Role of cAMP-responsive Element-binding Protein (CREB)-regulated Transcription Coactivator 3 (CRTC3) in the Initiation of Mitochondrial Biogenesis and Stress Response in Liver Cells

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Peroxisome proliferator-activated receptor α, coactivator 1α (PGC-1α) is the master regulator of mitochondrial biogenesis. PGC-1α expression is under the control of the transcription factor, cAMP-responsive element-binding protein (CREB). In searching for candidate transcription factors that mediate mitochondrial stress-initiated mitochondria-to-nucleus signaling in the regulation of mitochondrial biogenesis, we assessed the effect of silencing CREB-regulated transcription co-activators (CRTC). CRTC isoforms are co-activators of CREB-regulated transcription by a CREB phosphorylation-independent pathway. Using cultured HepG2 cells and primary mouse hepatocytes, we determined that mitochondrial stress imposed by the complex I inhibitor rotenone elicited mitochondrial biogenesis, which was dependent on an induction of PGC-1α, which was inhibited by silencing PGC-1α. PGC-1α induction in response to rotenone was inhibited by silencing the expression of CRTC3, which blocked downstream mitochondrial biogenesis. In contrast, silencing CRTC2 did not affect the induction of this pathway in response to rotenone. Thus, CRTC3 plays a selective role in mitochondrial biogenesis in response to rotenone.

The signal transduction pathway leading to expression of nuclear genes encoding mitochondrial proteins is believed to be regulated by Creb2-dependent transcription of Pgc-1α, a co-activator of these genes and master regulator of mitochondrial biogenesis (1–5). An important mechanism of Creb activation in this pathway is the activation and nuclear translocation of CRTC, a family of Creb co-activators that are known to respond to metabolic stress mediated through cAMP-dependent protein kinase (PKA) or AMP-activated protein kinase (AMPK) (6–8). Although mitochondrial biogenesis occurs in response to metabolic signals, we were interested in what mediates mitochondrial biogenesis and stress response when mitochondria are stressed. Using liver cells as our model system, we were particularly interested in exploring the possibility that mitochondrial stress by a mitochondrial-specific toxin, rotenone, which inhibits complex I (9), leads to a unique signaling pathway. Furthermore, because Crtc2 and Crtc3 are expressed in liver cells, we wished to test the hypothesis that their respective roles might be different in metabolic versus mitochondrial stress signaling in leading to Creb activation, induction of Pgc-1α, and subsequent up-regulation of nuclear genes encoding mitochondrial proteins including transcription regulators, electron transport components, and chaperones.

EXPERIMENTAL PROCEDURES

Cell Culture, Treatment, and Chemicals—Primary mouse hepatocytes (PMH) and HepG2 cells were cultured on collagen type I-coated dishes. Hepatocytes were isolated from male C57BL/6 mice 6–9 weeks of age and plated at 1.5 × 10⁶ cells/100-mm dish as described previously (10, 11). 3 h after plating, hepatocytes were washed and rested in serum-free Dulbecco’s modified Eagle’s medium/F-12 medium (10 ml/dish) overnight (~15 h). The incubation and following treatments were carried out in a 37 °C cell culture incubator with 5% CO₂. Chemical treatments were done in overnight culture medium for the indicated time except when otherwise specified. After the indicated treatment periods, cells were incubated with fresh serum-free Dulbecco’s modified Eagle’s medium/F-12 medium (10 ml/dish). Rotenone, tunicamycin, forskolin (FSK), and dimethyl sulfoxide (DMSO) were from Sigma.

