JPET Fast Forward. Published on December 19, 2006 as DOI: 10.1124/jpet.106.114835

Role of Hsp90 and Protein Degradation in Regulating Neuronal Levels of GRK3[1]

Samina Salim and Douglas C. Eikenburg

Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy,

University of Houston, Houston, TX 77204-5037

JPET Fast Forward. Published on December 19, 2006 as DOI: 10.1124/jpet.106.114835
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Regulation of GRK3 degradation

Corresponding author:

Douglas C. Eikenburg, Ph.D

Associate Professor

Department of Pharmacological and Pharmaceutical Sciences

College of Pharmacy, University of Houston

Houston, TX 77204-5037

Telephone: 713-743-1217 FAX: 713-743-1229

E-Mail: deikenburg@uh.edu

Number of Text pages: 12 Number of Tables: 0

Number of figures: 6 Number of References: 27

Word count:

Abstract: 250 Introduction: 512

Discussion: 1436

Abbreviations: G-protein coupled receptor kinases (GRK), Norepinephrine (NE), Epinephrine (EPI), adrenoceptor (AR), cyclic adenosine monophosphate (cAMP), human neuroblastoma cells (BE(2)-C cells), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Polyvinylidene Difluoride (PVDF), G-protein coupled receptors (GPCRs), Heat-shock protein 90 (Hsp90), Lactacystin (Lact), N-acetyl-l-leucyl-l-leucyl-l-norleucinol (ALLN), Geldanamycin (GA), Central nervous system (CNS).

Section: Cellular and Molecular

JPET Fast Forward. Published on December 19, 2006 as DOI: 10.1124/jpet.106.114835
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Cellular levels of GRK3 determine the sensitivity of the alpha$_{2A/B}$-adrenoceptor (α_2-AR) to agonist-induced down-regulation. Using human neuroblastoma BE(2)-C cells, this study examines how cellular GRK3 levels are affected by several mechanisms reported to influence stability and degradation of other GRKs. We first examined the interaction between the heat shock protein 90 (Hsp90) and GRK3; Hsp90 reportedly affects the maturation and stability of GRK2. In unstimulated cells, GRK3 co-immunoprecipitates (Co-IP) with Hsp90, suggesting a physical interaction. Moreover, when GRK3 protein expression was increased by 24h epinephrine (EPI) treatment, Hsp90 protein expression increased with a similar but slightly delayed time course. To investigate the influence of Hsp90 on GRK3 protein stability, we determined the effect of the Hsp90 inhibitor, geldanamycin (GA) on cellular GRK3 levels. GA eliminated the interaction between Hsp90 with GRK3 and produced a rapid, proteosome-mediated, 70% decrease in GRK3 levels within 24h. To investigate the influence of Hsp90 on up-regulation of GRK3 expression, we examined the effect of GA on EPI-induced up-regulation. GA reduced the absolute increase in GRK3; however, the percent increase in GRK3 by EPI was not significantly different in the absence vs. presence of GA (141±41% vs. 94±12%). Finally, we examined the influence of Ca^{+2}-activated proteases on cellular GRK3. Treatment with the calcium ionophore, ionomycin produced a rapid decrease in GRK3 levels that was inhibited by the calpain inhibitor, calpeptin. In conclusion, several mechanisms influence the degradation of GRK3, and therefore have the potential to affect GPCR signaling by regulating GRK3 levels in neurons.

INTRODUCTION

G-protein-coupled receptor kinases (GRKs) specifically interact with agonist-occupied G-protein-coupled receptors (GPCRs) to promote receptor phosphorylation. Receptor phosphorylation impairs receptor signaling by recruiting arrestins, uncoupling the receptor from the G-protein and promoting endocytosis of the receptor (Penn et al., 2000; Penela et al., 2006).

The GRKs are classified into three families. The GRK2/3 family is distributed through out the body and the central nervous system (CNS) and influences the function of many GPCRs (Penn et al., 2000; Penela et al., 2006). Within this family, GRK2 is generally expressed at higher levels in most tissues than is GRK3, and considerably more attention has been directed toward the function, regulation and role of GRK2 in cell signaling, almost to the complete exclusion of GRK3. However, recent evidence suggests that GRK3 may be particularly important in the regulation of signaling for specific GPCRs (Dautzenberg et al., 2001; Bawa et al., 2003; Desai et al., 2004; McLaughlin et al., 2004; Desai et al., 2005). For example, relatively small changes in GRK3 expression profoundly affect α_2-AR function and preferentially regulate neuronal α_{2A} - and α_{2B}-AR signaling (Bawa et al., 2003; Desai et al., 2004). In addition, CRF and dynorphin have been reported to cause selective up-regulation of GRK3 expression in cells expressing CRF-1 (Dautzenberg et al., 2001) and κ-opioid (McLaughlin et al., 2004) receptors, respectively, and inactivation of GRK3, but not GRK2, impairs the desensitization of α_2- and CRF-1 receptors (Dautzenberg et al., 2001; Desai et al., 2004)). Additionally, a single nucleotide polymorphism (SNP) in the putative promoter region of the human GRK3 gene has been reported (Niculescu et al., 2000; Barrett et al., 2003).

Lymphocytic cell lines derived from bipolar disorder (BPD) patients carrying this polymorphism exhibit a selective decrease in GRK3 but not GRK2 expression and the reduced levels of GRK3 are inversely related to the severity of patient symptoms (Niculescu et al., 2000).

There have been several reports describing mechanisms that regulate the synthesis, localization and degradation of GRK2 causing alterations in GPCR signaling. For example, Hsp90 has recently been reported to serve as a cellular chaperone for GRK2, participating in the maturation of newly synthesized GRK2 protein. Hsp90 also is reported to bind to and stabilize GRK2, limiting degradation of the GRK2 protein (Luo and Benovic, 2003). Finally, stimulation of GRK2 degradation has been reported to play an important role in regulating GPCR signaling in the immune system. Specifically, activation of lymphocytes leading to increased intracellular calcium results in activation of calpains, causing increased degradation of GRK2. Decreased GRK2 levels result in reduced cytokine receptor desensitization (Lombardi et al., 2002). To date, similar information is not available regarding the regulation of GRK3.

Given the important role that GRK3 plays in regulating the signaling of α_2-AR (Desai et al., 2004; Desai et al., 2005), CRF-1 (Dautzenberg et al., 2001) and κ-opioid (McLaughlin et al., 2004) receptors, and the data suggesting an association between reduction of GRK3 expression and BPD, the present study was initiated to determine the role of Hsp90, proteosomal degradation and calpain activation in the regulation of the levels of GRK3 in neuronal cells.

MATERIAL AND METHODS

Material: The following were purchased from the sources indicated:

(-) Epinephrine bitartrate (EPI), phenylmethylsulphonylflouride (PMSF), Dulbecco's Modified Eagles medium (DMEM), F12-DMEM, sodium metabisulphite, theophylline, (N-[2-Hydroxyethyl]piperazine-N`-[2-ethanesulfonic acid]) (HEPES), bovine serum albumin and poly-l-lysine hydrobromide, p-coumaric acid, luminol sodium salt, ALLN, ionomycin, lactacystin, hydrogen peroxide 30% (w:w) solution (Sigma Chemical Co., St. Louis, MO); fetal bovine serum and penicillin-streptomycin (Atlanta Biological, Norcross, GA); TEMED, Pre-stained SDS-PAGE protein marker (cat. # 1610324) and ammonium persulphate (BioRad); geldanamycin ([18S-(4E,5Z,8R*,9R*,10E,12R*,13S*,14R*,16S*)]- 9- [(aminocarbonyl)oxy]- 13-hydroxy- 8,14,19- trimetoxy- 4,10,12,16- tetramethyl- 2-azabicyclo[16.3.1.]docosa- 4,6,10,18,21- pentan- 3,20,22trion) (Biomol, Plymouth Meeting, PA); calpeptin (Calbiochem, San Diego, CA); anti-GRK3 rabbit IgG (cat # sc-563), goat anti-rabbit IgG HRP (cat # sc-2301), goat antimouse IgG HRP (cat # sc-2302), ProteinA/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA); and mouse anti-rabbit GAPDH IgG (cat # RDI-TRK4G4C5) (Research Diagnostic Inc, NJ). BE(2)-C cells were obtained from Dr. Kelly M Standifer, University of Houston, Houston, TX.

