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Role of 90-kDa Heat Shock Protein (Hsp 90) and Protein Degradation in Regulating Neuronal Levels of G Protein-Coupled Receptor Kinase 3

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ABSTRACT

Cellular levels of G protein-coupled receptor kinase (GRK)3 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 90-kDa heat shock protein (Hsp90) and GRK3; Hsp90 reportedly affects the maturation and stability of GRK2. In unstimulated cells, GRK3 coimmunoprecipitates with Hsp90, suggesting a physical interaction. Moreover, when GRK3 protein expression was increased by 24-h 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, proteasome-mediated, 70% decrease in GRK3 levels within 24 h. 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 percentage of increase in GRK3 by EPI was not significantly different in the absence versus presence of GA (141 ± 41 versus 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.

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 throughout the body and the central nervous system 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 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, 2005; McLaughlin et al., 2004). 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 in the

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ABBREVIATIONS: GRK, G protein-coupled receptor kinase; GPCR, G protein-coupled receptor; AR, adrenoceptor; CRF, corticotropin-releasing factor; Hsp, heat shock protein; EPI, epinephrine; ALLN, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinol; PAGE, polyacrylamide gel electrophoresis; GA, geldanamycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; BCA, bicinchoninic acid.

putative promoter region of the human GRK3 gene has been reported previously (Niculescu et al., 2000; Barrett et al., 2003). Lymphocytic cell lines derived from bipolar disorder 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, 90-kDa heat shock protein (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, 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 bipolar disorder, the present study was initiated to determine the role of Hsp90, proteasomal degradation, and calpain activation in the regulation of the levels of GRK3 in neuronal cells.

Materials and Methods

Materials. The following chemicals and agents were purchased from the sources indicated: (−)-epinephrine bitartrate (EPI), phenylmethylsulfonyl fluoride, Dulbecco's modified Eagle's medium, Ham's F-12/Dulbecco's modified Eagle's medium, sodium metabisulfite, theophylline, HEPES, bovine serum albumin, poly-*l*-lysine hydrobromide, *p*-coumaric acid, luminol sodium salt, ALLN, ionomycin, lactacystin, and hydrogen peroxide 30% (w/w) solution (Sigma-Aldrich, St. Louis, MO); fetal bovine serum and penicillin-streptomycin (Atlanta Biologicals, Norcross, GA); *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, prestained SDS-PAGE protein marker (catalog no. 1610324), and ammonium persulfate (Bio-Rad, Hercules, CA); geldanamycin ([18*S*-(4*E*,5*Z*,8*R**,9*R**,10*E*,12*R**,13*S**,14*R**,16*S**)]-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 Research Laboratories, Plymouth Meeting, PA); calpeptin (Calbiochem, San Diego, CA); anti-GRK3 rabbit IgG (catalog no. sc-563), goat anti-rabbit IgG horseradish peroxidase (catalog no. sc-2301), goat antimouse IgG horseradish peroxidase (catalog no. sc-2302), and protein A/G agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) IgG (catalog no. RDI-TRK4G4C5) (Research Diagnostics, Flanders, 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 nonessential amino acids and Ham's F-12 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 to 80% confluence.

Pretreatment. BE(2)-C cells were pretreated with vehicle (medium containing 0.1 mM ascorbate and 1 μM sodium metabisulfite) or vehicle containing 0.3 μM EPI for 1 to 24 h for GRK3 protein up-regulation time course experiments. Ascorbate and sodium metabisulfite 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 10 μM GA for 24 h followed by treatment with either 50 μM ALLN or 8 μM lactacystin for 6 h. 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 0.1 μM ionomycin for 4 h. In some cases, 100 μM calpeptin was present 1 h before 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 1× PBS, pH 7.4, and lysed immediately in 100 to 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 five to six passes through a 23-gauge needle. The cells were subsequently centrifuged at 1000 rpm for 10 min to remove cellular debris and nuclei. The protein concentrations of the lysates were determined using a protein detection kit (catalog no. 232009; Pierce Chemical, Rockford, IL) using BCA protein assay reagent A and reagent B. The cell lysates were diluted with 4× Laemmli buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 0.1 mg/ml bromphenol blue) and resolved by SDS-PAGE (10% gel). The proteins were transferred to polyvinylidene difluoride membranes (Hybond P; GE Healthcare, Little Chalfont, Buckinghamshire, UK). 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); therefore, we are confident that we are measuring interactions with GRK3. The same blot was cut at the bottom and probed for GAPDH as a loading control using mouse anti-rabbit GAPDH (1:8000). The blots were stripped and reprobed 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. Chemiluminescence was detected using an Alpha Innotech (San Leandro, CA) imaging system and quantified by densitometry using FluorChem FC8800 software.