Western Blotting—Whole cell or subcellular fraction (30–50 μg) was loaded on SDS-PAGE and then transferred to nitrocellulose membranes. The antibodies used for probing are specific for heat shock protein 60 (Hsp60), heat shock protein 10 (Hsp10) (StressGen), cytochrome c oxidase subunit IV (COX IV) (Cell Signaling), CREB (Cell Signaling), cytochrome c (Cyt c) (BD Pharmingen), pan-TORC (Calbiochem), CRTC2 (Calbiochem), CRTC3 (Cell Signaling), prohibitin1 (PHB1) (Cell Signaling), and β-actin (Sigma). The immunoreactive bands were visualized by chemiluminescence detection systems (Santa Cruz Biotechnology) or SuperSignal West Pico...
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chemiluminescent substrate (Pierce). The probed membranes were restored after treatment with stripping buffer (Pierce) when needed.

siRNA Transfection and Adenoviral shRNA Transduction—HepG2 cells were transfected with siRNA pool targeting to CRT2 (siCRT2), CRT3 (siCRT3), or scrambled control siRNA (siControl). siRNAs were purchased from Dharmacon, and the manufacturer’s protocol was followed. We used the BLOCK-i-T adenoviral RNAi expression system (Invitrogen) to generate expression constructs for shRNAs targeting lacZ (shlacZ) and mouse Crtc2 (shCrtc2), Crtc3 (shCrtc3), and Pgc-1α (shPgc-1α), respectively. shRNA sequences are listed in supplemental Table S1. A male C57BL/6 mouse 6–9 weeks of age was tail vein-injected with 3 × 10⁶ infectious units of adenovirus expressing shRNA, and PMH were isolated 10 days after injection.

Quantitation of mRNA—Total RNA was extracted using an RNeasy RNA extraction kit (Qiagen). 0.5 μg of total RNA was reverse-transcribed using an Omniscript reverse transcription kit (Qiagen) supplemented with 10 μM random hexamer (Applied Biosystems). The resulting cDNA (volume equivalent to 5–10 ng of total RNA in a reverse transcription reaction) was subjected to quantitative real-time PCR analysis with the SYBR Green PCR master mix (Qiagen) and ABI Prism 7900HT sequence detection system (Applied Biosystems). Primer pairs used in quantification of gene expressions were from Primer Bank (Harvard University), and primer ID numbers are available on request. The relative quantification of mRNA expression was analyzed by the ΔΔCT method or standard curve method following the manufacturer’s instructions (Applied Biosystems). Data were analyzed using the SDS2.1 software (Applied Biosystems). All data were normalized by endogenous GAPDH expression, and the relative mRNA levels of untreated controls were set as 1.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed as described previously (12) with modifications. PMH were switched to serum-free Dulbecco’s modified Eagle’s medium/F-12 medium for 15 h and then either left untreated or treated with rotenone or FSK for the indicated times. After cross-linking with 1% paraformaldehyde 37 °C for 30 min, cells were washed, collected, and lysed on ice in hypotonic buffer by homogenizing with pellet pestle. Nuclei from 30 min, cells were washed, collected, and lysed on ice in hypotonic buffer, sonicated, and centrifuged. The supernatant chromatin was precleared, diluted, and divided. Chromatin samples were immunoprecipitated overnight with 3 μl of anti-CREB (Cell Signaling), 5 μl of anti-pTORC or 5 μl of anti-CRTC2 (Calbiochem), or rabbit normal serum as negative control. The immune complex was collected with 60 μl of 50% slurry protein A-agarose preblocked with 1% BSA and 2 μg of salmon sperm DNA, washed, and eluted. Cross-links on immunoprecipitated and input chromatin were then reversed, and DNAs were purified with the DNAzol genomic DNA extraction reagent (Invitrogen). Immunoprecipitated DNAs (undiluted) and input DNAs (1% of the immunoprecipitation input) (diluted 1:10) were subjected to 35–40 cycles of PCR amplification using a PCR master mix kit (Qiagen catalog number 201443). The primer pair used in quantification of the gene-specific promoter region is summarized in supplemental Table S2. The set of oligonucleotides was designed to amplify and detect a specified promoter region encompassing CRE sites. PCR products were visualized in 2% agarose gel with ethidium bromide. For the reChIP assay, CREB antibody immunoprecipitated chromatin were eluted and re-immunoprecipitated with anti-pTORC.