Cell Culture: The BE(2)-C cells were maintained in a humidified atmosphere (6% CO_2:94% air) in a 1:1 mixture of Eagle's minimal essential medium with non-essential amino acids and Ham's F12 medium supplemented with 10 % fetal bovine serum, 100 U/ml penicillin G and 0.1 mg/ml streptomycin sulfate. Throughout the study EPI-treatment experiments were initiated when cells reached 70-80% confluence.

Pretreatment: BE(2)-C cells were pretreated with vehicle (medium containing 0.1 mM Ascorbate and 1 μM Sodium metabisulphite), or vehicle containing 0.3 μM EPI for 1h-24h for GRK3 protein up-regulation time course experiments. Ascorbate and Sodium metabisulphite are antioxidants included to minimize autoxidation of EPI during the treatments. Inclusion of these agents is standard procedure for experiments involving treatment with catecholamines.

Proteosomal Inhibitor Experiment: BE(2)-C cells were treated with/without GA (10μM) for 24h followed by treatment with either ALLN (50μM) or lactacystin (8μM) for 6h. The cells were washed with PBS, harvested in hypotonic lysis buffer, subjected to SDS-PAGE and probed with anti-GRK3 antibody.

Ionomycin Treatment: BE(2)-C cells were treated with Ionomycin (0.1μM) for 4h. In some cases Calpeptin (100μM) was present 1h prior to and during ionomycin/vehicle treatment. The cells were washed with PBS, harvested in hypotonic lysis buffer, subjected to SDS-PAGE and probed with anti-GRK3 antibody.

Western blot analysis: Cells were washed once with 1X PBS (pH 7.4), lysed immediately in 100-200 μl of hypotonic lysis buffer (50 mM Tris-HCl pH 7.4, 4 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin) followed by 5-6 passes through a 23-gauge needle and subsequently centrifuged at 1000 rpm for 10 min to remove cellular debris and nuclei. The protein concentrations of the lysates were determined using a Pierce protein detection kit (Pierce, Rockford, IL Cat# 232009) using BCA protein assay reagent A and reagent B.. The cell lysates were diluted with 4X Laemmli buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1 mg/ml bromophenol blue), resolved by SDS-PAGE

(10% gel) and the proteins transferred to PVDF membranes (Amersham Pharmacia Biotech, Buckinghamshire, England). The levels of GRK3 were determined by immunoblotting using anti-GRK3 antibody (1:1000). We have previously used this antibody to selectively detect GRK3 in samples containing both GRK2 and GRK3 (Bawa et al., 2003; Desai et al., 2004) and therefore are confident that we are measuring interactions with GRK3. The same blot was cut at the bottom and probed for GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) as a loading control using mouse anti-rabbit GAPDH (1:8000). The blots were stripped and re-probed for Hsp90 using anti-Hsp90 antibody (1:1000). The immunoreactive bands were detected by a horseradish peroxidase-conjugated secondary antibody and the blots were developed using chemiluminescence reagent prepared by adding p-coumaric acid and luminol in 100 mM Tris-HCl, pH 8.5 and hydrogen peroxide solution. Chemiluniscence was detected using an Alpha Innotech imaging system and quantified by densitometry using Fluorchem FC8800 software.

Protein estimation: Protein concentrations were determined by the Pierce bicinchoninic acid protein detection kit (Pierce, Rockford, IL Cat# 232009) using BCA protein assay reagent A (Cat# 23223) and reagent B (Cat# 23224) (Smith et al., 1985).

Immunoprecipitation: BE(2)-C cells were grown in 10-cm plates until ~70% confluent, then treated with vehicle /EPI (0.3 μM) for 5-60 min or 2-24 h as applicable. The cells were washed once with cold 1X PBS buffer (pH 7.4) and cell lysates prepared as described above. Immunoprecipitaion was conducted as published (Asghar et al., 2001) with some modifications. Anti-GRK3 antibody was used to immunoprecipitate proteins. Briefly, the cell lysates were added to the IP buffer containing (in mM) 50 mm tris-HCl,

pH 8.0, 150 NaCl, 1 EDTA, 1 EGTA, I DTT, 1 PMSF, 1% Triton-X 100, and protease inhibitor cocktail and incubated overnight with anti-GRK3 antibody. The antigen-antibody complex thus formed was incubated with protein A/G agarose beads for 2h. The ternary complex of antigen-antibody-protein A/G agarose was settled down by centrifugation, washed once with IP buffer and then with a buffer containing (in mM) 50 Tris-HCl, pH 8.0, 250 NaCl, 1 EDTA, and 0.1% Triton X-100. The complex was finally washed with another buffer containing (in mM) 50 Tris-HCl, pH 8.0, 250 NaCl. Bound proteins were eluted from proteinA/G agarose beads by addition of 25 μl of SDS sample buffer followed by boiling for 5 min. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and immunoblotted with anti-GRK3 or anti-Hsp90 antibody.

Data Analysis: The values are expressed as mean ± SEM. Comparisons between groups were made either by Student's t-test or one-way ANOVA followed by Tukey's post-hoc test where appropriate (GraphPad software, Inc. San Diego, CA), and groups were considered significantly different if $p < 0.05$.

RESULTS

Cellular levels of GRK3 were determined by western blot analysis in BE(2)-C cells pretreated with vehicle or 0.3 μM EPI for 1-24h (**Figure 1A**). The cellular levels of GRK3 were significantly increased following 2, 6, 8 and 24h of pretreatment with 0.3 μM EPI as compared to their respective vehicle controls, $p<0.05$, n=3. Interestingly, the highly conserved protein chaperone, heat shock protein 90 (Hsp90) also shows an up-regulation upon EPI (0.3 μM) treatment in the same samples (**Figure 1B**). The internal protein loading control, GAPDH, remains unaltered. The Hsp90 protein levels are significantly increased at 6h with only a slight increase observed at 4h of EPI treatment as compared to their respective vehicle controls, $p<0.05$, n=3. Furthermore, using an immunoprecipitation assay we detected direct interaction between Hsp90 and endogenous GRK3 protein (**Figure 2**). EPI treatment induced an increase in the interaction between Hsp90 and GRK3 with increasing time as compared to the vehicle.

Next, to assess the effect of the Hsp90 interaction on endogenous GRK3 protein expression, BE(2)-C cells were treated with 10 μM Geldanamycin (GA) for 0.5-24h, and GRK3 protein levels were analyzed by immunoblotting using anti-GRK3 antibody. GA significantly reduced GRK3 protein expression at 2h and GRK3 remained at this reduced level through out 24h treatment (**Figure 3**). In agreement with a previous report (Luo and Benovic, 2003), we also detected increased degradation of GRK2 in our cells upon GA treatment (data not shown). Pretreatment of cells with GA for 12h followed by EPI (24h) treatment leads to an increase in GRK3 protein as compared to pretreatment with GA alone (**Figure 4A and B).** GA treatment completely disrupts the GRK3-Hsp90 complex, as we did not detect any Hsp90 protein in the GRK3 immunoprecipitates after GA

treatment (**Figure 4C**). EPI treatment caused 141% increase in GRK3 protein levels in vehicle as compared to a 94% increase in GRK3 protein levels in GA pre-treated cells. While the relative increase in the presence of GA is less, the percent increase between the two groups (141±41 and 94±12) are not significantly different (**Figure 4D**). Hence, Hsp90 inhibition reduces the absolute but not the relative increase in GRK3 levels produced by EPI.

