Activation of Orphan Nuclear Constitutive Androstan
terone Requires Subnuclear Targeting by Peroxisome
Proliferator-activated Receptor \( \gamma \) Coactivator-1\( \alpha \)

A POSSIBLE LINK BETWEEN XENOBIOTIC RESPONSE AND NUTRITIONAL STATE*

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In contrast to the classical nuclear receptors, the constitutive androstan receptor (CAR) is transcriptionally active in the absence of ligand. In the course of searching for the mediator of CAR activation, we found that ligand-independent activation of CAR was achieved in cooperation with the peroxisome proliferator-activated receptor \( \gamma \) coactivator-1\( \alpha \) (PGC-1\( \alpha \)). PGC-1\( \alpha \), a PGC-1\( \alpha \) homologue, also activated CAR to less of an extent than PGC-1\( \alpha \). Coexpression of the ligand-binding domain of a heterodimerization partner, retinoid X receptor \( \alpha \), enhanced the PGC-1\( \alpha \)-mediated activation of CAR, although it had a weak effect on the basal activity of CAR in the absence of PGC-1\( \alpha \). Both the N-terminal region, with the LXXLL motif, and the C-terminal region, with a serine/arginine-rich domain, in PGC-1\( \alpha \) were required for full activation of CAR. Pull-down experiments using recombinant proteins revealed that CAR directly interacted with both the LXXLL motif and the RS domain. Furthermore, we demonstrated that the RS domain of PGC-1\( \alpha \) was required for CAR localization at nuclear speckles. These results indicate that PGC-1\( \alpha \) mediates the ligand-independent activation of CAR by means of subnuclear targeting through the RS domain of PGC-1\( \alpha \).

Nuclear receptors comprise a large family of ligand-regulated transcription factors that mediate various physiological responses (1). Nuclear receptors are composed of highly conserved structural domains, including a DNA-binding domain (DBD) and a ligand-binding domain (LBD). In response to ligand binding, the LBDs of nuclear receptors undergo a structural rearrangement, resulting in the recruitment of transcriptional coactivators, such as CBP, cAMP-response element-bind-

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‡ The abbreviations used are: DBD, DNA-binding domain; LBD, ligand-binding domain; CBP, cAMP-response element-binding protein; P/CAB, p300/CBP-associated factor; PPAR\( \gamma \), peroxi-
some proliferator-activated receptor \( \gamma \); PGC-1\( \alpha \), PPAR\( \gamma \) coactivator-1\( \alpha \); CAR, constitutive androstan receptor; PXR, pregnane X receptor; andro-
stanol, 5a-androst-3\( \alpha \)-ol (androstenol) and 5\( \alpha \)-androst-16-en-3\( \alpha \)-ol (androstenol) (7). Yet, it is still unclear whether or not these steroids are true natural ligands for CAR, because a superphysiological concentration was required to inhibit the CAR activity. Thus, the elucidation of the mechanism of CAR activation is a current issue. During a search for a coactivator coupled with CAR, we found that the transcriptional coactivator PGC-1\( \alpha \) mediated the ligand-independent activation of CAR. PGC-1\( \alpha \) was originally identified as a PPAR\( \gamma \)-binding protein in a cell line derived from brown adipose tissue (8) and subsequently was shown to couple with several nuclear receptors (9–15). Unlike other transcriptional coactivators, PGC-1\( \alpha \) has the distinctive structural features of a serine/arginine-rich domain (RS domain) and an RNA recognition motif (RRM) at the C-terminal end, which are both observed in many splicing factors (16). We demonstrate here that the RS domain of PGC-1\( \alpha \) plays an important role for CAR activation by interacting with CAR and targeting CAR to nuclear speckles. The significance of the interaction of CAR with PGC-1\( \alpha \), which is considered to be a key modulator in glucose homeostasis, is also discussed.

EXPERIMENTAL PROCEDURES

Plasmids—The plasmids pCMX-FLAG-mPGC1\( \alpha \), pCMX-FLAG-

mPGC1\( \beta \), pCMX-GAL4-hERR3 (amino acids 173–436), pCMX-GAL4-
hPPAR\( \gamma \)2 (amino acids 204–506), pCMX-GAL4-hPXR (amino acids

