the same assay system. **C.** Western blot showing increased RGS2 protein expression by ouabain and digoxin in transiently transfected HEK-293T cells. **D.** Ouabain and digoxin increases endogenous RGS2 protein expression in rat primary aortic VSMC as demonstrated by Western blot. All treatments were overnight. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  using one-way ANOVA with Bonferroni's post hoc test for multiple comparisons.

**Figure 3. RGS2 protein is stabilized by ouabain and digoxin.** HEK-293 cells stably transfected with RGS2-PL were treated overnight with 100 nM ouabain, 100 nM digoxin or 10  $\mu$ M MG-132. Following the treatment cycloheximide (10  $\mu$ g/ml) was added to block protein translation at different time points to determine RGS2 protein half-life. **A.** Chemiluminescence

corresponding to protein levels of RGS2 was detected with the PathHunter™ ProLabel assay. The half-life of RGS2 under control conditions is  $17.5 \pm 5.8$  min. Both ouabain and digoxin increases the half-life of RGS2 to  $77.9 \pm 43.4$  and  $54.4 \pm 26.1$  min, respectively (quantified in **B**). Treatment with MG-132 completely stabilizes RGS2 expression.  $*p < 0.05$ ;  $**p < 0.01$  using one-way ANOVA with Bonferroni's post hoc test for multiple comparisons.

**Figure 4. CTS increase RGS2 protein expression through actions on the Na/K-ATPase.**

Endogenous RGS2 protein expression was detected LLC-PK cells after overnight treatment with 100 nM ouabain or 100 nM digoxin. In cells with normal expression of the Na/K-ATPase (AAC-19) the ouabain/digoxin-induced increase in RGS2 protein expression is maintained. This increase is lost in cells where the Na/K-ATPase levels have been reduced by shRNA (PY-17; Na/K-ATPase null). **A**. Representative Western blot from 4 experiments. **B**. Quantification of RGS2 levels normalized to an actin loading control.  $**p < 0.01$ ;  $***p < 0.001$  using two-way ANOVA with Bonferroni's post hoc test for multiple comparisons.

**Figure 5. Functional effect of increased RGS2 protein expression on GPCR signaling.**

HEK-293T cells were transiently transfected with the  $M_3$  muscarinic receptor alone or together with RGS2-HA. 48 h after transfection carbachol-induced ERK1/2 phosphorylation was measured by Western blot with or without overnight pre-treatment with digoxin. Carbachol induces strong phosphorylation of ERK1/2 after 10 min. In the presence of RGS2-HA there is a trend towards suppression of the signal which is significantly enhanced in the presence of 100 nM digoxin ( $*p < 0.05$  using one-way ANOVA with Bonferroni's post hoc test for multiple comparisons). In cells with no RGS2-HA digoxin has no effect on carbachol-induced ERK1/2 phosphorylation. The response in the presence of RGS2 is significantly different between digoxin-treated and non-treated using Student's paired t-test ( $*p < 0.05$ ).

**Figure 6. Up-regulation of RGS2 protein levels by digoxin *in vivo*.** Male C57BL/6 mice were treated with either vehicle or 2 µg/kg digoxin for 7 days via osmotic minipumps. Levels of RGS2 protein expression in tissue were then analyzed by Western blot. Treatment with digoxin induced a significant increase in RGS2 protein immunoreactivity in both heart (**A**) and kidney (**B**) compared to control. In contrast, the closely related RGS4 showed no increase in heart after digoxin treatment (**C**). Representative blots and quantification from 8 animals in each group.

\*\* $p < 0.01$ ; \*\*\* $p < 0.001$  using Student's unpaired t-test.

## Tables

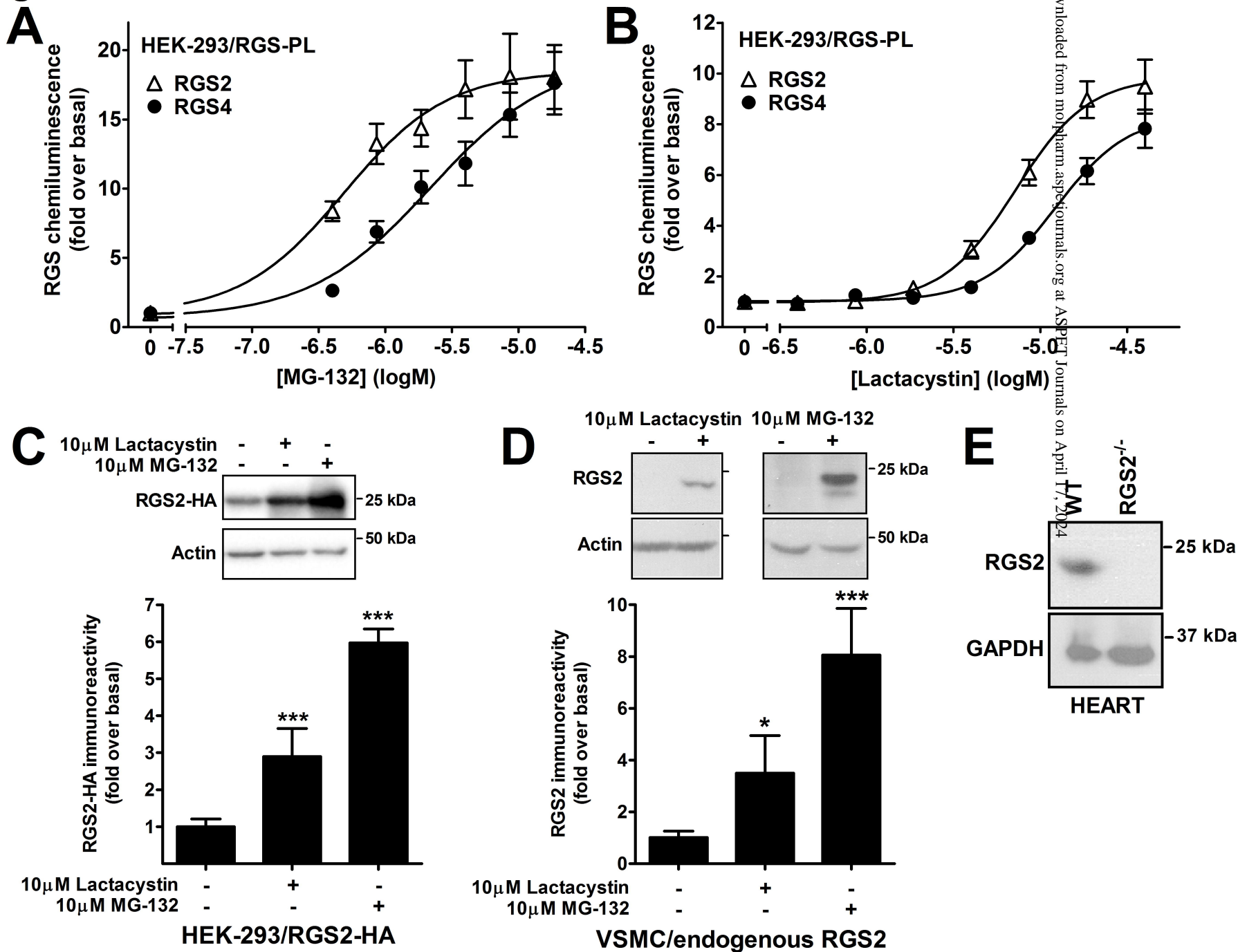
**Table 1. RGS2 mRNA expression in vascular smooth muscle cells**