Protein Estimation. Protein concentrations were determined by the Pierce BCA detection kit (catalog no. 232009; Pierce Chemical) using BCA protein assay reagent A (catalog no. 23223) and reagent B (catalog no. 23224) (Smith et al., 1985).

Immunoprecipitation. BE(2)-C cells were grown in 10-cm plates until ~70% confluent, then they were treated with vehicle/EPI at 0.3 μM for 5 to 60 min or for 2 to 24 h as applicable. The cells were washed once with ice-cold 1× PBS buffer, pH 7.4, and cell lysates were prepared as described above. Immunoprecipitation was conducted as published previously (Asghar et al., 2001) with some modifications. Anti-GRK3 antibody was used to immunoprecipitate proteins. In brief, the cell lysates were added to the immunoprecipitation buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 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 2 h. The ternary complex of antigen-antibody-protein A/G agarose was settled down by centrifugation, washed once with immunoprecipitation buffer, and then with a buffer containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1 mM EDTA, and 0.1% mM Triton X-100. The complex was finally washed with another buffer

containing 50 mM Tris-HCl, pH 8.0, and 250 mM NaCl. Bound proteins were eluted from protein A/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 ± S.E.M. Comparisons between groups were made either by Student's *t* test or one-way analysis of variance followed by Tukey's post hoc test where appropriate (GraphPad Software Inc., San Diego, CA), and groups were considered significantly different if $p < 0.05$.