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before transfection, the cells were plated in 24-well plates at a density of 10^5 cells per well. The cells were incubated with 10% fetal calf serum (Hyclone Laboratories). One day after transfection, the medium was changed to phenol red-free Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% charcoal/dextran-treated fetal calf serum (Invitrogen) containing 150 mM NaCl, 0.1 mM EDTA, and 10% FBS.
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The large fragment was recircularized by ligation, giving rise to the EcoRI-EcoRI mutant. The small fragment was circularized by self-ligation, giving rise to the PGC-1-EcoRI fragment of TFIIB, excised from phIIB (obtained from Dr. D. Reinberg, Harvard University), into XbaI-BamHI-digested pCMX-GAL4 DBD. The pCMX-GAL4-TFIIb plasmid was constructed by inserting an NheI-BamHI fragment of TFIIb, excised from phIIb (obtained from Dr. D. Reinberg, Harvard University), into XbaI-BamHI-digested pcDNA (obtained from Dr. K. Simon). The HindIII-KpnI fragment containing a FLAG peptide sequence was excised from pCMX-GAL4-FLAG1p and inserted into HindIII-KpnI-digested pCMX-GAL4 RXRα, giving rise to pCMX-GAL4-RXRα.

For the enhanced green fluorescent protein (EGFP) fusion protein, the EcoRI-BamHI fragment of hCAR LBD was cloned in-frame with NheI and XhoI, and the large fragment was recircularized by ligation, giving rise to the PGC-1-Nhe deletion mutant. The large fragment was blunted by Klenow DNA polymerase and was circularized by self-ligation, giving rise to the PGC-1-Nhe/XhoI mutant. The pCMX-FLAG-mPGC1α plasmid was digested with XhoI, blunted by Klenow DNA polymerase, and circularized by self-ligation, giving rise to the PGC-1-XhoI mutant. The pCMX-FLAG-mPGC1α plasmid was digested with EcoRI, and the large fragment was circularized by self-ligation, giving rise to the PGC-1-EcoI mutant. The EcoRI-MfeI fragment containing the RS domain of PGC-1α was inserted into the EcoRI-digested PGC-1-EcoI mutant, giving rise to the PGC-1-MfeI mutant.

The EcoRI-Ncol fragment of hCAR LBD was cloned in-frame with the glutathione S-transferase (GST)-encoding sequence of pEGXP61 (Amersham Biosciences). Deletion mutants of GST-fused PGC-1α were constructed in a similar manner as described above. For the S tag fusion protein, PGC-1α was digested with the indicated restriction enzymes and cloned into pET30 (Novagen).

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PGC-1α-mediated Activation of CAR

RESULTS

Functional Coupling of PGC-1α with CAR—To find the mediator of CAR activation, we examined the coupling of various nuclear receptor coactivators with CAR (Fig. 1A). The CAR LBD was fused with the GAL4 DBD and was recruited to the promoter region with the GAL4 binding element, to drive the expression of the luciferase reporter gene. After transfection of the respective plasmids into COS-7 cells, transcriptional activation was measured by the luciferase activity. Without any exogenous coactivator, CAR showed the constitutive activity (white bar). Cotransfection of various coactivators with CAR revealed that PGC-1α strongly enhanced the CAR activity. PGC-1α also stimulated CAR activity to a lower extent than CAR-1a. Other coactivators, such as Tip60, TFIID, and P/CaF, did not stimulate the CAR activity. Next, we compared the magnitude of CAR activation by PGC-1α with that of other nuclear receptors (Fig. 1B). PGC-1α also enhanced the ERR3 and PPARγ activities in the absence of their ligands, as well as the CAR activity. A similar level of transcriptional enhancement by PGC-1α was observed with agonist-stimulated ERα.

Effects of Putative Ligands on PGC-1α-mediated CAR Activation—The sex steroids androstened and androstenol reportedly inhibit CAR activity (7, 17). In fact, we observed that androstened (10 μM) partially inhibited the basal activity of CAR (Fig. 2A). In contrast, androstened (10 μM) weakly activated PXR, which is consistent with the previous report (17).

Effects of Heterodimerization with RXRs on PGC-1α-mediated CAR Activation—RXRs is known to heterodimerize with CAR to enhance the constitutive activity of CAR (7, 18) and its androstenedol sensitivity (7, 19). We therefore investigated the effect of heterodimerization with RXRs on the PGC-1α-mediated activation of CAR (Fig. 3). Because RXRs itself was slightly activated by PGC-1α, as shown in Fig. 1, we used the construct of RXRs LBD that was not fused with GAL4 DBD, to avoid the direct recruitment of PGC-1α by RXRs to the promoter. In fact, PGC-1α did not couple with the RXRs LBD without the GAL4 DBD (lane 2). Coexpression of the RXRs LBD had a weak effect on the basal activity of CAR but enhanced the PGC-1α-mediated CAR activation (lanes 3 and 4). The RXRs LBD had no effect on the PXR basal activity and a very weak effect on the PXR activity when coexpressed with PGC-1α (lanes 5 and 6). Although RXRs enhanced the PGC-1α-mediated activation of CAR, the coexpression of the RXRs LBD did not alter the required domains of PGC-1α for CAR activation (Fig. 4 and data not shown). Then we further characterized the PGC-1α-mediated CAR activation with RXRs.