mRNA levels of endogenously expressed RGS2 was determined using RT-PCR. Only overnight treatment with 10 $\mu$ M MG-132 has significant effects on RGS2 mRNA expression.

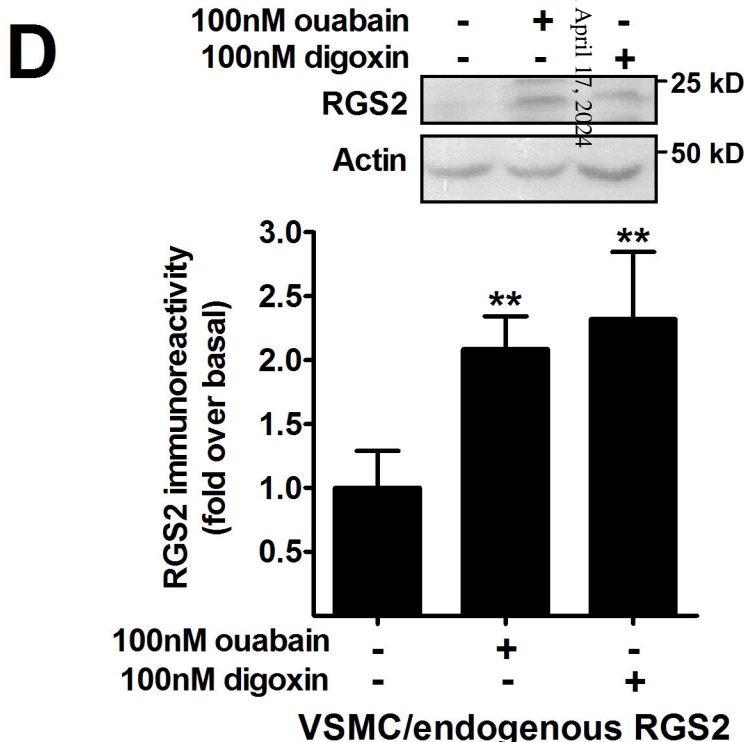
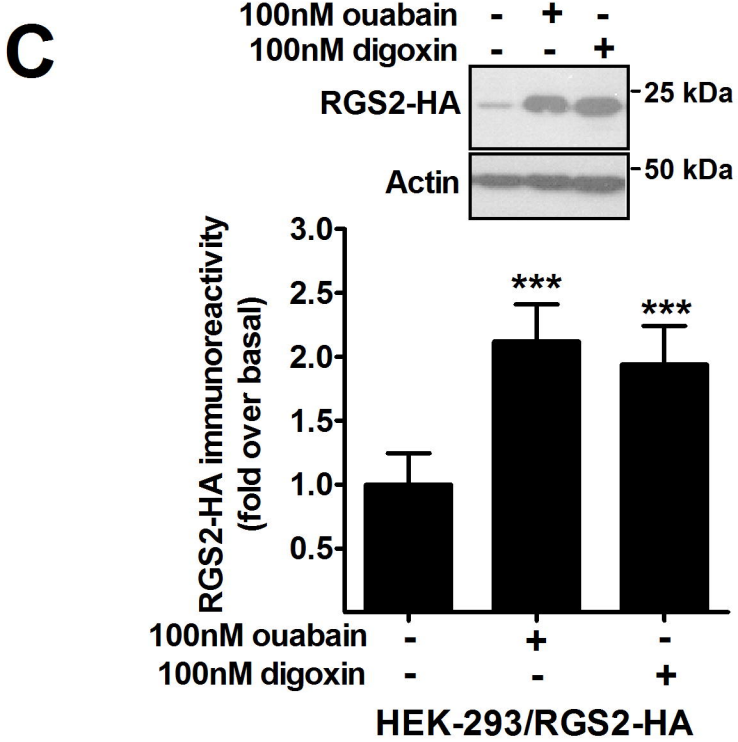
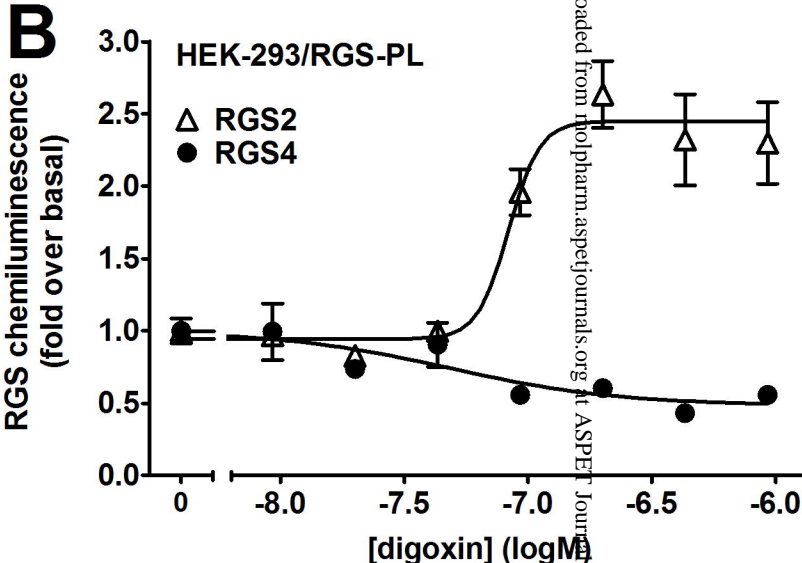
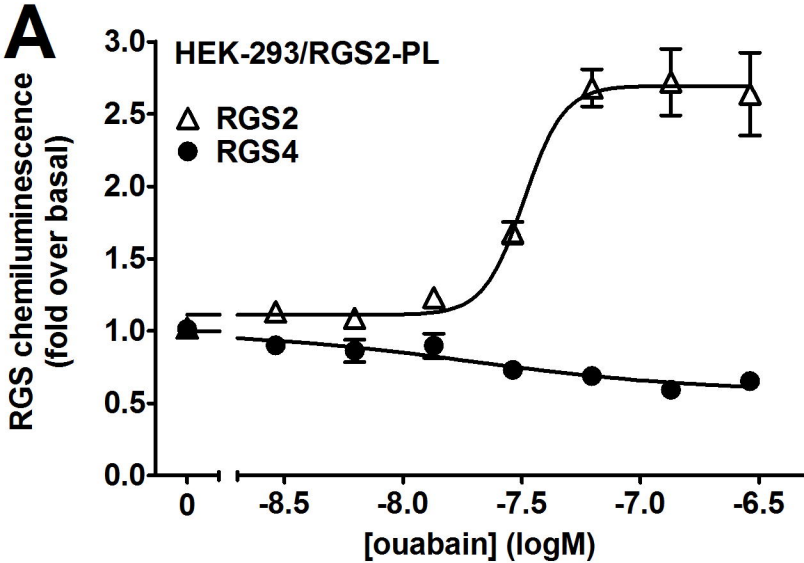
| Treatment              | RGS2 mRNA (fold vs. control) |                     |
|------------------------|------------------------------|---------------------|
|                        | 3h                           | o/n                 |
| 100nM digoxin          | -                            | 1.31 $\pm$ 0.71     |
| 100nM ouabain          | -                            | 0.91 $\pm$ 0.17     |
| 10 $\mu$ M lactacystin | 0.80 $\pm$ 0.06              | 1.94 $\pm$ 1.92     |
| 10 $\mu$ M MG-132      | 0.56 $\pm$ 0.15              | 10.07 $\pm$ 5.12*** |