To examine which degradation pathway contributes to the loss of GRK3 when the interaction with Hsp90 is disrupted, we treated BE(2)-C cells with/without GA (10μM) for 24h followed by treatment with either the cysteine protease inhibitor, ALLN (50μM; at this concentration the proteasomal pathway is also inhibited) or a specific proteasome inhibitor, lactacystin (8μM) for 6h. Twenty-four hour pretreatment of the cells with GA resulted in a significant reduction (~40%) of GRK3 protein. Treatment of the cells with ALNN or lactacystin in the presence of GA reversed the degradation of GRK3 protein. The level of GRK3 protein is comparable to the control but greater than the level in the GA treated sample (**Figure 5**).

Finally, the potential contribution of the calcium-activated calpain pathway in the degradation of GRK3 was examined. BE(2)-C cells were pre-treated with calpeptin (100μM) for 1h and subsequently treated with ionomycin (0.1μM) for 4h. The 4h time point was chosen as the cell morphology was representative of normal healthy cells. Ionomycin treatment caused significant degradation of GRK3 protein as compared to the control while calpeptin treatment prevented GRK3 degradation, $p<0.05$ n=3 **(Figure 6A)**. Ionomycin (0.1μM) treatment for 4h minimally affects BE(2)-C cell morphology whereas 6h of ionomycin treatment severely alters cell morphology. Therefore, GRK3 levels are

not reduced due to cell toxicity that would be evident as abnormal cell morphology. **(Figure 6B)**.

DISCUSSION

In this study, we report a coordinated up-regulation of GRK3 and Hsp90 protein expression in response to EPI treatment and present evidence of a direct interaction between endogenous GRK3 and Hsp90 protein in human neuroblastoma BE(2)-C cells. Disruption of the interaction between Hsp90 and GRK3 targets GRK3 for degradation via the proteosomal pathway. Additionally, we present evidence that increased intracellular calcium activates calpain-dependent degradation of GRK3, significantly reducing neuronal GRK3 levels.

The observations presented herein suggest that GRK3, like GRK2, interacts with the chaperone protein, Hsp90 in neuronal cells and that this interaction is important in the maintenance of steady state levels as well as agonist-promoted increases in GRK3 protein expression. The participation of heat shock proteins in the regulation of signaling molecules is an emerging concept. Hsp90 has been reported to participate in the regulation of several kinases including GRK2, Erb2, Akt/PKB and Raf-1 (Pratt and Toft, 2003). In all cases, inhibition of the interaction between these client proteins and Hsp90 by agents like geldanamycin results in enhanced degradation of the client proteins (Pratt and Toft, 2003). Therefore, one role of Hsp90 appears to be client protein stabilization. Another proposed role of Hsp90 is assistance in the maturation of newly synthesized GRK2 protein (Luo and Benovic, 2003).

In the present study EPI treatment produced a significant increase in GRK3 expression. Some of our data would support a role for Hsp90 in the synthesis of endogenous GRK3 in neuronal cells. The observed increase in GRK3 protein could result from increased transcription/translation, reduced protein degradation, or a

combination of these two factors. We have previously reported that antisense (Desai et al., 2004) and MEK1/2/ inhibitor (Salim et al., under review) treatment eliminates the EPI-induced increase in GRK3 protein and mRNA expression, supporting a major role for increased GRK3 synthesis in GRK3 protein up-regulation. Hsp90 could participate in the maturation of this newly synthesized GRK3. Two observations in the present study also suggest the possibility that a Hsp90-mediated increase in GRK3 protein stability could contribute. First, as previously discussed, Hsp90 clearly stabilizes GRK3. Second, the interaction between Hsp90 and GRK3 is significantly increased by EPI. Within 5 min of EPI treatment the association between Hsp90 and GRK3 doubles and this might be expected to confer greater stability on GRK3 protein. However, while the increased association is almost immediate, it takes at least 6 hours before GRK3 protein levels are significantly increased. Therefore, it seems unlikely that the increase in GRK3/Hsp90 association produced by EPI treatment confers significant additional stability to GRK3. This conclusion is supported by the similar relative increase in GRK3 whether the Hsp90/GRK3 interaction is present or not. Nevertheless, the percent increase in GRK3 is smaller, although not significantly, in the presence of geldanamycin and this could suggest a minor contribution by Hsp90 to GRK3 maturation or stability in the overall process. At present we do not know either the cause or the significance of the increase in association between GRK3 and Hsp90 observed in response to EPI, but a similar rapid, agonist-induced increase in association between Hsp90 and eNOS has been reported (Joy et al., 2006). Future experiments will examine the individual influences of Hsp90 on the synthesis, maturation and degradation of GRK3, as well as on GRK3 activity, to clarify this aspect of cellular GRK3 regulation.

In addition to the increase in GRK3 protein expression in response to EPI, there also was an increase in Hsp90 protein expression that lagged slightly behind the increase in GRK3. The reason for the delay between the increase in GRK3 expression and the increase in Hsp90 expression is unclear at present, but we hypothesize that the increases in expression of the two proteins are separate but related events. Hsp90 serves as a chaperone for many cellular proteins. Therefore, it is possible that the initially available pool of Hsp90 is sufficient to participate in maturation of the newly synthesized GRK3. Additionally, it takes hours before a measurable increase in GRK3 protein is observed after EPI. Thus, it seems unlikely that the increased utilization of Hsp90 to assist in the maturation of newly synthesized GRK3 would deplete the intracellular pool of GRK3, necessitating increased Hsp90 synthesis. Alternatively, the increase in association between the cellular pool of GRK3 and Hsp90 after EPI treatment would occupy a greater fraction of the total cellular pool of Hsp90. This could decrease free Hsp90 availability in the cell and, if sustained, might trigger an increase in Hsp90 synthesis. Future metabolic experiments with pulse-chase labeling will clarify these issues.

Another objective of our study was to understand the nature of the enhanced degradation of GRK3 caused by GA. For GRK2, ubiquitination of the kinase and subsequent proteosomal degradation appears to be responsible for GRK2 degradation (Penela et al., 1998). When the interaction between Hsp90 and GRK2 is inhibited, GRK2 levels rapidly fall and inhibition of the proteosomal pathway results in the accumulation of polyubiquitinated GRK2 (Luo and Benovic, 2003). Our results suggest that GRK3 also undergoes rapid proteosomal degradation after GA treatment. A specific inhibitor of the proteosomal pathway eliminated the negative effects of GA on GRK3 protein levels.

However, it remains unclear if GRK3, like GRK2, is polyubiquitinated as part of this process. A lysine residue in GRK2 is reported to be the site of ubiquitination and an analogous residue is present in GRK3. Detailed experiments to demonstrate such ubiquitination for GRK3 have not been reported. However, one observation in the present study suggests that GRK3 is not polyubiquitinated. In the presence of GA and proteosome inhibitors, no laddering of high molecular weight GRK3-immunoreactive bands was observed in western blot analysis. This is in contrast to GRK2; laddering of high molecular weight GRK2 immnoreactive bands has been reported in samples from cells treated with GA and proteosome inhibitors (Luo and Benovic, 2003). Therefore, it appears that Hsp90 stabilizes GRK3 from rapid proteosomal degradation but the role of ubiquitination remains to be determined.

A final consideration in the present study was the susceptibility of GRK3 to degradation resulting from the activation of proteolytic pathways, in addition to those described above. In particular, oxidative stress is reported to stimulate GRK2 degradation via calpain in T-lymphocytes (Lombardi et al., 2002; Penela et al., 2003). The decrease in cellular GRK2 levels in lymphocytes during activation has been suggested to intensify cytokine signaling in these cells (Lombardi et al., 2002). Structural features, such as the PEST score, suggested it was unlikely that calpains would exert an effect on the degradation of GRK3 (Rechsteiner, 1990; Barnes and Gomes, 1995; Tompa et al., 2004). However, treatment of neuronal cells with the calcium ionophore, ionomycin, resulted in the rapid loss of GRK3 protein in our neuronal cell model and this loss was prevented by the calpain inhibitor, calpeptin.