Functional Domains within PGC-1α Required for CAR Activation—PGC-1α has a putative nuclear receptor interaction region containing the LXXL motif, which is conserved in several nuclear receptor coactivators, the RS domain, and the RRM, the latter two of which are observed in many non-snRNP splicing factors (16). To define the functional domains of PGC-1α required for CAR activation, we made a series of deletion mutants of PGC-1α (Fig. 4A) and investigated their ability to activate CAR using the luciferase assay (Fig. 4B, panel a). Removal of the LXXL motif (PGC-1α-Nhe/Xba) de-
increased but did not abolish the ability of PGC-1α to activate CAR. The deletion of RRM (PGC-1-Mfe) did not affect the CAR activation, whereas the deletion of both the RRM and RS domains (PGC-1-Xba and PGC-1-Eco) further reduced the ability. A deletion mutant lacking all three regions (PGC-1-Nhe) completely lost the ability to activate CAR. These results indicated that both the LXXLL motif and the RS domain in PGC-1α are required for the full activation of CAR.

Next we investigated whether the requirement of these domains for the enhancement was specific to CAR. We took advantage of ERR3, which was activated by cotransfection of mains for the enhancement was specific to CAR. We took ad-

The regions within PGC-1α that interact with CAR LBD—We asked whether PGC-1α would physically interact with the CAR LBD and which domains were required for the interaction. Deletion mutants of GST-fused PGC-1α were expressed and purified from Escherichia coli (Fig. 5A, panels a and b). The CAR LBD was purified as a GST-fused protein, and then the GST portion was removed by PreScission cleavage. Panel α, the complex was separated by SDS-PAGE and was visualized by silver staining. Panel b, the PGC-1α fragment was detected by Western blotting using an anti-His antibody.
and visualized by Western blotting using an anti-CAR antibody (Fig. 5A, panel c). The CAR LBD was able to bind to PGC-1-Eco (lane 5), PGC-1-Xba (lane 6), and PGC-1-full (lane 7) but not to PGC-1-Nhe (lane 4). The difference in the CAR binding ability between PGC-1-Eco and PGC-1-Nhe indicated that the region containing the LXXLL motif was required for the association with the CAR LBD. A tighter interaction was observed in PGC-1-Xba (lane 6) and PGC-1-full (lane 7) than in PGC-1-Eco (lane 5). Although PGC-1-Xba and PGC-1-full were contaminated with many lower bands (Fig. 5A, panel b), the CAR interaction was not correlated with those lower bands. These results suggest that PGC-1α interacts with the CAR LBD through the C-terminal region in addition to the LXXLL motif.

We produced additional deletion mutants lacking the LXXLL motif, as His- and S-tagged (His-Stag) proteins, to locate other regions of PGC-1α interacting with the CAR LBD, besides the LXXLL motif region (Fig. 5B, panels a and b). The CAR LBD was mixed with the Stag-PGC-1α proteins immobilized onto S-protein beads, and the bound CAR LBD was pulled down and visualized by Western blotting using an anti-CAR antibody (Fig. 5B, panel c). The CAR LBD was able to bind to these His-Stag PGC-1α proteins (Fig. 5B, panel c), indicating that multiple regions of PGC-1α directly interact with CAR. It was notable that the NeoI-MfeI fragment, containing the RS domain, directly bound to CAR (lane 5), which possibly reflects the fact that the RS domain had a crucial effect on CAR activation (Fig. 4B, panel a).

Purification of the Recombinant CAR LBD-CCP-1α RS Domain Complex—To prove that the CAR LBD directly interacts with the RS domain of PGC-1α, we coexpressed the GST-fused CAR LBD and the His-tagged RS domain in E. coli and purified the complex of these two proteins. Serial affinity purification allowed us to isolate the CAR LBD-CCP-1α RS domain complex (Fig. 5C). The complex was eluted from glutathione-Sepharose beads by cleaving off the GST tag by PreScission protease, which ensured the direct binding of PGC-1α to the CAR LBD rather than nonspecific binding to the column (Fig. 5C). Aliquots of the eluate were subjected to SDS-PAGE and were analyzed by silver staining (Fig. 5D, panel a). The protein appeared as a 29-kDa band, corresponding to the CAR LBD. The coeluted 37-kDa protein was identified as a PGC-1α fragment by Western blotting using an anti-His antibody (Fig. 5D, panel b).