RESULTS

Cell Model of Mitochondrial Biogenesis in Response to Mitochondrial Stress—To address the upstream mechanisms of activation of mitochondrial biogenesis in response to mitochondria-specific stress, we treated PMH and HepG2 cells with a mitochondria-specific toxin, rotenone, which inhibits complex I (9), as compared with FSK, which activates cAMP-dependent protein kinase A (PKA) in cytosol (13), or the endoplasmic reticulum (ER)-specific stress inducer, tunicamycin, which inhibits glycosylation of proteins in the ER (14). PMH were treated with rotenone for 3 h, FSK for 24 h, or tunicamycin for 6 h. Mitochondrial biogenesis and stress response were then determined at 24 h. HepG2 cells, which are known to be less sensitive to rotenone-induced cell death, kept in freshly made culture medium consisting of DMEM with 4.5 g/liter glucose supplemented with 4 mM l-glutamine, 2% fetal bovine serum (15–17), were treated with rotenone continuously for 24 h. Rotenone inhibits ~85% of mitochondrial electron transport at 20 μM in HepG2 cells (18). ~50% of ATP level is maintained in primary hepatocytes and other primary cells when treated with 20–60 μM rotenone (19–24). We found that with short exposure of primary hepatocytes to rotenone, the cells remained alive and nuclear gene expression could be studied. As shown in Fig. 1A, mitochondrial respiratory proteins such as Cyt c and COX IV and mitochondrial chaperones such as Hsp10, PHB1, and Hsp60 were induced in a dose-dependent fashion in rotenone-treated PMH. In contrast, mitochondrial protein expression in FSK- or tunicamycin-treated PMH did not change. As a loading control of the whole cell lysates, β-actin levels did not increase under these conditions. mRNA of nuclear encoded mitochondrial genes such as Cyt c, COX IV, and Hsp10 (Fig. 1B) were up-regulated at 12 h after rotenone treatment. A similar, albeit less robust, response was observed in HepG2 cells treated with rotenone but not with FSK (Fig. 1C). These results indicate that rotenone treatment induced mitochondrial biogenesis (increased expression of mitochondrial respiratory proteins) and mitochondrial stress response (increased expression of mitochondrial chaperones) in both PMH and HepG2 cells.

PGC-1α Is an Early Response and Essential Gene in Mitochondrial Stress-induced Mitochondrial Biogenesis—Over the past decade, several transcription factors and co-activators have been identified that control mitochondrial biogenesis and function with PGC-1α being a key regulator (25–29). We examined the time course of changes in gene expression of a set of key transcription factors for nuclear encoded mitochondrial proteins in PMH after rotenone-induced mitochondrial stress. As shown in Fig. 2A, nuclear respiratory factor-1 (Nrf-1) and the CREB transcription complex (Crb1, Crebbp, Crtc2, and Crtc3) were up-regulated 3–6-fold beginning 9 h after mitochondrial stress. In contrast, induction of Pgc-1α expression occurred earlier at 6 h after treatment. Rotenone-induced
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Pgc-1α induction contrasts with the known rapid activation of Pgc-1α by Crtc2-Creb within 1–2 h after FSK (3, 30). However, in contrast to FSK-induced increase in cAMP, rotenone is not a direct activator of signaling pathways, and therefore, presumably the delayed response represents the time necessary for mitochondrial dysfunction to reach a threshold to activate retrograde signaling. This is somewhat analogous to the time scale of tunicamycin-induced expression of ER stress-responsive genes (31). Our results suggest that Pgc-1α is an early response transcriptional regulator of mitochondrial stress-induced mitochondrial biogenesis and stress response. Furthermore, rotenone-induced increased mRNA expression of Pgc-1α-regulated genes such as Nrf-1, Cyt c, COX IV, and Hsp10 was blocked in shRNA-mediated Pgc-1α-silenced PMH (Fig. 2B). The results confirm that Pgc-1α is a key mediator of rotenone-induced mitochondrial biogenesis.