Calpain-mediated degradation of GRK3 in neuronal cells has significant potential importance, particularly in relation to excitotoxic neuronal cell injury and death (Araujo and Carvalho, 2005). The role of calpains in neuronal injury and death is receiving increasing attention, with calpain inhibitors being examined as potential neuroprotection agents. Furthermore, α_2-AR agonists confer neuroprotection in several models of ischemic/excitotoxic neuronal death (Marien et al., 2004; Jellish et al., 2005), suggesting that α_2-AR play a significant role in neuroprotection within the CNS (Marien et al., 2004; Jellish et al., 2005). Reduced GRK3 levels could significantly modulate this action by reducing α_2-AR desensitization and enhancing the protective effects of α_2-AR activation. Moreover, one can envision a mechanism that would enable adjustment of α_2-AR responsiveness in response to changes in cell status. Under normal conditions, GRK3 levels would appropriately modulate α_2-AR signaling into the cell. However, under excessive electrical activation and depolarization, associated with increased intracellular calcium accumulation, GRK3 levels and α_2-AR desensitization would be reduced, permitting maximum neuroprotection. Oxidative stress is a common trait of hypertension and plays an important role in hypertension-evoked brain injury by increasing the tissue concentrations of neurotransmitters and increasing cellular calcium ions influx (Nishigaya et al., 1991; Brown et al., 2004; Poulet et al., 2006). Therefore, calpain activation could cause increased degradation of GRK3 and render the α_2-AR resistant to desensitization by NE and EPI.

In conclusion, the present study identifies several mechanisms by which the degradation of GRK3 is regulated, including the stabilizing influence of the protein chaperone, Hsp90 to inhibit GRK3 degradation and the ability of calcium-activated

calpain degradation of GRK3 to rapidly decrease GRK3 levels. Whether Hsp90 participates in the folding and maturation of GRK3 during protein synthesis remains to be determined. Future studies also will investigate the role of calcium-activated GRK3 degradation by calpains as a potential mechanism to modify neuronal levels of GRK3 under physiological or pathophysiological conditions.

ACKNOWLEDGEMENTS

The authors express their appreciation to Dr. Kelly M Standifer for her constructive criticism and Dr. Aarti Desai for her suggestions during the course of this investigation.

REFERENCES

Araujo IM and Carvalho CM (2005) Role of nitric oxide and calpain activation in neuronal death and survival. *Curr Drug Targets CNS Neurol Disord* **4**:319-324.

Asghar M, Hussain T and Lokhandwala MF (2001) Activation of dopamine D(1)-like receptor causes phosphorylation of alpha(1)-subunit of Na(+),K(+)-ATPase in rat renal proximal tubules. *Eur J Pharmacol* **411**:61-66.

Barnes JA and Gomes AV (1995) PEST sequences in calmodulin-binding proteins. *Mol Cell Biochem* **149-150**:17-27.

Barrett TB, Hauger RL, Kennedy JL, Sadovnick AD, Remick RA, Keck PE, McElroy SL, Alexander M, Shaw SH and Kelsoe JR (2003) Evidence that a single nucleotide polymorphism in the promoter of the G protein receptor kinase 3 gene is associated with bipolar disorder. *Mol Psychiatry* **8**:546-557.

Bawa T, Altememi GF, Eikenburg DC and Standifer KM (2003) Desensitization of alpha 2A-adrenoceptor signalling by modest levels of adrenaline is facilitated by beta 2-adrenoceptor-dependent GRK3 up-regulation. *Br J Pharmacol* **138**:921-931.

Brown RC, Mark KS, Egleton RD and Davis TP (2004) Protection against hypoxia-induced blood-brain barrier disruption: changes in intracellular calcium. *Am J Physiol Cell Physiol* **286**:C1045-1052.

Dautzenberg FM, Braun S and Hauger RL (2001) GRK3 mediates desensitization of CRF1 receptors: a potential mechanism regulating stress adaptation. *Am J Physiol Regul Integr Comp Physiol* **280**:R935-946.

Desai AN, Standifer KM and Eikenburg DC (2004) Simultaneous alpha2B- and beta2-adrenoceptor activation sensitizes the alpha2B-adrenoceptor for agonist-induced down-regulation. *J Pharmacol Exp Ther* **311**:794-802.

Desai AN, Standifer KM and Eikenburg DC (2005) Cellular G protein-coupled receptor kinase levels regulate sensitivity of the {alpha}2b-adrenergic receptor to undergo agonist-induced down-regulation. *J Pharmacol Exp Ther* **312**:767-773.

Jellish WS, Murdoch J, Kindel G, Zhang X and White FA (2005) The effect of clonidine on cell survival, glutamate, and aspartate release in normo- and hyperglycemic rats after near complete forebrain ischemia. *Exp Brain Res* **167**:526-534.

Joy S, Siow RC, Rowlands DJ, Becker M, Wyatt AW, Aaronson PI, Coen CW, Kallo I, Jacob R and Mann GE (2006) The isoflavone Equol mediates rapid vascular relaxation: Ca2+-independent activation of endothelial nitric-oxide synthase/Hsp90 involving ERK1/2 and Akt phosphorylation in human endothelial cells. *J Biol Chem* **281**:27335-27345.

Lombardi MS, Kavelaars A, Penela P, Scholtens EJ, Roccio M, Schmidt RE, Schedlowski M, Mayor F, Jr. and Heijnen CJ (2002) Oxidative stress decreases G protein-coupled receptor kinase 2 in lymphocytes via a calpain-dependent mechanism. *Mol Pharmacol* **62**:379-388.

Luo J and Benovic JL (2003) G protein-coupled receptor kinase interaction with Hsp90 mediates kinase maturation. *J Biol Chem* **278**:50908-50914.

Marien MR, Colpaert FC and Rosenquist AC (2004) Noradrenergic mechanisms in neurodegenerative diseases: a theory. *Brain Res Brain Res Rev* **45**:38-78.

McLaughlin JP, Myers LC, Zarek PE, Caron MG, Lefkowitz RJ, Czyzyk TA, Pintar JE and Chavkin C (2004) Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. *J Biol Chem* **279**:1810-1818.

Niculescu AB, 3rd, Segal DS, Kuczenski R, Barrett T, Hauger RL and Kelsoe JR (2000) Identifying a series of candidate genes for mania and psychosis: a convergent functional genomics approach. *Physiol Genomics* **4**:83-91.

Nishigaya K, Yoshida Y, Sasuga M, Nukui H and Ooneda G (1991) Effect of recirculation on exacerbation of ischemic vascular lesions in rat brain. *Stroke* **22**:635-642.

Penela P, Murga C, Ribas C, Tutor AS, Peregrin S and Mayor F, Jr. (2006) Mechanisms of regulation of G protein-coupled receptor kinases (GRKs) and cardiovascular disease. *Cardiovasc Res* **69**:46-56.

Penela P, Ribas C and Mayor F, Jr. (2003) Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases. *Cell Signal* **15**:973-981.

Penela P, Ruiz-Gomez A, Castano JG and Mayor F, Jr. (1998) Degradation of the G protein-coupled receptor kinase 2 by the proteasome pathway. *J Biol Chem* **273**:35238-35244.

Penn RB, Pronin AN and Benovic JL (2000) Regulation of G protein-coupled receptor kinases. *Trends Cardiovasc Med* **10**:81-89.

Poulet R, Gentile MT, Vecchione C, Distaso M, Aretini A, Fratta L, Russo G, Echart C, Maffei A, De Simoni MG and Lembo G (2006) Acute hypertension induces oxidative stress in brain tissues. *J Cereb Blood Flow Metab* **26**:253-262.