\*\*\* $p$ <0.001 using one-way ANOVA with Bonferroni's post hoc test for multiple comparisons.

**Figure 1**



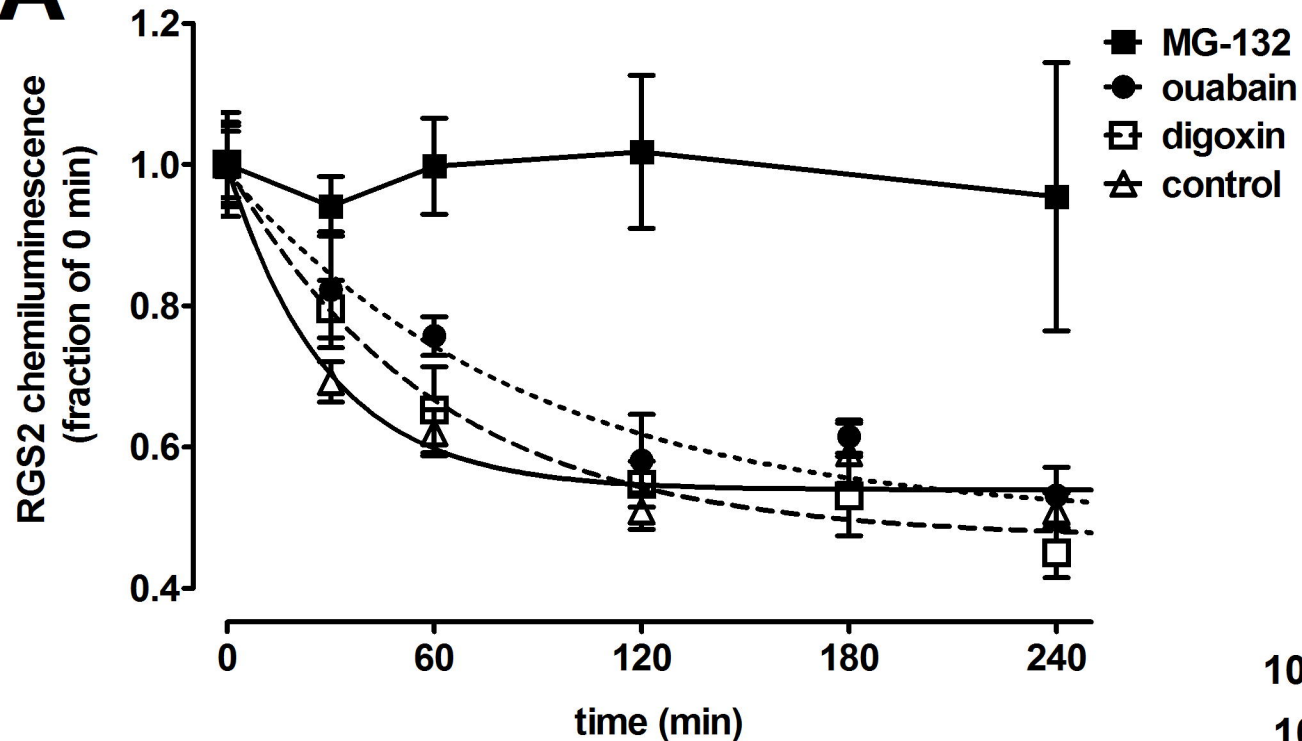
**Figure 2**



