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Oxidants and Ca^{+2} induce PGC-1α degradation through calpain

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ABSTRACT

Peroxisome proliferator activator receptor γ coactivator 1α (PGC-1α) is a transcriptional coactivator known to mediate mitochondrial biogenesis. Whereas PGC-1α transcription is regulated by a variety of signaling cascades, the mechanisms of PGC-1α degradation have received less investigation. Thus, we investigated the mechanisms responsible for PGC-1α degradation in renal proximal tubular cells (RPTC). Amino acid sequence analysis of the PGC-1α protein revealed three PEST sequence-rich regions, predictive of proteolysis by calpains and/or the proteasome. Under basal conditions, treatment with the protein synthesis inhibitor cycloheximide resulted in rapid degradation of PGC-1α ($t_{1/2}$ = 38 min), which was blocked by the proteasome inhibitor epoxomicin, but not the calpain inhibitor calpeptin. Oxidant exposure resulted in the degradation of both endogenous and adenovirally over-expressed PGC-1α, which was inhibited by calpeptin but not epoxomicin. Thapsigargin-induced release of ER Ca^{2+} also stimulated calpain-dependent, epoxomicin-independent degradation of PGC-1α. Finally, Ca^{2+} addition to lysates of RPTC over-expressing PGC-1α resulted in calpeptin-sensitive, epoxomicin-insensitive degradation of PGC-1α. In summary, we suggest two distinct mechanisms regulate PGC-1α: basal PGC-1α turnover by proteasome degradation and oxidant- and Ca^{2+}-mediated PGC-1α degradation through calpain.

Because mitochondrial dysfunction is associated with a variety of disease states and acute organ injury, elucidating the mechanisms responsible for the regulation of mitochondrial function is essential for development of new therapeutic strategies [1–13]. Mitochondrial biogenesis ameliorates a variety of disease processes such as neurodegeneration and muscular dystrophy, and accelerates the recovery of mitochondrial and cellular functions in injured renal cells [12,14,15]. Strategies have been developed to screen for novel small molecule activators of the mitochondrial biogenesis regulator PGC-1α [16]. Whereas theses strategies have focused on identifying molecules that regulate the transcription of PGC-1α, other groups have focused on targeting post-translational modifications known to increase the expression and activity of PGC-1α [17–19]. Small molecule flavone, isoflavone, and stilbene structures have been identified as activators of SIRT1, a known deacetylase of PGC-1α and an activator thereof [17–21].

Activation of p38 MAPK results in the phosphorylation of PGC-1α at several critical residues within its repression domain, resulting in the dissociation of a myb160 binding protein and increases in the transcriptional activity of PGC-1α [22]. Phosphorylation of PGC-1α by p38 also increases the $t_{1/2}$ of PGC-1α from approximately 2 to 6 h [23]. These data support the idea that modulating p38 MAPK activity and other mechanisms directed towards extending the $t_{1/2}$ of PGC-1α may be a viable therapeutic option to promote mitochondrial biogenesis and treat diseases associated with mitochondrial dysfunction.

Several groups have shown that PGC-1α is rapidly turned over by a proteasomal dependent pathway and during periods of oxidative stress, PGC-1α translocates to the nucleus in an SIRT1-dependent manner to stimulate a compensatory increase in mitochondrial biogenesis prior to proteasomal degradation [24]. In these studies the total pool of PGC-1α protein was not altered as the depletion of PGC-1α was offset by the increased transcription of PGC-1α.

Sano et al. identified several regions of PGC-1α that are enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) (PEST sequences) [25]. These sequences are known target sequences for a variety of cysteine proteases, including the Ca^{2+}-activated cysteine protease calpain [26–33]. Thus, we hypothesized that PGC-1α may also be degraded by a calpain during conditions of Ca^{2+} overload. The goal of this study was to examine the various mechanisms mediating degradation of PGC-1α in renal cells under basal and pathologic conditions.

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Materials and methods

Reagents

Anti-GAPDH and anti-PGC-1α (H300) antibodies were purchased from Fitzgerald Antibodies (Concord, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. A Myc antibody was obtained from Cell Signaling (Danvers, MA). The calpain substrate, suc-Leu-Leu-Val-Tyr-AMC (sLLVY-AMC), was purchased from Bachem (King of Prussia, PA). Calpeptin and purified calpain I from porcine kidney were obtained from EMD Biosciences (La Jolla, CA). All secondary antibodies were purchased from Pierce Biotechnology (Rockford, IL). Anti-PGC-1α was used at 1:300, whereas all other antibodies were used at 1:1,000. All other chemicals were purchased from Sigma (St. Louis, MO).

Isolation and culture of renal proximal tubules

Female New Zealand White rabbits (2 kg) were purchased from Myrtle's Rabbitry (Thompson Station, TN). RPTC were isolated using the iron oxide perfusion method and grown in 35-mm tissue culture dishes under improved conditions as previously described [34]. The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 (without glucose, phenol red, or sodium pyruvate) supplemented with 15 mM HEPES buffer, 2.5 mM L-glutamine, 1 μM pyridoxine HCl, 15 mM sodium bicarbonate, and 6 mM lactate. Hydrocortisone (50 nM), selenium (5 ng/ml), human transferrin (5 μg/ml), bovine insulin (10 nM), and L-ascorbic acid-2-phosphate (50 μM) were added daily to fresh culture medium. Confluent RPTC were used for all experiments. RPTC monolayers were treated with various compounds or diluent (DMSO) for 24 h.