Pratt WB and Toft DO (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. *Exp Biol Med (Maywood)* **228**:111-133.

Rechsteiner M (1990) PEST sequences are signals for rapid intracellular proteolysis. *Semin Cell Biol* **1**:433-440.

Salim S, Standifer KM and Eikenburg DC (under review) ERK1/2 Mediated Transcriptional Regulation of GRK3 Expression In Neuronal Cells. *J Pharmacol Exp Ther*.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**:76-85.

Tompa P, Buzder-Lantos P, Tantos A, Farkas A, Szilagyi A, Banoczi Z, Hudecz F and Friedrich P (2004) On the sequential determinants of calpain cleavage. *J Biol Chem* **279**:20775-20785.

FOOTNOTES

[1] This research was supported by a grant from the American Heart Association, Texas Afffiliate Inc, #0555032Y awarded to DCE. Some of the data presented in this manuscript were presented at the Society for Experimental Biology meeting in San Francisco, CA, April 2006 and the Keystone Syposium G-Protein Coupled Receptors: Evolving Concepts and New Techniques in Keystone, CO, February, 2006.

LEGENDS FOR FIGURES

Figure 1. Time course for up-regulation of GRK3 and Hsp90 in BE(2)-C cells pretreated with 0.3 μM EPI. Cellular levels of GRK3 **(A)** and Hsp90 **(B)** were determined by western blot analysis in BE(2)-C cells pretreated with vehicle or 0.3 μM EPI for 1-24h. The cells were washed with PBS, harvested in hypotonic lysis buffer, subjected to SDS-PAGE and immunoblotted for GRK3, and Hsp90 proteins. The bottom part of the blot was cut away and probed with anti-GAPDH antibody to determine GAPDH levels as an internal loading control. Levels of GRK3 and Hsp90 are presented normalized against GAPDH. The cellular levels of GRK3 are significantly increased (*) following 4,6,8, and 24h of pretreatment with 0.3 μM EPI as compared to their respective vehicle controls, $p<0.05$. The levels of Hsp90 are significantly increased (*) following 6,8 and 24h of pretreatment with 0.3 μM EPI as compared to their respective vehicle controls, $p<0.05$. Shown in A and B are representative immunoblots of at least 4-6 different experiments. The levels of GRK3 or Hsp90 protein were quantified by densitometry on an Alpha Innotech imaging system using FC8800 software.

Figure 2. Direct interaction between endogenous GRK3 and Hsp90 in BE(2)-C cells determined by immunoprecipitation. Cell lysates from BE(2)-C cells after vehicle/EPI treatment were subjected to immunoprecipitation using an anti-GRK3 antibody as described under “Experimental Procedures”. The GRK3 immunoprecipitates were subjected to SDS-PAGE and immunoblotted for Hsp90 (upper panel) and GRK3 (lower panel) proteins. The interaction between GRK3 and Hsp90 is demonstrated by detection

of Hsp90 in the GRK3 immunoprecipitates. Shown is a representative blot of at least four independent experiments.

Figure 3. Inhibition of Hsp90 with Geldanamycin (GA, 10 μM) causes a time-dependent decrease in basal cellular levels of GRK3. BE(2)-C cells were treated with the specific Hsp90 inhibitor, geldanamycin (GA) for the indicated period of time at 37ºC. The cells were washed with PBS, harvested in hypotonic lysis buffer, subjected to SDS-PAGE and immunoblotted for GRK3 and GAPDH (loading control) proteins. GRK3 levels are presented normalized against GAPDH. Shown is an immunoblot representative of at least three independent experiments. The cellular levels of GRK3 are significantly decreased (*) following 2, 6 and 24h of GA treatment as compared to the non-GA-treated control sample (0 h), $p<0.05$, n=3.

Figure 4. Effect of EPI treatment on GRK3 protein in vehicle- and Geldanamycin (10 μM) -pretreated BE(2)-C cells. A and B: BE(2)-C cells were treated with EPI (0.3 μM) or vehicle for 24h following 12h pretreatment with the specific Hsp90 inhibitor geldanamycin GA (10 μM) or vehicle. GA remained present throughout the EPI treatment. The cells were washed with PBS, harvested in hypotonic lysis buffer, subjected to SDS-PAGE and immunoblotted for GRK3 and GAPDH (loading control) proteins. GRK3 levels are presented normalized against GAPDH. GA pretreatment (12h) significantly decreased cellular GRK3 content ($), $p<0.05$, n=3. EPI (0.3 μM, 24h) treatment significantly increased the cellular level of GRK3 protein compared to the vehicle control in the absence (*) or presence (#) of GA pretreatment, $p<0.05$. n=3. **C:**

Cell lysates from cells treated as described in A/B were immunoprecipitated with anti-GRK3 antibody, the immunoprecipitates were electrophoresed by SDS-PAGE and then probed with both anti-GRK3 and anti-Hsp90 antibodies. Hsp90 antibody failed to detect Hsp90 protein (right panel) in the GRK3 immunoprecipitates, indicating absence of the GRK3-Hsp90 complex in the presence of GA treatment. The panels are arranged according to molecular size for comparison**. D:** The data in panel B are presented here as percent increase in GRK3 protein after 24h EPI treatment. EPI (0.3μM; 24h) induced an increase in GRK3 protein expression in the absence (open bar) or presence (filled bar) of GA (10 μM for 12 h), expressed as percent of vehicle in the absence or presence of GA.

Figure 5. Hsp90 inhibition induces GRK3 degradation via a proteosome pathway. BE(2)-C cells were treated with/without GA (10μM) for 24h, followed by treatment with either ALLN (50μM) or lactacystin (8μM) for 6h in the continued presence of GA. The cells were washed with PBS, harvested in hypotonic lysis buffer, subjected to SDS-PAGE and immunoblotted for GRK3 and GAPDH (loading control) proteins. GRK3 levels are presented normalized against GAPDH. Shown is an immunoblot representative of three different experiments. The degradation caused by Hsp90 inhibitor GA (*) is reversed in the presence of either ALLN or lactacycin with GA (#), $p<0.05$, $n=3$.

Figure 6. Ionomycin treatment causes calpain-mediated degradation of GRK3. A: BE(2)-C cells (~80% confluent) were treated with ionomycin (0.1μM) or vehicle for 4h.

In some cases Calpeptin (100μM) was present 1h before and throughout ionomycin/vehicle treatment. The cells were washed with PBS, harvested in hypotonic lysis buffer, subjected to SDS-PAGE and immunoblotted for GRK3 and GAPDH (loading control) proteins. GRK3 levels are presented normalized against GAPDH. Ionomycin treatment significantly decreased GRK3 levels (*) and this effect was prevented by the calpain inhibitor, calpeptin (#), $p<0.05$, $n=3$. **B:** Ionomycin (0.1μM) treatment for 4h does not drastically affect BE(2)-C cell morphology whereas 6h treatment severely alters cell morphology as viewed in phase-contrast under the light microscope.