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Figure 3

**A**



**B**

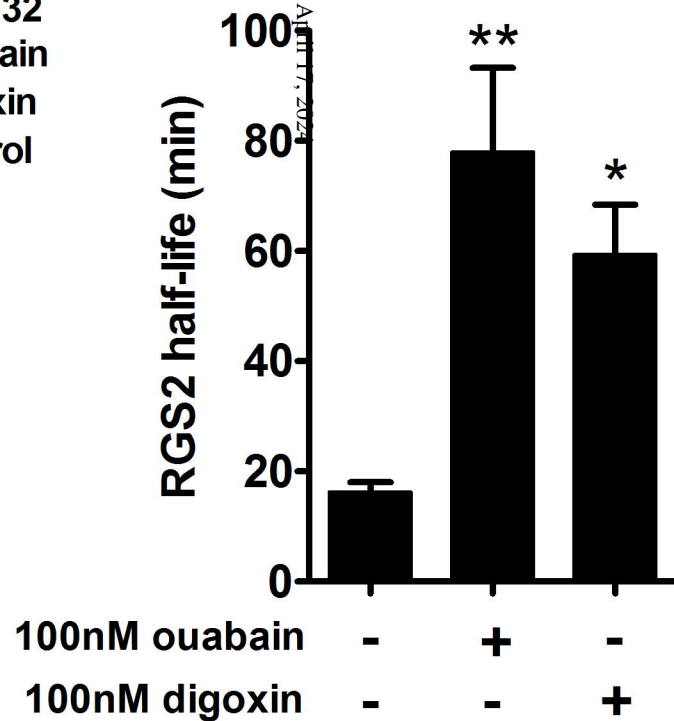
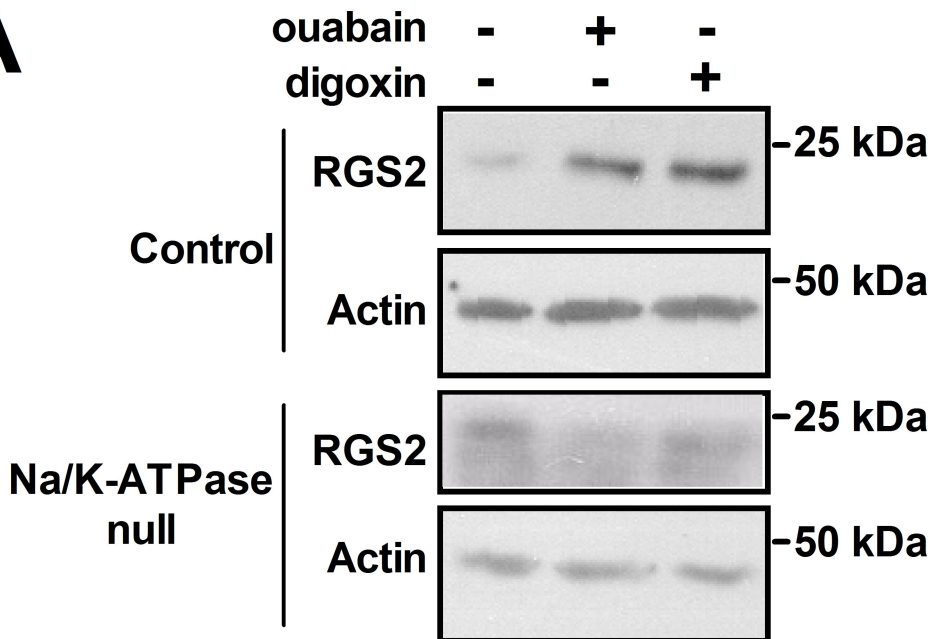
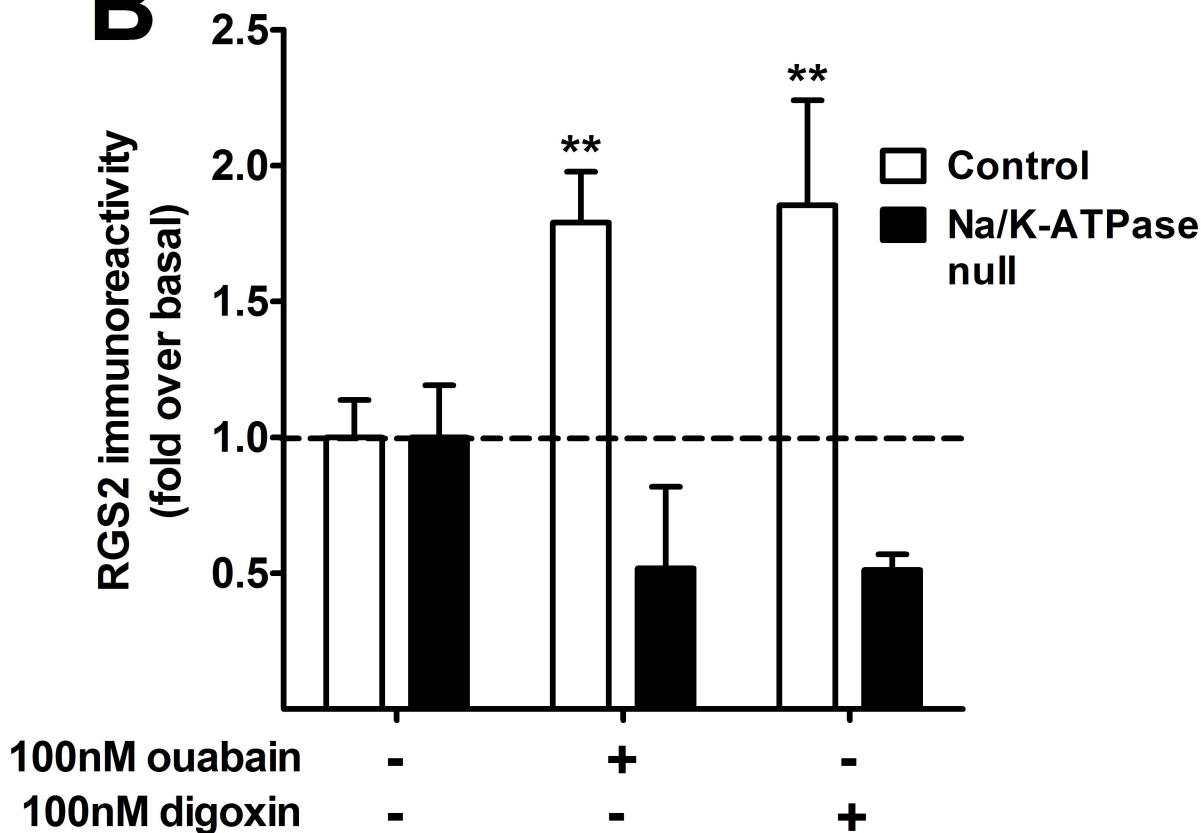