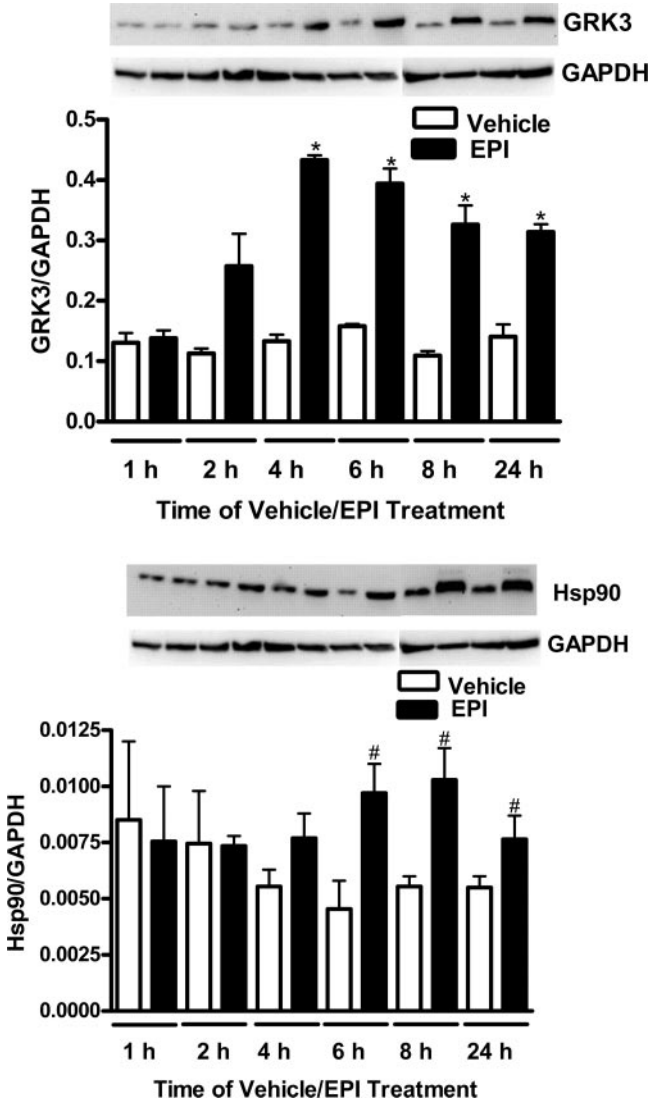


Fig. 1. Time course for up-regulation of GRK3 and Hsp90 in BE(2)-C cells pretreated with 0.3 μM EPI. Cellular levels of GRK3 (A) and Hsp90 (B) were determined by Western blot analysis in BE(2)-C cells pretreated with vehicle or 0.3 μM EPI for 1 to 24 h. The cells were washed with PBS, harvested in hypotonic 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 24 h of pretreatment with 0.3 μM EPI compared with their respective vehicle controls ($p < 0.05$). The levels of Hsp90 are significantly increased (*) following 6, 8, and 24 h of pretreatment with 0.3 μM EPI compared with their respective vehicle controls ($p < 0.05$). Shown in A and B are representative immunoblots of at least four to six different experiments. The levels of GRK3 or Hsp90 protein were quantified by densitometry on an Alpha Innotech imaging system using FluorChem FC8800 software.

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 to 24 h (Fig. 1A). The cellular levels of GRK3 were significantly increased following 2, 6, 8, and 24 h of pretreatment with 0.3 μM EPI compared with their respective vehicle controls ($p < 0.05$; $n = 3$). Interestingly, the highly conserved protein chaperone Hsp90 also shows an up-regulation upon 0.3 μM EPI treatment in the same samples (Fig. 1B). The internal protein loading control GAPDH remains unaltered. The Hsp90 protein levels are significantly increased at 6 h with only a slight increase observed at 4 h of EPI treatment compared with their respective vehicle controls ($p < 0.05$; $n = 3$). Furthermore, using an immunoprecipitation assay, we detected direct interaction between Hsp90 and endogenous GRK3 protein (Fig. 2). EPI treatment induced an increase in the interaction between Hsp90 and GRK3 with increasing time compared with 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 GA for 0.5 to 24 h, and GRK3 protein levels were analyzed by immunoblotting using anti-GRK3 antibody. GA significantly reduced GRK3 protein expression at 2 h, and GRK3 remained at this reduced level throughout 24-h treatment (Fig. 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 12 h followed by EPI (24-h) treatment leads to an increase in GRK3 protein compared with pretreatment with GA alone (Fig. 4, A and B). GA treatment completely disrupts the GRK3-Hsp90 complex, because we did not detect any Hsp90 protein in the GRK3 immunoprecipitates after GA treatment (Fig. 4C). EPI treat-

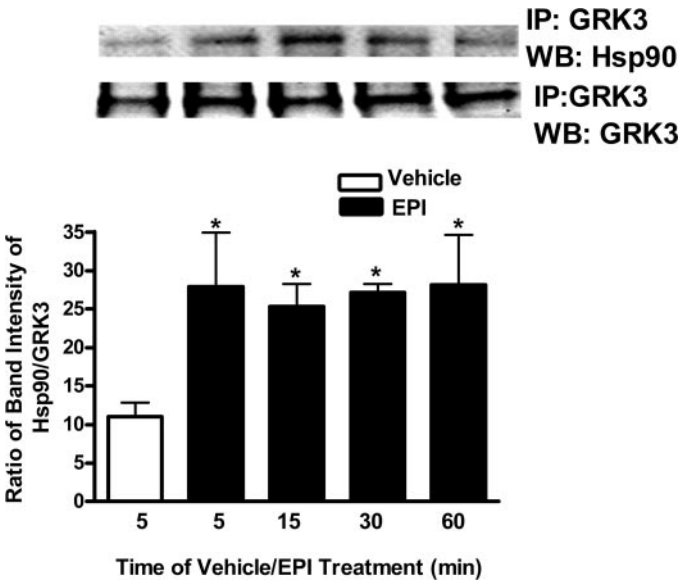


Fig. 2. Direct interaction between endogenous GRK3 and Hsp90 in BE(2)-C cells determined by immunoprecipitation. Cell lysates from BE(2)-C cells after vehicle/EPI treatment were subjected to immunoprecipitation using an anti-GRK3 antibody as described under *Materials and Methods*. The GRK3 immunoprecipitates were subjected to SDS-PAGE and immunoblotted for Hsp90 (top) and GRK3 (bottom) 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.