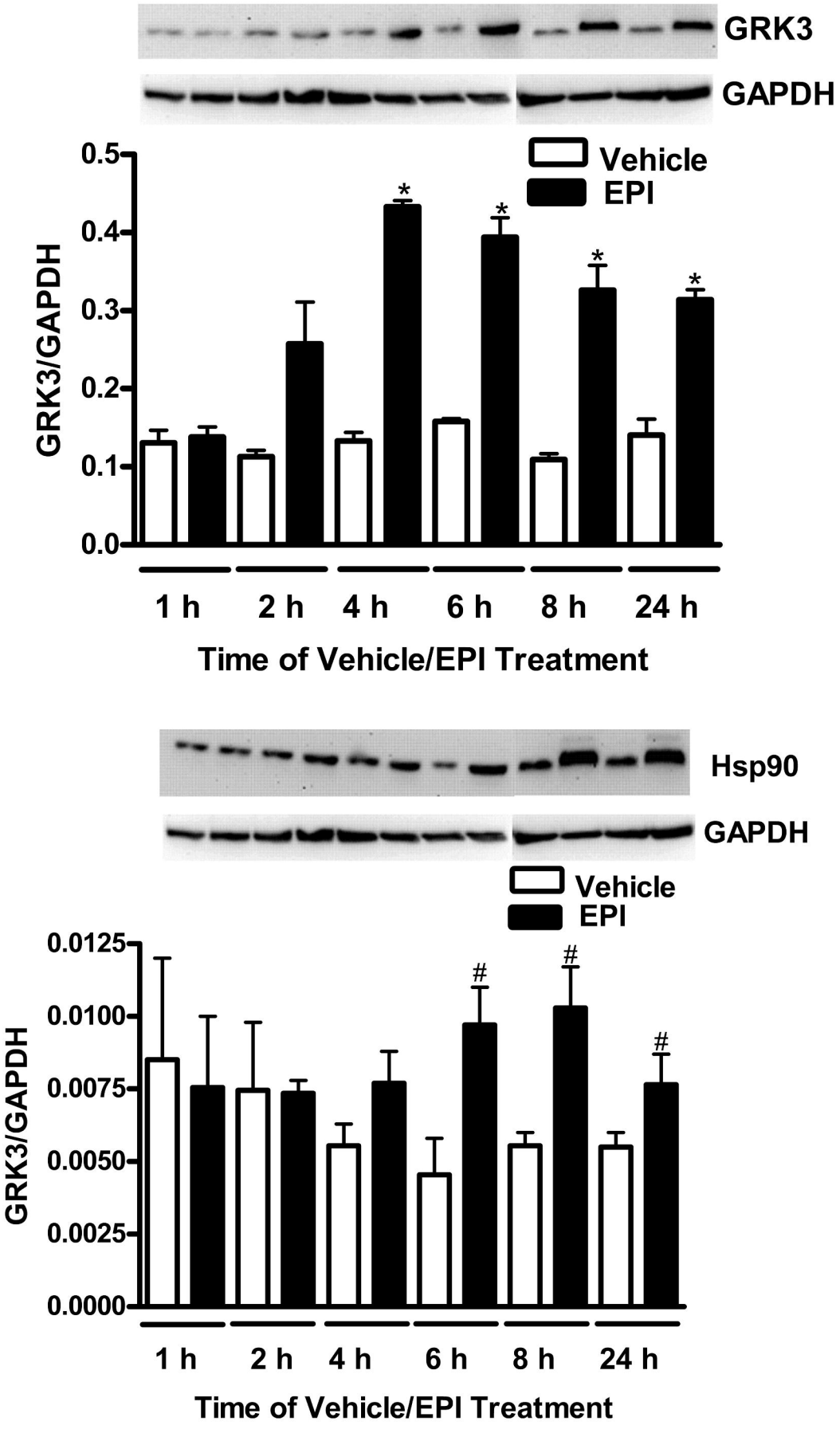
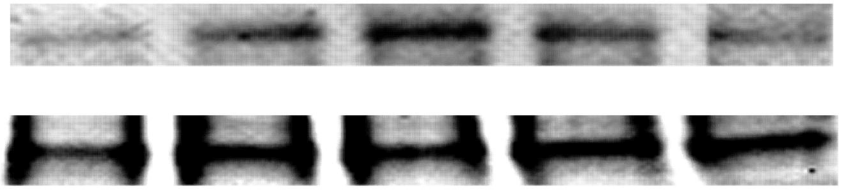

GRK3
GAPDH
Vehicle
EPI
GRK3/GAPDH
0.5
0.4
0.3
0.2
0.1
0.0
*
1 h
2 h
4 h
6 h
8 h
24 h
Time of Vehicle/EPI Treatment
Hsp90
GAPDH
Vehicle
EPI
GRK3/GAPDH
0.0125
0.0100
0.0075
0.0050
0.0025
0.0000
#
1 h
2 h
4 h
6 h
8 h
24 h
Time of Vehicle/EPI Treatment

Figure 1


IP: GRK3
WB: Hsp90
IP:GRK3
WB: GRK3

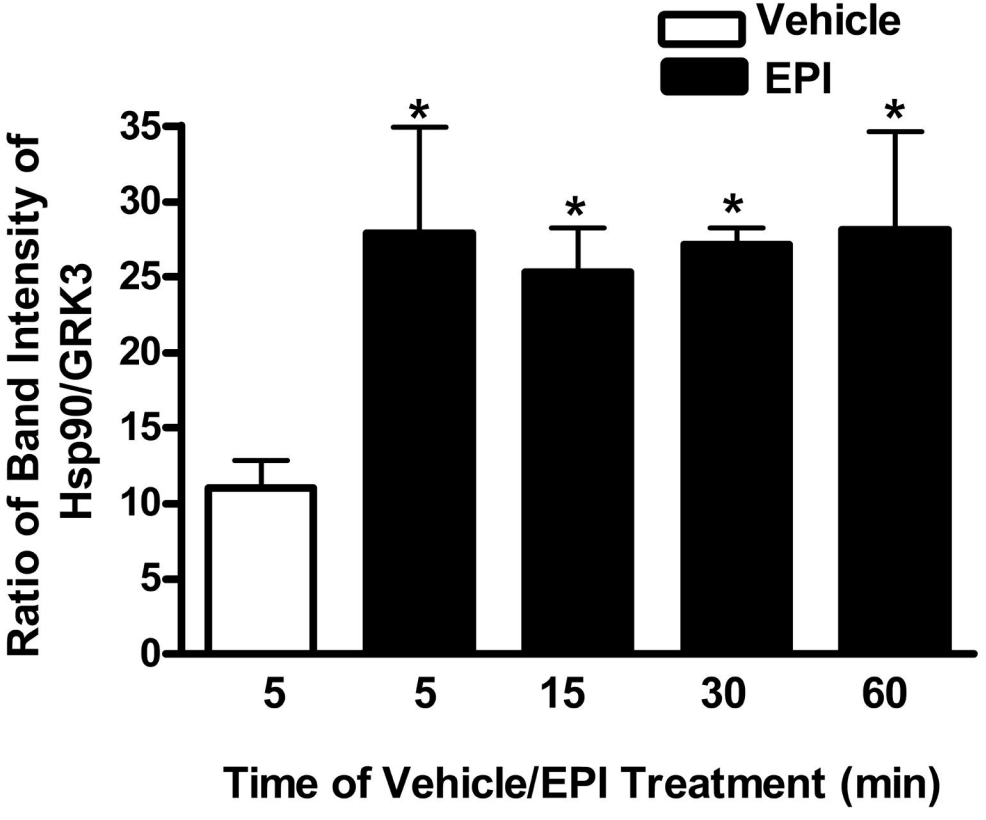

Vehicle
EPI
Ratio of Band Intensity of Hsp90/GRK3
35
30
25
20
15
10
5
0
*
*
*
*
5
5
15
30
60
Time of Vehicle/EPI Treatment (min)