Role of CRTC2 Versus CRTC3 in Response to Mitochondria-specific Stress Induced by Rotenone—Mitochondrial biogenesis in mouse muscle cells has been shown to be increased by adenovirus-mediated expression of human CRTC genes (4). In human liver, CRTC2 mRNA expression was found to be greater than CRTC3, whereas CRTC1 was not expressed (4). We assessed whether the mitochondrial biogenesis and stress response to rotenone are dependent on endogenous Crtc2 or Crtc3 in PMH. C57BL/6 mice (8–10 weeks) were injected with adenovirus expressing shRNA specific to mouse Crtc2, Crtc3, or lacZ (as a control) through the tail vein. Crtc2 or Crtc3 protein and mRNA expression was decreased to ≤20% of shlacZ control (Fig. 3, A and B). PMHs were isolated 10 days after adenovirus injection and challenged with rotenone as described for Fig. 1A, and expression of mRNA of nuclear encoded mitochondrial genes was determined by real-time quantitative PCR. As shown in Fig. 3C, PMH isolated from mice that were treated with shlacZ or shCrtc2 exhibited robust induction of nuclear encoded mitochondrial genes in response to rotenone at 12 h after treatment. In comparison, the response of nuclear encoded mitochondrial gene expression to rotenone was markedly inhibited in cells in which human CRTC2 or CRTC3 protein and mRNA expression was silenced by siRNA (Fig. 4, A and B), and then real-time quantitative PCR of mRNA expression of representative genes involved in mitochondrial biogenesis over time in response to continuous nonlethal exposure to rotenone was performed. Both siControl-treated and siCRTC2-treated HepG2 cells exposed to rotenone showed increased expression of nuclear encoded mitochondrial biogenesis-related genes, COX IV and cytochrome c, and the mitochondrial chaperone, Hsp10 (Fig. 4C). In contrast, these responses to rotenone were markedly inhibited in cells in which CRTC3 expression had been silenced. This increase or inhibition of nuclear encoded mitochondrial gene expression was paralleled by a corresponding increase or decrease in protein

FIGURE 1. Mitochondrial stress-induced mitochondrial biogenesis. A, isolated PMH were rested overnight, treated with rotenone for 3 h or treated with tunicamycin (Tm, 10 µM) for 6 h, and then cultured in toxin-free medium for 24 h. FSK (10 µM) treatment was for 24 h. 30 µg of protein extract was used for Western blot for immunodetection of mitochondrial proteins such as Cyt c, COX IV, Hsp10, PHB1, and Hsp60; β-actin was used as a loading control. B, total mRNA from PMH as in A was extracted as described at the indicated times, and real-time quantitative PCR was performed for Cyt c, COX IV, and Hsp10 gene transcription status. Data are presented as mean ± S.E. (n = 3). Asterisk, p < 0.05, Student’s t test. C, HepG2 cells were treated for 24 h, and mitochondrial biogenesis and chaperone expression were examined as in A.
expression in siCRTC2 or siCRTC3 transfected HepG2 cells (Fig. 4D). Taken together, these results suggest that endogenous Crtc3 plays a selective and critical role in the initial response of liver cells to mitochondria-specific stress, leading to increased expression of nuclear encoded mitochondrial genes. In contrast, endogenous Crtc2 is dispensable for the response to rotenone.

As shown in Fig. 5A (PMH) and Fig. 5B (HepG2 cells), rotenone-induced mitochondrial stress led to increased Pgc-1α mRNA expression in control, which was not altered when Crtc2 was silenced. In contrast, silencing Crtc3 blunted Pgc-1α gene induction in response to rotenone-induced mitochondrial stress. Thus, our results indicated that Crtc3 is necessary for the early induction of Pgc-1α in response to rotenone.
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Recruitment of Crib-Crtc3, but Not Crtc2, as Pgc-1α Promoter in Mitochondrial Stress-induced Mitochondrial Biogenesis—Pgc-1α gene transcription is initiated by the Crib transcription complex (4, 32). To determine that the Crib-Crtc(s) complex is involved in the initial up-regulation of Pgc-1α gene expression in rotenone-induced mitochondrial stress, we performed a ChIP assay of the CRE site of the Pgc-1α gene. Crtc3 recruitment was determined using pan-TORC antiserum as other Crtc3 antisera were not suitable for ChIP assay. Crtc2 antiserum is specific for Crtc2, whereas pan-TORC recognizes Crtc2 and Crtc3, as demonstrated by immunoprecipitation (not shown). At 6 h after removal of rotenone from PMH, one of the Crtc isoforms (Crtc2 or Crtc3 by pan-TORC antibody) and Crib were associated with each other at the CRE site, whereas Crtc2 was not (Fig. 6). Because pan-TORC, but not CRTC2 antisera, identified CRTC at the promoter, we conclude that Crib was associated more likely with Crtc3 at the Pgc-1α promoter after rotenone-induced mitochondrial stress. When Crtc3 and Crib were bound to the Pgc-1α promoter at 6 h after mitochondrial stress, Pgc-1α mRNA was induced by nearly 3-fold and was followed by increased expression of