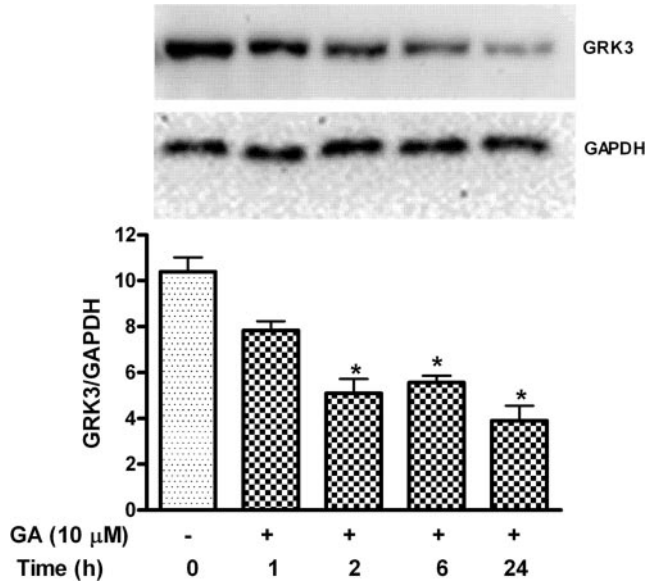


Fig. 3. Inhibition of Hsp90 with 10 μM GA causes a time-dependent decrease in basal cellular levels of GRK3. BE(2)-C cells were treated with the specific Hsp90 inhibitor GA for the indicated period at 37°C. The cells were washed with PBS, harvested in hypotonic lysis buffer, subjected to SDS-PAGE, and 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 24 h of GA treatment compared with the non-GA-treated control sample (0 h) ($p < 0.05$; $n = 3$).

ment caused 141% increase in GRK3 protein levels in vehicle compared with a 94% increase in GRK3 protein levels in GA-pretreated cells. Although the relative increase in the presence of GA is less, the percentage of increase between the two groups (141 ± 41 and 94 ± 12) is not significantly different (Fig. 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 10 μM GA for 24 h followed by treatment with either the cysteine protease inhibitor ALLN at 50 μM (at this concentration, the proteasomal pathway is also inhibited) or a specific proteasome inhibitor, lactacystin, at 8 μM for 6 h. A 24-h 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 with the control but greater than the level in the GA-treated sample (Fig. 5).

Finally, the potential contribution of the calcium-activated calpain pathway in the degradation of GRK3 was examined. BE(2)-C cells were pretreated with 100 μM calpeptin for 1 h and subsequently treated with 0.1 μM ionomycin for 4 h. The 4-h time point was chosen because the cell morphology was representative of normal, healthy cells. Ionomycin treatment caused significant degradation of GRK3 protein compared