Figure 2

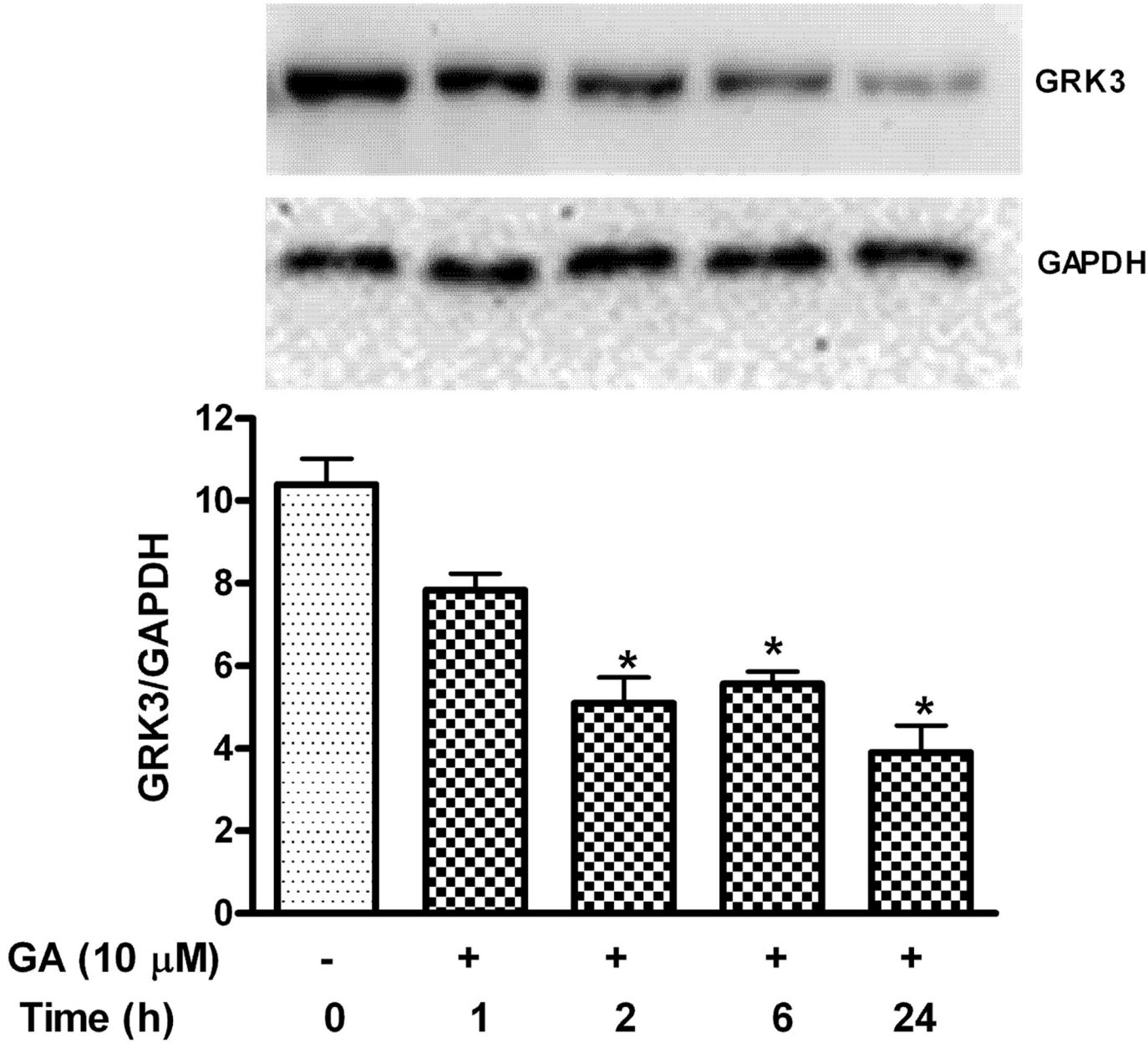


Figure 3

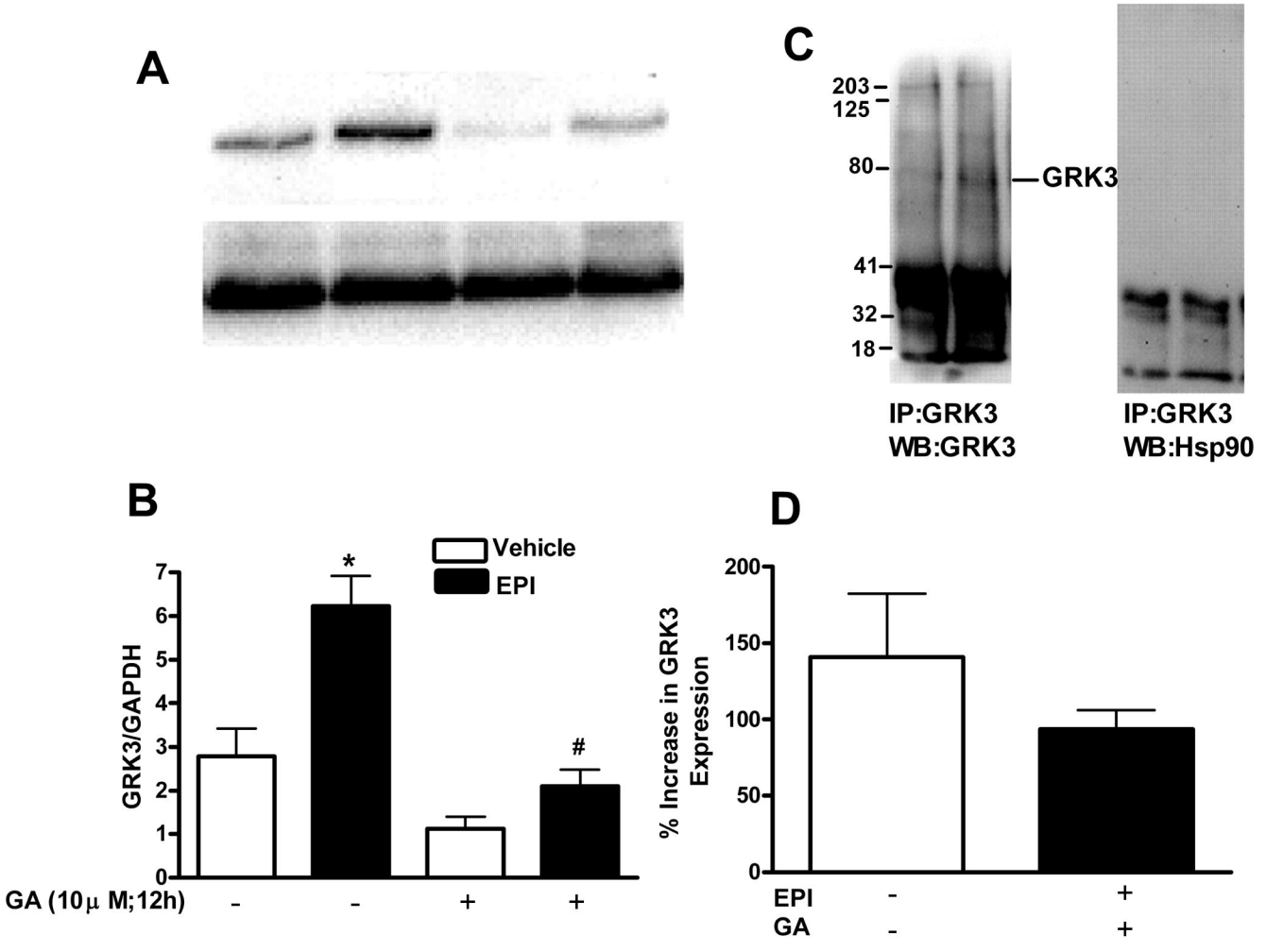

A
C
203
125
80
41
32
18
GRK3
IP:GRK3
WB:GRK3
IP:GRK3
WB:Hsp90
B
Vehicle
EPI
GRK3/GAPDH
*
#
GA (10μ M;12h)
D
% Increase in GRK3 Expression
EPI
GA