Nrf-1, Crib transcription complex, cytochrome c, COX IV, or Hsp10 (Figs. 1B and 2A), suggesting that an initial induction of Pgc-1α by Crtc3 and Crib is involved in response to mitochondrial stress-induced mitochondrial biogenesis.

DISCUSSION

Stress responses to oxidants/redox perturbations and protein malfolding have been increasingly recognized as crucial adaptive mechanisms in balancing the promotion of and resistance to cellular injury (33–35). Such responses are mediated by the activation of transcription factors that regulate the expression of sets of genes that control organelle biogenesis as well as organelle-specific protective chaperones. Specific responses to stress in cellular compartments are mediated by distinct transducers. Nuclear translocation of heat shock factor (HSF), hypoxia-inducible factor (HIF), and nuclear respiratory factor-2 (Nrf-2) mediate heat shock (36), hypoxia-inducible (37), and oxidative stress responses in cytosol (38), and release of several transcription factors such as activating transcription factor 6 (ATF6) from ER controls the unfolded protein/ER response (39).

Induction of Pgc-1α and mitochondrial oxidative phosphorylation genes in response to transient decrease of ATP after mitochondrial uncoupler treatment of fibroblasts is mediated by CREB-CRTC signaling complex activation (8). Activated CREB has proven to be an important regulator of PGC-1α promoter in liver and muscle (40–42). In liver cells, basal PGC-1α expression is low, and the transcriptional up-regulation of PGC-1α is crucial in initiating mitochondrial biogenesis (25–29, 32, 42, 43). The expression of Pgc-1α is mediated by the activation of the transcription factor Crib either through a direct effect of PKA on the phosphorylation of Crib or through an indirect effect mediated by the CREB activator, CRTC (1–5). The CRTC family, formerly referred to as transducer of regulated CREB (TORC) protein, includes Crtc1, which is expressed mainly in brain and lymphocytes, and not in liver, and Crtc2 and Crtc3, which are ubiquitous (4). Crtc2 is localized in cytosol and mitochondria by CRTC nuclear exclusion (30). Additionally, Pin1 associates with Crtc2 at serine 136 in the nuclear localization signal and thereby attenuates FSK-induced nuclear localization of Crtc2 and binding with Crib (44). The subcellular localization and mechanism of activation of Crtc3 are less well characterized, but it appears to have a similar distribution as Crtc2 (1, 45). The N-terminal half (1–298 amino acids) of the full-length protein is a functional domain for the CREB-Tax-dependent HTLV1-LTR activation system (46), where Bcl3 is a negative regulator, by interacting with Crtc3 (47).

The regulation of the production of mitochondria and its chaperones is of great interest in understanding the control of energy metabolism and the susceptibility to hepatotoxicity. It is well known that the Crib-dependent transcriptional up-regulation of Pgc-1α is critical in mitochondrial biogenesis.
as most clearly demonstrated by Pgc-1α knock-out, which prevents mitochondrial biogenesis (7, 48–50). We confirmed the importance of Pgc-1α in mitochondrial biogenesis in response to rotenone by showing that Pgc-1α induction preceded mitochondrial biogenesis and that silencing Pgc-1α blocked the downstream expression of mitochondrial transcription factors and structural genes. The signaling mechanisms for Creb activation in metabolic scenarios involves cAMP-dependent release of Crtc2 from 14-3-3 as well as direct effects of PKA on Creb (3, 5). Our work is unique in addressing the effect of mitochondrial stress on the CREB-PGC-1α pathway. We were interested in the question of whether mitochondrial biogenesis in response to mitochondrial stress induced by mitochondrial toxin, rotenone, is dependent on CRTC, and particularly whether there is a differential contribution of Crtc2 versus Crtc3 in mediating the response. We examined this question by silencing the expression of Crtc2 or Crtc3 and then exposing primary mouse hepatocytes and HepG2 cells to rotenone and determining the expression of nuclear genes encoding transcription factors, mitochondrial structural proteins, as well as mitochondrial chaperones such as Hsp10 and Hsp60, which reflect mitochondrial biogenesis. Most importantly, when Crtc3 was silenced, mitochondrial biogenesis in response to a
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forms or a decrease in total Crtc2 or Crtc3 in cytosol in response to rotenone (not shown). However, we did identify a small fraction of Crtc3, but not Crtc2, in mitochondria (not shown), the significance of which is uncertain at present. Thus, the mechanism of selective Crtc3 activation may reflect some signal from mitochondria induced by rotenone (e.g. reactive oxygen species), which activates a subtraction of Crtc3 in cytosol, or it may reflect release of Crtc3 from mitochondria; this mitochondrial Crtc3 presumably would need to be modified so as to resist the sequestration mechanism known for the cytoplasmic pool of Crtc3.