PGC-1α-dependent Arrangement of CAR Subnuclear Localization—In the course of the experiment investigating the co-localization of CAR with PGC-1α in cells, we found that CAR moved to nuclear speckles when PGC-1α was coexpressed. We used the EGFP-fused CAR LBD to determine the localization of CAR (Fig. 6A, center panel), which showed a distribution similar to that of the GAL4 DBD-fused CAR, as determined by immunofluorescence (Fig. 6A, left panel). Coexpression of RXRa did not affect the distribution of CAR (Fig. 6B). When PGCA-1α was coexpressed, CAR colocalized at nuclear speckles with PGC-1α (Fig. 6C, panel a). In all of the 50 total cells counted, colocalization in nuclear speckles was observed (100%). A significant amount of CAR was still present in the cytoplasm even when PGC-1α was coexpressed, suggesting that the subnuclear distribution, but not the cytoplasm-nuclear shuttling of CAR, is important for the activity. PGC-1α expressed without CAR localized at nuclear speckles; however, a diffusely distributed signal was observed in the entire nucleus (Fig. 6C, panel a, PGC-1α alone). Coexpression of CAR with PGC-1α seemed to weaken the diffuse distribution of PGC-1α in the nucleus and to intensify the signal in nuclear speckles, suggesting that the binding of CAR and PGC-1α might cause a mutual effect on each other.

We used the deletion mutants of PGC-1α to investigate which domain of PGC-1α was responsible for the subnuclear targeting of CAR. The PGC-1-Nhe/Xba mutant lacking the LXXLL motif was able to shift the localization of CAR to the nuclear speckles (Fig. 6C, panel b). Of 44 total cells counted, 30 cells showed colocalization of CAR and PGC-1α in nuclear speckles (68%). The PGC-1-Mfe mutant lacking RRM was not able to localize in nuclear speckles by itself (Fig. 6C, panel c, PGC-1 alone). This observation is consistent with the previous report (20). Interestingly, the localization of PGC-1α in nuclear speckles was observed even in the absence of RRM but only when CAR was coexpressed (Fig. 6C, panel c). Colocalization of CAR and PGC-1α was observed in 32 of the 38 total cells counted (84%). Using the PGC-1-Xba mutant lacking both the RS domain and RRM, neither CAR nor PGC-1α localized in the nuclear speckles (Fig. 6C, panel d). The PGC-1-Eco mutant no longer showed exclusive nuclear localization, because of the absence of a nuclear localization signal (Fig. 6, C, panel e, and D). A comparison between the activity and the localization suggested that the colocalization in nuclear speckles may be a limited process for PGC-1α-mediated CAR activation.

DISCUSSION

In this study, we report that the transcriptional coactivator PGC-1α mediates the ligand-independent activation of the nuclear receptor CAR. Our results showed that the N-terminal region containing the LXXLL motif and the C-terminal region containing the RS domain participate in the interaction and the activation with CAR. Both regions were necessary for full activation. In addition, the RS domain was required for colocalization of PGC-1α in nuclear speckles with CAR. These data indicated that the ligand-independent activation of CAR by PGC-1α is achieved by subnuclear targeting through the RS domain of PGC-1α.

CAR shows constitutive activity in the absence of exogenous ligand when transfected into cell lines, such as HepG2 and CV-1 cells; however, it is reportedly inactive in primary cultured hepatocytes because of its cytoplasmic localization (21). This study gave us the idea that an additional factor may be required for the constitutive activity of CAR. In the course of searching for a coactivator coupling with CAR, we found that PGC-1α greatly enhanced the CAR activity in the absence of ligand. This activation was specific to PGC-1α, because several other coactivators we examined did not show the enhancement. This link of CAR with PGC-1α surprised us, because the liver is not the major organ of PGC-1α expression (8). However, important experimental data have recently described how PGC-1α expression in the liver is dynamically regulated by the nutritional state of the body; PGC-1α is induced by starvation or fasting and plays a critical role in gluconeogenesis (12, 22).