Figure 4

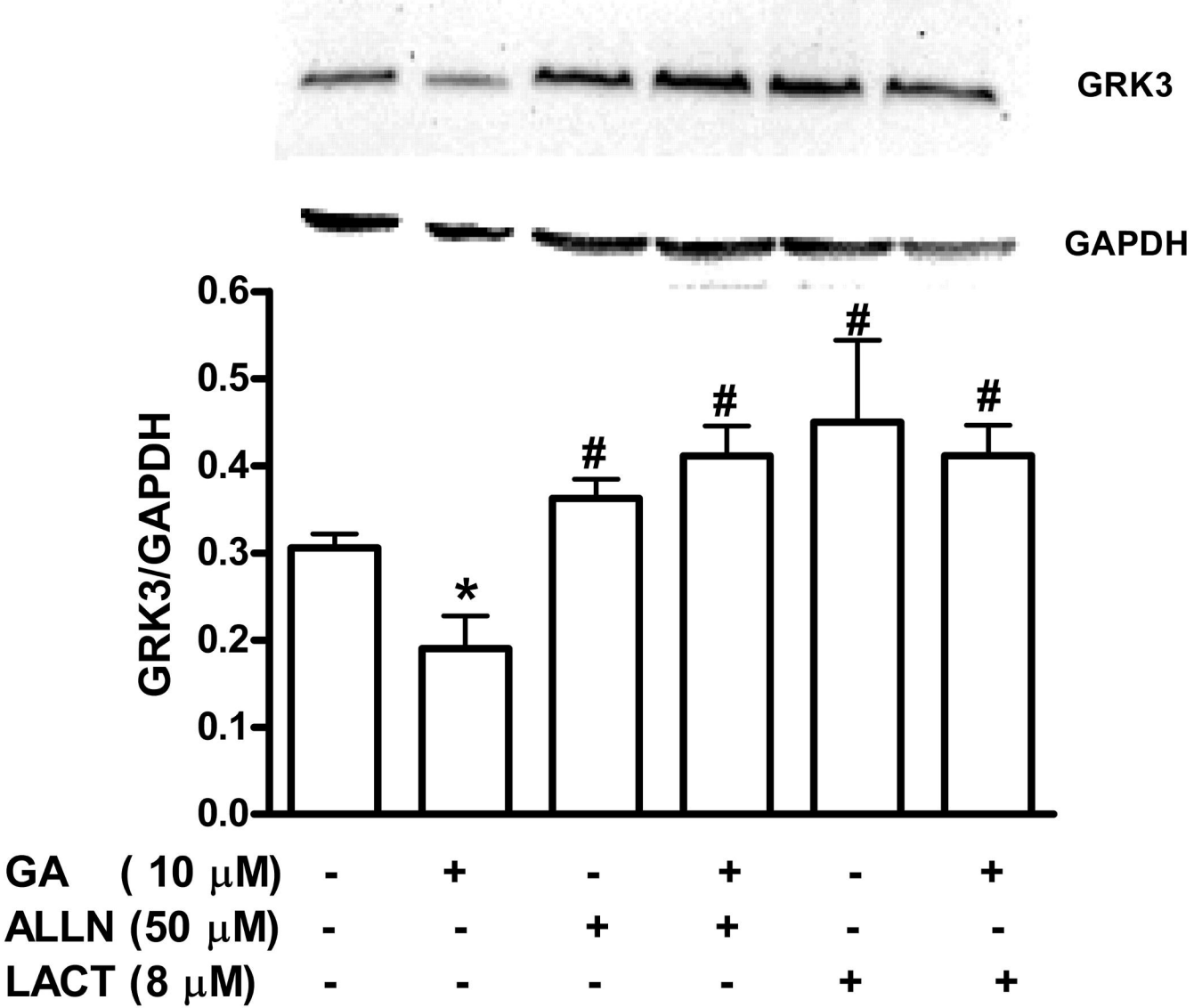

GRK3
GAPDH
GRK3/GAPDH
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
#
#
#
#
GA (10 μM) - + - + - +
ALLN (50 μM) - - + + - -
LACT (8 μM) - - - - + +

Figure 5

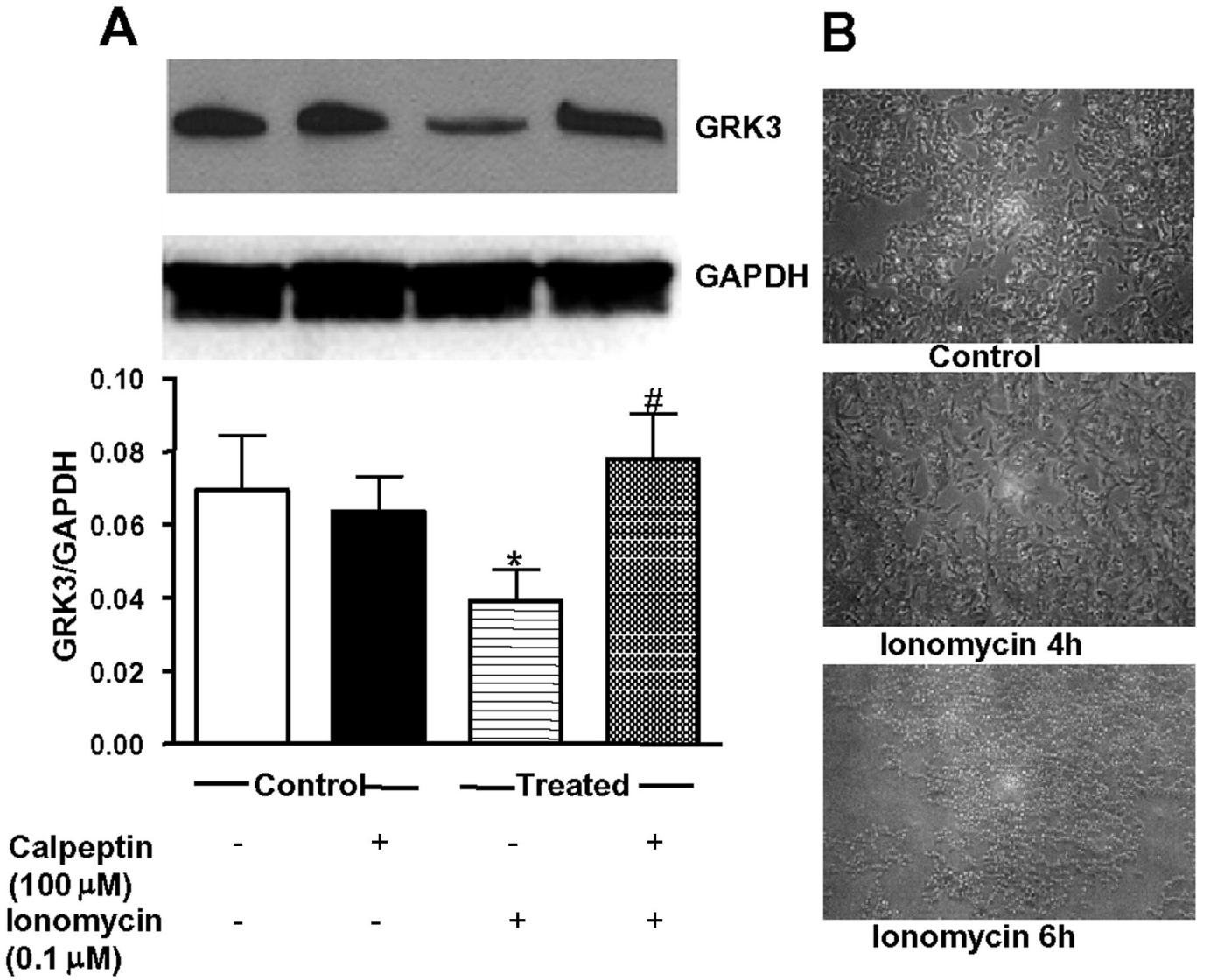

A
GRK3
GAPDH
GRK3/GAPDH
0.10
0.08
0.06
0.04
0.02
0.00
*
#
Control
Treated
Calpeptin (100 μM)
Ionomycin (0.1 μM)
- + - +
- - + +
B
Control
Ionomycin 4h
Ionomycin 6h

Figure 6