Figure 4

**A**



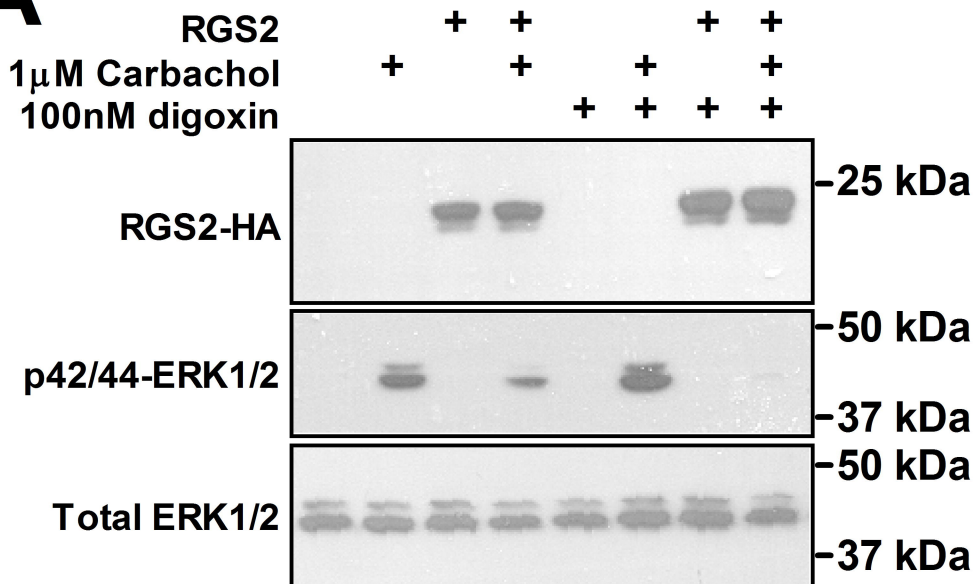
**B**



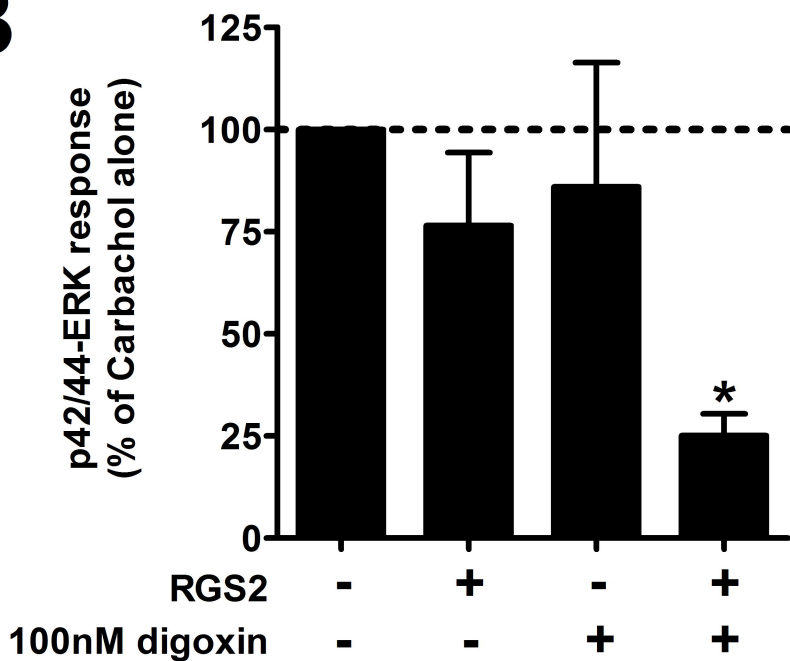


# Figure 5

## A

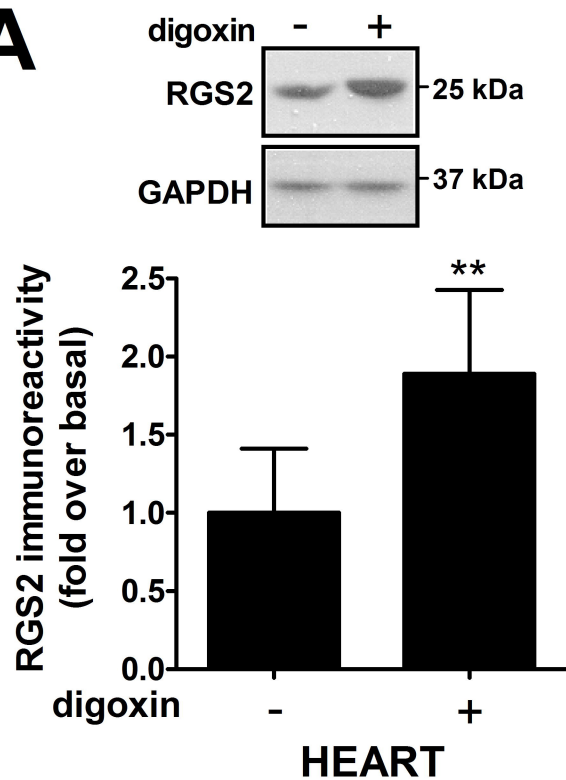


## B

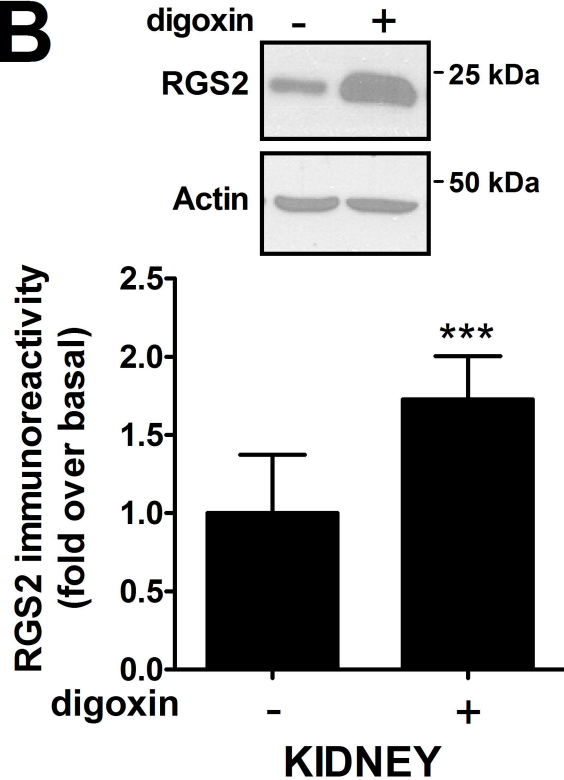


**Figure 6**

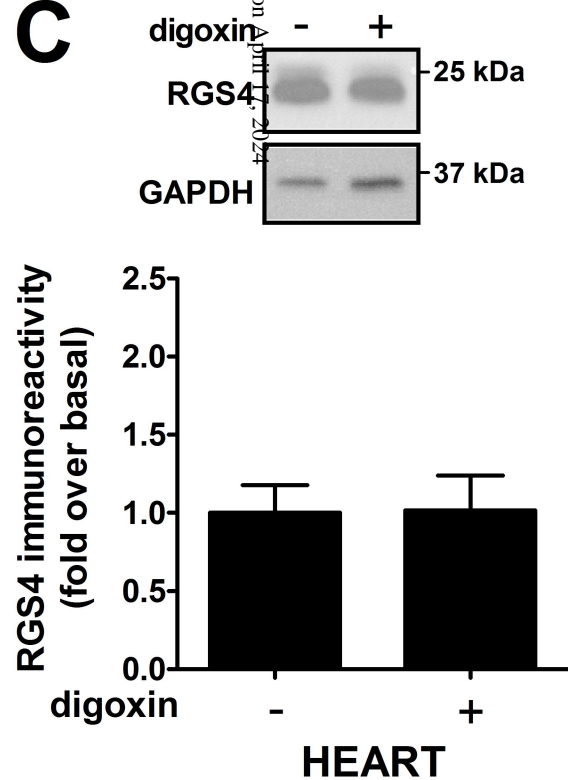
**A**



**B**



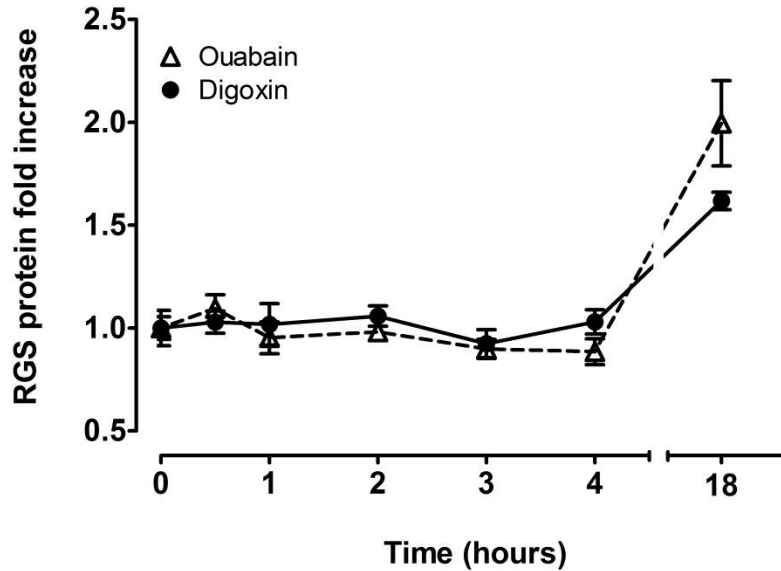
**C**



## Cardiotonic steroids stabilize RGS2 protein levels

*B. Sjögren, S. Parra, L. J. Heath, K. B. Atkins, Z-J. Xie, R. R. Neubig*

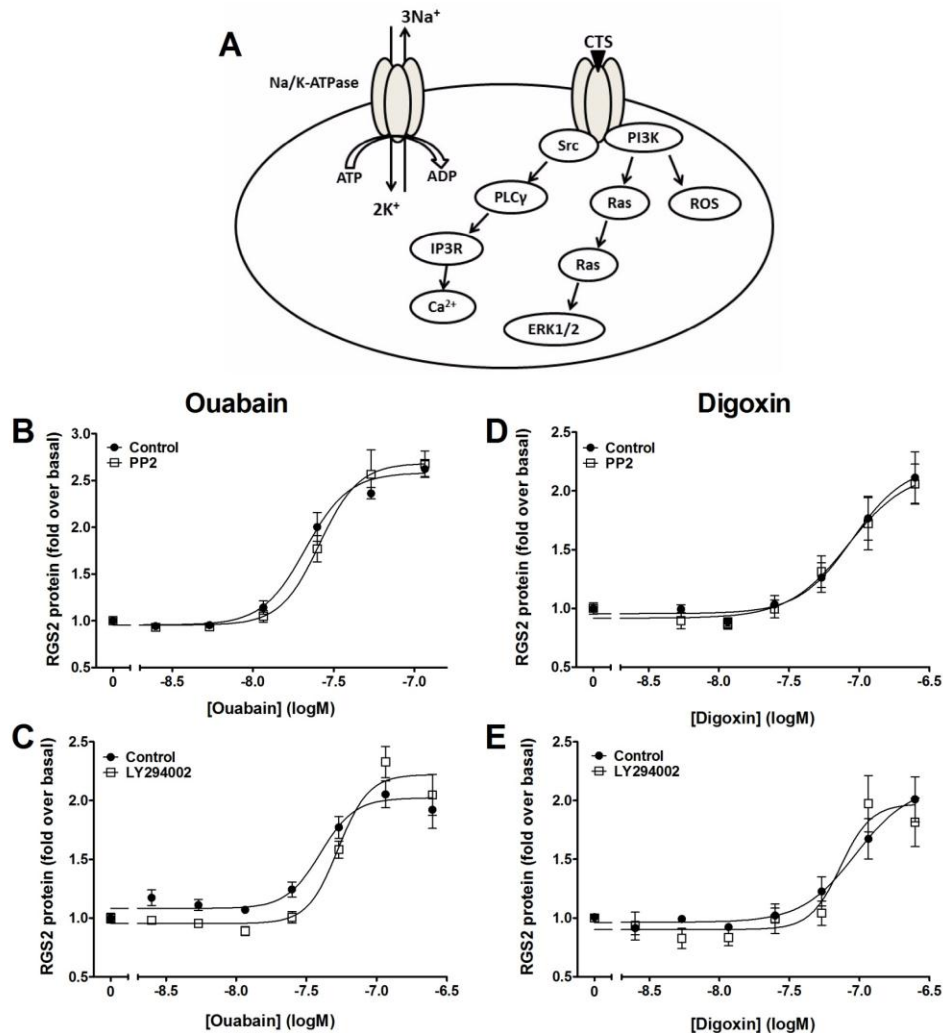
### Supplemental Figure 2



**Supplemental figure 1. Time-dependent increase of RGS2 protein expression by ouabain and digoxin.