Recently, the crystal structures of several orphan nuclear receptors, including ultraspiracle, hepatocyte nuclear factors (HNF4α and HNF4γ), retinoic acid-related orphan receptor β, ERR3, and PXR, have been solved (23–28). Notably, the crystals of the ultraspiracle, HNF4α, HNF4γ, and retinoic acid-related orphan receptor β LBDs prepared from E. coli contained lipid ligands, presumably derived from cell lipid constituents, in the hydrophobic pocket. According to these studies, one possible difference between the cell lines and the primary hepatocytes was that CAR might be activated by an unknown endogenous ligand produced by the cell lines. Alternatively, a natural ligand, which exists in the primary hepatocytes but not in the established cell lines, may repress the CAR activity, and its repression might be modulated by the interaction with PGC-1α. The CAR reverse agonist, androstenol, did not completely inhibit the PGC-1α-mediated CAR activation, although the human CAR, which we used, is less sensitive to...
Because CAR forms a heterodimer with RXRα (7, 18, 19), we assessed the effect of the heterodimerization between CAR and RXRα on the PGC-1α coactivation. RXRα itself slightly coupled with PGC-1α in the absence of ligand, but the enhancement of PGC-1α-mediated CAR activation by the RXRα LBD was not additive but synergistic, suggesting that the CAR-RXR heterodimer may stabilize PGC-1α binding to CAR rather than CAR and RXRα binding to PGC-1α independently. Using deletion mutants of PGC-1α, we observed that the coexpression of the RXRα LBD did not change the domains of PGC-1α required for CAR activation (data not shown), supporting the idea that RXRα stabilizes the binding. These results suggest that the heterodimerization with RXRα does not explain the constitutive activity of CAR. This idea does not contradict the in vivo observation that RXRα remains at the nucleus, even when CAR is inactive in the cytosol in hepatocytes (21).
PGC-1α reportedly couples with several other nuclear receptors in ligand-dependent and ligand-independent manners (8–15). PGC-1α interacts with these nuclear receptors through the LXXLL motif in its N-terminal region. In this study, we observed that CAR also directly interacted with the region containing the LXXLL motif. In addition, we found that another region containing RS domain in PGC-1α also interacted with CAR by pull-down experiments and probed the complex formation using purified proteins. The C-terminal fragment containing the RS domain and RRM reportedly interacts with an ERα hinge region in a ligand-independent manner (10). Taken together, the RS domain in PGC-1α may provide a novel interface to nuclear receptors. The interaction between PGC-1α and nuclear receptors through multiple domains may confer the specificity of the coactivation.

The RS domain as well as RRM in PGC-1α is shared with SR proteins, which are essential splicing factors that participate in mRNA processing through their RS domains and RRMs (20, 29). PGC-1α diffusely localized at the nucleus and concentrated in the nuclear speckles, where splicing factors are colocalized (20). Nuclear speckles of splicing factors are considered to be a site for storage and assembly of splicing factors (30). Nuclear speckles are highly dynamic structures. Upon gene activation, the splicing factors, visualized by GFP fusions, are released from the nuclear speckles to transcriptionally active sites (31). In the absence of RRM, PGC-1α did not reside in the nuclear speckles by itself; however, when CAR was coexpressed, the PGC-1α moved to nuclear speckles together with CAR, suggesting that the coexpression of CAR and PGC-1α has a mutual effect on their localization (Fig. 6D). This targeting activity was totally dependent on the RS domain and was observed even in the absence of the LXXLL motif in PGC-1α. The RS domains in the splicing factors are reportedly required for both targeting to and dissociation from nuclear speckles (32–34). These results strongly suggest that CAR binds to PGC-1α through the RS domain and rearranges the domain to acquire the ability to move to the nuclear speckles for transcriptional activation or for altering mRNA processing. It is interesting that the CAR-mediated transcriptional activation of cytochrome P450 by xenobiotics in hepatocytes is abolished by the serine/threonine-specific phosphatase inhibitor, okadaic acid (21), which is also known to prevent the movement of splicing factors into nuclear speckles (35). The involvement of the serine/threonine-specific phosphatases in regulating PGC-1α-mediated CAR activation will be investigated in the future.

Although the physiological relevance of PGC-1α-mediated CAR activation will be needed for further experiments, our data strongly suggest that PGC-1α regulates the activity of CAR in hepatocytes. Microarray data have suggested that CAR regulates several enzymes catalyzing fatty acid or carbohydrate metabolism (36). Thus, it would be intriguing to investigate the CAR activity in the liver under different nutritional conditions and the PGC-1α levels after exposure to xenobiotics, which would provide clues to the relationships between these important pathways, drug catalysis and metabolic conditions.

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