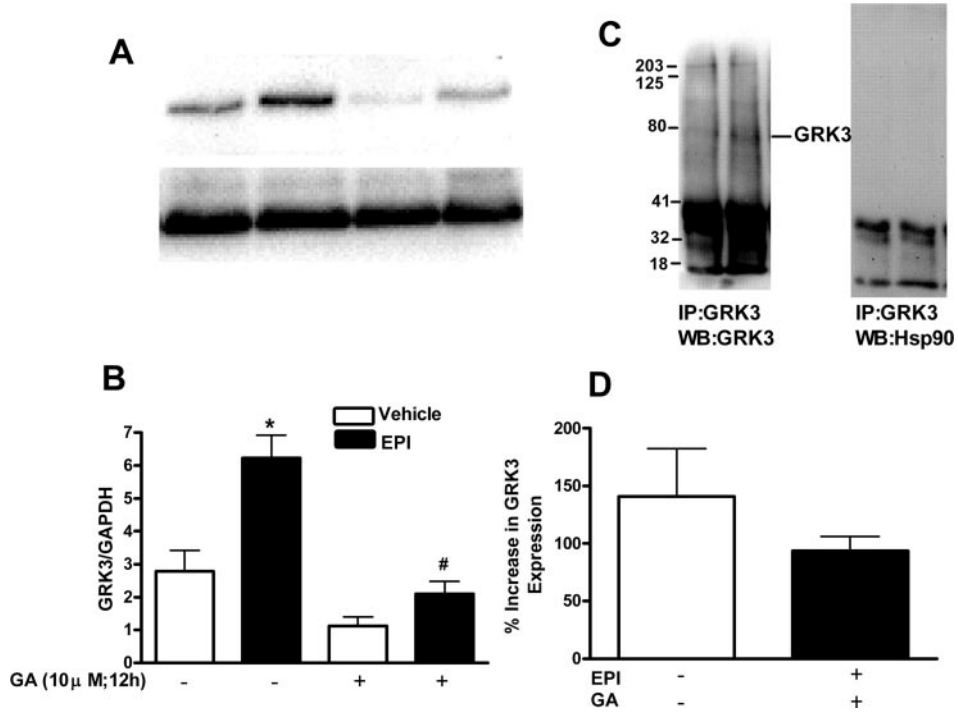


Fig. 4. Effect of EPI treatment on GRK3 protein in vehicle- and 10 μM geldanamycin-pretreated BE(2)-C cells. A and B, BE(2)-C cells were treated with 0.3 μM EPI or vehicle for 24 h following 12-h pretreatment with the specific Hsp90 inhibitor GA at 10 μM) or vehicle. GA remained present throughout the 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 (12 h) significantly decreased cellular GRK3 content ($p < 0.05$; $n = 3$). EPI (0.3 μM; 24 h) treatment significantly increased the cellular level of GRK3 protein compared with 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 and 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) 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, data in B are presented here as percentage of increase in GRK3 protein after 24-h EPI treatment. EPI (0.3 μM; 24 h) induced an increase in GRK3 protein expression in the absence (open bar) or presence (closed bar) of GA (10 μM; 12 h), expressed as percentage of vehicle in the absence or presence of GA.

with the control, whereas calpeptin treatment prevented GRK3 degradation ($p < 0.05$; $n = 3$) (Fig. 6A). Ionomycin (0.1 μM) treatment for 4 h minimally affects BE(2)-C cell morphology, whereas 6 h 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 (Fig. 6B).