** HEK-293 cells stably transfected with RGS2-PL were analyzed using the PathHunter™ ProLabel assay. 100nM ouabain or digoxin was added at indicated time points. Only overnight (18h) treatment with these CTS results in increased RGS2 protein levels. Results from two experiments run in quadruplicates.

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**Supplemental figure 3. Ouabain and digoxin do not increase RGS2 protein levels through actions of Src or PI-3-kinase.**

**A.** Schematic of cellular events mediated through the Na/K-ATPase. CTS are known to inhibit pump function as well as initiating signal transduction cascades mediated through Src and PI-3-kinase (Adapted from Liu J and Xie ZJ, 2010; *Biochim Biophys Acta* **1802**(12):1237-1245). **B-E.** HEK-293 cells stably transfected with RGS2-PL or RGS4-PL were analyzed using the PathHunter™ ProLabel assay. Cells were pre-treated for 1h with the Src inhibitor PP2 (3 $\mu$ M; **B, D**) or the PI-3-kinase inhibitor LY294002 (10 $\mu$ M; **C, E**) followed by overnight treatment with increasing concentrations of ouabain (**B, C**) or digoxin (**D, E**). Neither inhibitor had any effect on CTS-induced RGS2 protein level increase. Each graph represents an average of three experiments run in triplicate.