We also found that the expression of Crtc2 and Crtc3 increased at later times after the initial Crtc3-mediated activation of Creb and induction of Pgc-1α expression. It seems likely that the later transcripational up-regulation of Crtc2 and Crtc3, possibly in a redundant fashion, is a feed-forward amplification mechanism in mitochondrial biogenesis. At present, it is clear that the response to mitochondria-specific toxic stress does not occur in the absence of Crtc3 but that Crtc2 is dispensable.

In the future, our findings will need to be considered in the context of the proposed mitochondrial stress response in which chaperone induction is mediated by C/EBP-homologous protein (51–53) and perhaps proteolytic peptides released from mitochondria (54). We did observe induction of mitochondrial chaperones in addition to mitochondrial biogenesis. The previous studies (55) of mitochondrial stress response did not address mitochondrial biogenesis. Whether Crtc3 activation participates in the context of the response to malfolding of mitochondrial proteins will need to be addressed in future studies.

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FIGURE 5. Effect of silencing of Crtc2 versus Crtc3 on rotenone-induced Pgc-1α induction. A, after 3 h in plating medium, PMH from shRNA-treated mice were rested overnight in serum-free DMEM/F12 medium, rotenone (20 μM) was added for 3 h, and then PMH was continued in culture with rotenone-free/serum-free medium for the indicated times. Pgc-1α mRNA expression was analyzed quantitatively using real-time PCR. B, Crtc2 and Crtc3 siRNA transfected HepG2 cells as described under “Experimental Procedures” were treated with rotenone (20 μM) for the indicated times, and PGC-1α mRNA expression was analyzed as described. Data are presented as mean ± S.E. (n = 3). Asterisk, p < 0.05. Student’s t test. D = DMSO.

FIGURE 6. Crtc3 recruitment to Pgc-1α promoter in PMH after rotenone treatment. A, ChIP assay of recruitment of Crtc2, Crtc3, and Creb protein to the CRE site of the Pgc-1α promoter, 6 h after rotenone treatment, PMH were fixed in paraformaldehyde, and a ChIP assay was performed. Creb and Crtc3, but not Crtc2, were recruited to the Pgc-1α promoter 6 h after rotenone treatment when Pgc-1α gene expression was increased. Simultaneous recruitment of Creb and Crtc3 was confirmed in the reChIP assay. B, recruitment of Crtc2 to Pgc-1α promoter by FSK (10 μM) treatment was observed using anti-Crtc2. NS = rabbit normal serum. Representative data of three experiments are shown.

mitochondrial toxin was inhibited, whereas silencing Crtc2 did not inhibit this response. Crtc3, but not Crtc2, was associated with CREB at the Pgc-1α promoter (ChIP assay), indicating that Crtc3 translocates to this site at the time Pgc-1α expression is induced in response to rotenone.

We do not have a complete understanding of the mechanism for the selectivity of the response of Crtc3. Both Crtc2 and Crtc3 are mainly sequestered in cytosol as phosphorylated forms bound to 14-3-3 (1), and a small fraction of both is found in the ER. We did not find an increase in nonphosphorylated
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