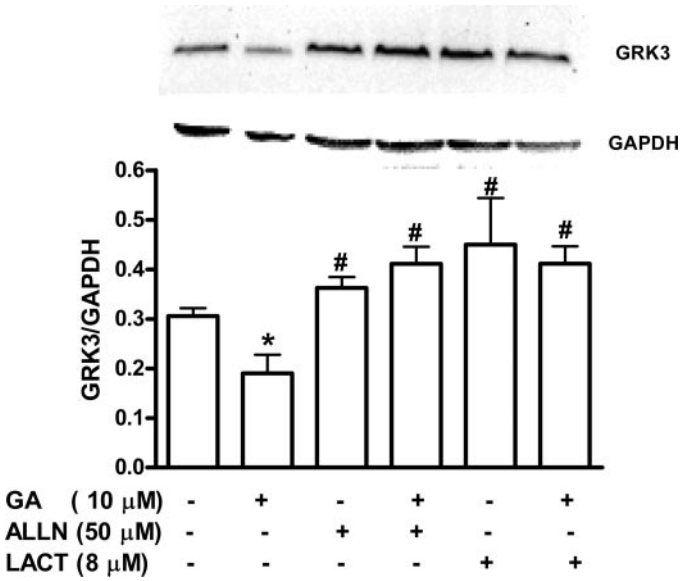


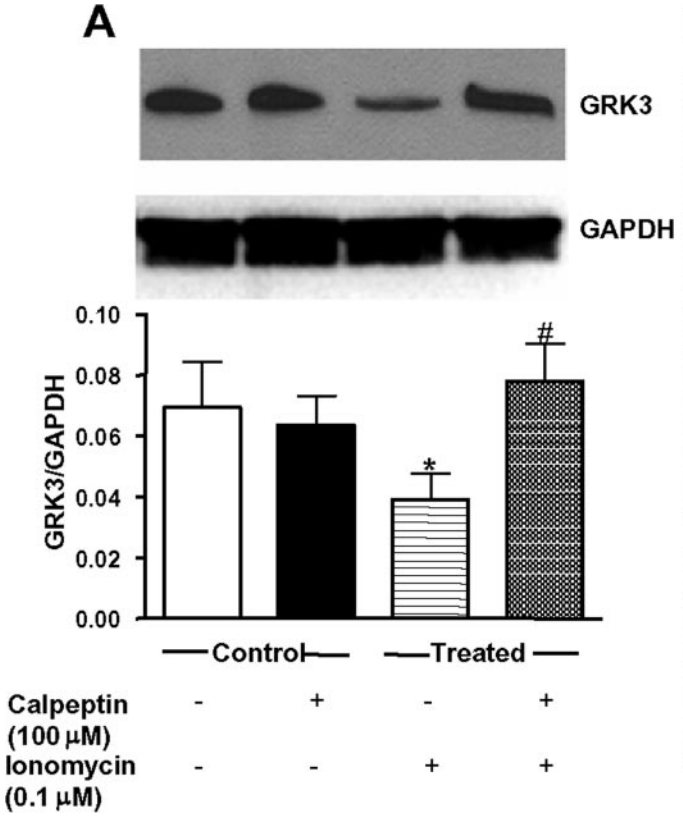
Fig. 5. Hsp90 inhibition induces GRK3 degradation via a proteasome pathway. BE(2)-C cells were treated with/without 10 μM GA for 24 h, followed by treatment with either 50 μM ALLN or 8 μM lactacystin for 6 h in the continued presence of GA. The cells were washed with PBS, harvested in hypotonic lysis 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 lactacystin with GA (#) ($p < 0.05$; $n = 3$).

Discussion

In this study, we report a coordinated up-regulation of GRK3 and Hsp90 protein expression in response to EPI treatment, and we 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 proteasomal pathway. Furthermore, 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 such as geldanamycin results in enhanced degradation of the client proteins (Pratt and Toft, 2003). Therefore, one role of Hsp90 seems 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



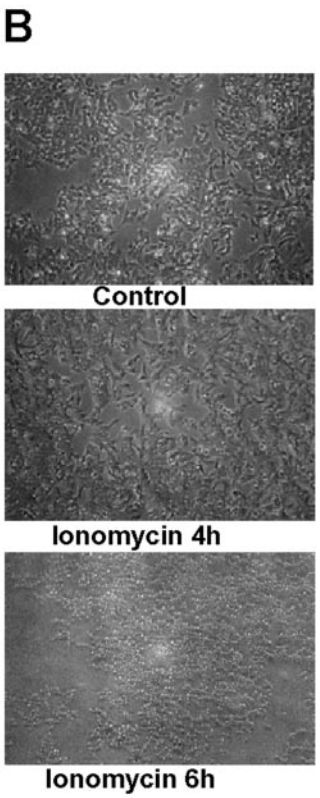


Fig. 6. Ionomycin treatment causes calpain-mediated degradation of GRK3. A, BE(2)-C cells (~80% confluent) were treated with 0.1 μM ionomycin or vehicle for 4 h. In some cases, 100 μM calpeptin was present 1 h before and throughout ionomycin/vehicle treatment. The cells were washed with PBS, harvested 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, 0.1 μM ionomycin treatment for 4 h does not drastically affect BE(2)-C cell morphology, whereas 6-h treatment severely alters cell morphology as viewed in phase contrast under the light microscope.

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 mitogen-activated protein kinase kinase 1/2 inhibitor (Salim et al., 2007) 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 an Hsp90-mediated increase in GRK3 protein stability could contribute. First, as discussed previously, 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, although the increased association is almost immediate, it takes at least 6 h 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 percentage of 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 endothelial nitric-oxide synthase has been reported previously (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. Furthermore, it takes hours before a measurable increase in GRK3 protein is observed after EPI. Thus, it seems unlikely that the increased use 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, it 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 proteasomal degradation seem 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 proteasomal pathway results in the accumulation of polyubiquitinated GRK2 (Luo and Benovic, 2003). Our results suggest that GRK3 also undergoes rapid proteasomal degradation after GA treatment. A specific inhibitor of the proteasomal pathway eliminated the negative effects of GA on GRK3 protein levels. However, it remains unclear whether 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 proteasome 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 immunoreactive bands has been reported in samples from cells treated with GA and proteasome inhibitors (Luo and Benovic, 2003). Therefore, it seems that Hsp90 stabilizes GRK3 from rapid proteasomal 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 proline (P), glutamic acid (E), serine (S), and threonine (T), i.e., 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-ARs play a significant role in neuroprotection within the central nervous system (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 in-

tracellular 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 norepinephrine 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.

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