Calpain 1 activity

Calpain 1 activity was determined by a fluorometric assay using purified calpain 1 and the calpain substrate sLLVY-AMC as previously described [35]. Briefly, calpain 1 (final concentration 0.76 μg/ml) was added to assay buffer containing various concentrations of calpain inhibitors and Ca^{2+} (final concentrations: 20 mM HEPES, 150 mM KCl, 5 mM DTT, 6 mM Ca^{2+}, pH 7.4). After a brief period of mixing, sLLVY-AMC (final concentration 50 μM) was added. Activity was measured under linear conditions as a function of AMC hydrolysis using an excitation wavelength of 355 nm and an emission wavelength of 444 nm.

Preparation of cell lysates and immunoblot analysis

RPTC were washed twice with PBS without Ca^{2+} and Mg^{2+} and harvested in cell lysis buffer from BioVision (Mountain View, CA). Immunoprecipitations were performed by incubating lysates (1 mg cellular protein) overnight with Protein G coated agarose beads to preclear non-specific proteins. Lysates were incubated with antibody for 12 h, followed by a 3 h incubation with protein G coated agarose beads, and then centrifugation. Samples were subsequently boiled and prepared for electrophoresis. After protein disruption by sonication for 30 s, equal amounts of protein lysates were separated by SDS–PAGE and transferred to nitrocellulose membranes. After treatment with 5% skim milk or BSA at 4 °C overnight, membranes were incubated with various antibodies for 2 h and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody for 1 h. Bound antibodies were visualized by chemiluminescence detection on an Alpha Inotech imaging system (San Leandro, CA).

Replication-deficient adenovirus infection

Recombinant adenovirus expressing PGC-1α or a nuclear GFP control were a generous gift from Bruce Spiegelman (Harvard Medical School) [36]. Viral titers were determined by plaque formation assay using HEK293 cells and expressed as plaque-forming units (pfu)[1]. RPTC were infected with each virus at a multiplicity of infection (MOI) of 30 for 24 h at 37 °C in a humidified, 5% CO_2 incubator. Afterward, the cultures were placed in normal culture media for an additional 24 h, at which point all measurements were taken. At an MOI of 30, 99% of the cells showed expression of the viral gene insert as indicated by flow cytometry (data not shown).

Statistical analysis

Data are presented as means ± SE and were subjected to one- or two-way ANOVA as appropriate. Multiple means were compared using the Student–Newman–Keuls test, with $p < 0.05$ considered statistically significant. RPTC isolated from an individual rabbit represent a single experiment ($n = 1$). Experiments were performed with 2–5 plates of cells and repeated until an n of at least 3 was reached.

Results

We observed that the basal level of PGC-1α in RPTC was low. To characterize the degradation of PGC-1α in RPTC, PGC-1α was over-expressed using an adenoviral vector and degradation was evaluated in the presence of the protein synthesis inhibitor cycloheximide (100 μM). Adenovirally over-expressed PGC-1α was degraded with a calculated $t_{1/2}$ of ~38 min (Fig. 1), suggesting that PGC-1α has a rapid turnover rate in RPTC under control conditions.

We and others [25] determined that PGC-1α has numerous PEST sequences (Fig. 2). Because cysteine proteases are known to cleave at PEST sequences, we examined several cysteine protease inhibitors for their ability to block PGC-1α degradation. MG-132 (10 μM), a prototypical proteasome inhibitor, inhibited the activity of purified calpain 1, consistent with reports of MG-132 cross reacting with a variety of cysteine proteases (Fig. 3) [37]. The calpain-specific inhibitor, calpeptin (100 μM), also inhibited calpain 1 activity. In contrast, the more specific proteasome inhibitor, epoxomicin (10 μM) [38–40], had no effect on calpain 1 activity.

RPTC over-expressing PGC-1α were treated with cycloheximide to block PGC-1α synthesis, permitting the observation of PGC-1α degradation in isolation. Prior to the addition of cycloheximide, RPTC were pretreated with DMSO, MG-132 (10 μM), epoxomicin (10 μM), or calpeptin (100 μM). Approximately 55% of PGC-1α protein was degraded within the first hr with total degradation occurring 2 h after the addition of cycloheximide (Fig. 4). This degradation was inhibited by pretreatment with the proteasome/calpain inhibitor MG-132. Similarly, epoxomicin blocked PGC-1α degradation at 1 h and approximately 80% at 2 h. In contrast, calpeptin had no effect on PGC-1α at 1 h and blocked approximately 20% at 2 h. In a complimentary experiment, MG-132, epoxomicin, or calpeptin were added to RPTC in which PGC-1α had been over-expressed in the absence of cycloheximide. Adenoviral-induced PGC-1α accumulated within 1 h of treatment with both MG-132 and epoxomicin, but did not accumulate in the presence of calpeptin (Fig. 5). We suggest that basal degradation of PGC-1α is mediated by the proteasome and not through calpains.

[1] *Abbreviations used:* RPTC, renal proximal tubular cells; pfu, plaque-forming units; MOI, multiplicity of infection; TBHP, *tert*-butyl hydroperoxide; P, proline; S, serine; T, threonine.