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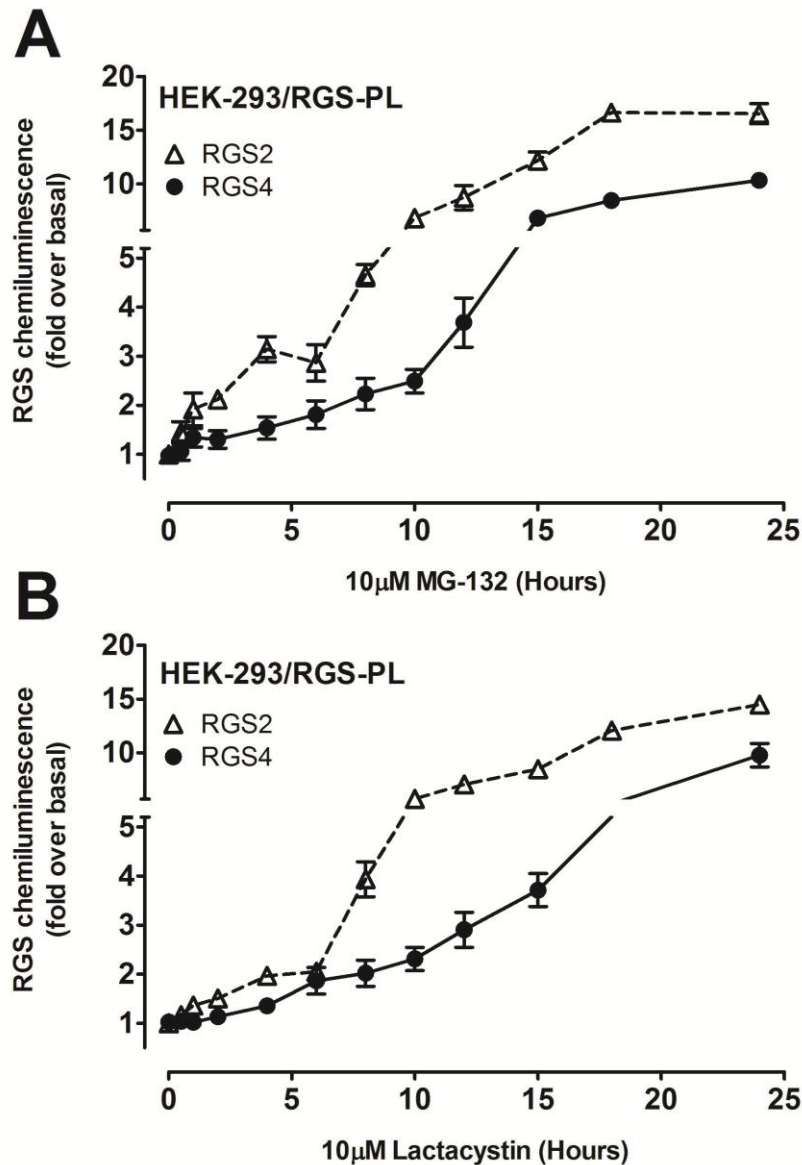
**Supplemental Table 1.** Summary of hits from high-throughput screen of the Microsource Spectrum 2000 and the Biofocus NCC (NIH collection of FDA approved drugs) for compounds that increase RGS2 protein expression. The hits from the RGS2 screen can be divided into categories based on their previously known pharmacology. Note that digoxin was not a primary hit but was chosen for follow-up based on similarity to other CTS and that the majority of hits have not been confirmed in follow-up studies.

| <b>NAME</b>               | <b>Viability<br/>(% of control)</b> | <b>RGS2 increase<br/>(% above control)</b> | <b>DRUG CLASS/USES</b>                            |
|---------------------------|-------------------------------------|--|---|
| Gitoxin                   | 103.1                               | 81.9                                       | Cardiotonic<br>steroids                           |
| Sarmentogenin             | 95.1                                | 59.4                                       |   |
| Ouabain                   | 90.1                                | 51.1                                       |   |
| Gitoxigenin               | 90.1                                | 40.4                                       |   |
| Lanatoside C              | 92.3                                | 26.5                                       |   |
| Vinblastine               | 90.6                                | 83.3                                       | Chemotherapy                                      |
| Mercaptopurine            | 98.2                                | 59.7                                       |   |
| 5-Azacytidine; Vidaza     | 80.2                                | 48.2                                       |   |
| Retusoquinone             | 79.4                                | 38.1                                       |   |
| Camptothecin              | 107.5                               | 29.9                                       |   |
| Teniposide                | 97.2                                | 26.8                                       |   |
| Mesna                     | 93.3                                | 22.8                                       |   |
| Crinamine                 | 109.5                               | 40.8                                       | HIV-1<br>Protease<br>inhibitors                   |
| Nelfinavir Mesylate       | 87.3                                | 28.5                                       |   |
| Indinavir Sulphate        | 101.2                               | 28.3                                       |   |
| 3-Hydroxyflavone          | 77.0                                | 26.1                                       | Flavonoids  |
| Acacetin Diacetate        | 76.6                                | 25.7                                       |   |
| Mundulone                 | 95.6                                | 25.1                                       |   |
| Deguelin                  | 104.9                               | 24.0                                       |   |
| Megestrol                 | 89.5                                | 29.3                                       | Synthetic Steroid hormones                        |
| Ethinyl Estradiol         | 111.1                               | 24.8                                       |   |
| Metaproterenol            | 95.2                                | 24.7                                       | Corticosteroids<br>Asthma/Allergy                 |
| Fluticasone<br>Propionate | 93.7                                | 21.4                                       |   |
| Flumethazone<br>Pivalate  | 102.5                               | 20.3                                       |   |
| Tacrolimus                | 98.7                                | 30.4                                       |   |
| Thimerosal                | 68.3                                | 47.2                                       | Antiseptic agent                                  |
| Monesin A                 | 69.1                                | 31.3                                       | Antibiotic  |
| Mexamine                  | 89.2                                | 24.8                                       | Serotonin derivative                              |
| Nisoldipine               | 87.0                                | 24.1                                       | Hypertension;<br>Ca <sup>2+</sup> channel blocker |
| Dichlorophene             | 92.4                                | 23.7                                       | Antimicrobial agent                               |
| Hydroxychloroquine        | 92.6                                | 22.5                                       | Malaria   |
| Flufenamic Acid           | 90.8                                | 21.3                                       | NSAID   |

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**Supplemental Figure 1**



**Supplemental figure 1. Time-dependent increase of RGS2 and RGS4 protein expression by proteasome inhibitors.** HEK-293 cells stably transfected with RGS2-PL or RGS4-PL were analyzed using the PathHunter™ ProLabel assay. 10µM MG-132 or Lactacystin was added at indicated time points. Both proteasome inhibitors increase both RGS2 and RGS4 protein chemiluminescence in a time-dependent manner. The difference in magnitude between the two proteins could be due to the fact that single clones were used. Results from two experiments run in quadruplicates.