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Activation of peroxisome proliferator-activated receptors (PPARs) by their ligands and protein kinase A activators

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ABBREVIATIONS

AF, activation domain;

Bro, bromopalmitate;

8-Br-cAMP, 8-Bromoadenosine 3':5'-cyclic monophosphate;

CAT, chloramphenicol acetyl-transferase;

CT: cholera toxin;

DBD, DNA binding domain;

FSK, forskolin;

GST, glutathione S-transferase;

H89, N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl;

IBMX, isobutyl methylxanthine;

LBD, ligand binding domain;

PAGE, polyacrylamide gel electrophoresis;

PKA, protein kinase A;

PPAR, peroxisome proliferator-activated receptors;

RXR, retinoid X receptor;

TK, thymidine kinase;

ABSTRACT

The nuclear peroxisome proliferator-activated receptors (PPARs) α, β and γ activate the transcription of multiple genes involved in lipid metabolism. Several natural and synthetic ligands have been identified for each PPAR isotype but little is known about the phosphorylation state of these receptors. We show here that activators of protein kinase A (PKA) can enhance mouse PPAR activity in the absence and the presence of exogenous ligands in transient transfection experiments. The activation function 1 (AF-1) of PPARs was dispensable for transcriptional enhancement, whereas the activation function 2 (AF-2) was required for this effect. We also show that several domains of PPAR can be phosphorylated by PKA *in vitro*. Moreover, gel experiments suggest that PKA stabilizes binding of the liganded PPAR to DNA. PKA inhibitors decreased not only the kinase dependent induction of PPARs but also their ligand-dependent induction, suggesting that the ligands may also mobilize the PKA pathway to lead to maximal transcriptional induction by PPARs. Moreover, comparing PPARα KO with PPARα wild-type mice, we show that the expression of the ACO gene can be regulated by PKA-activated PPARα in liver. These data demonstrate that the PKA pathway is an important modulator of PPAR activity and we propose a model associating this pathway in the control of fatty acid β-oxidation under conditions of fasting, stress and exercise.

INTRODUCTION

PPARα, β and γ (NR1C1, NR1C2, NR1C3)(1), which are encoded by separate genes and exhibit distinct tissue-distribution, belong to the nuclear receptor superfamily (2). This family includes receptors for sexual and adrenal steroids, retinoids, thyroid hormone, vitamin D, ecdysone, and a number of receptors whose ligands are still unknown and therefore are called orphan receptors (3, 4). PPARs were first shown to be activated by substances that induce peroxisome proliferation (5, 6). PPARs share a common structure of 5 domains named A/B, C, D, E (the F domain is absent from PPARs compared with other members of the superfamily) (7). Key functions have been assigned to each of these domains (for review, (2, 8)). The N-terminal A/B domain contains the ligand-independent transcription activation function 1 (AF-1) (9). The C domain has a characteristic helix-loop-helix structure stabilized by two zinc atoms and is responsible for the binding to peroxisome proliferator response elements (PPREs) in the promoter region of target genes. The D domain is a hinge region which can modulate the DNA binding ability of the receptor and which is involved in corepressors binding (10). The E domain has a ligand binding function and exhibits a strong ligand-dependent activation function (AF-2). In a classical manner, the ligand binding domain facilitates the heterodimerization of PPAR with the retinoid X receptor (RXR). In its active state, this heterodimer is able to associate with coactivators (11-13) and when bound to a PPRE to modulate the expression of target genes.

It was also recently demonstrated that phosphorylation can modulate PPAR activity (14-21), but there are no data available concerning the phosphorylation of PPAR by the protein kinase A (PKA) pathway. PKA activity is enhanced in the liver under conditions of stress, fasting or exercise (22, 23). Under these conditions, PPAR target genes such as AcylCoA Oxidase, are up-regulated (24-26).

The aim of this work was to investigate whether PKA activators, mimicking stress, fasting or exercise, could directly modulate the activity of PPARs. We performed a detailed analysis of the effects of PKA on the activity of the mouse PPARα, β and γ isotypes. We observed a PKA-dependent enhancement of the activity of all three isotypes in a ligand-independent and ligand-dependent manner on two distinct promoters. Several but not all PKA activators were able to produce this effect. Moreover, PKA inhibitors were able to repress the action of PKA activators. Very interestingly, PKA inhibitors were able to repress the effect of PPAR ligands by 50 to 75%, suggesting that these ligands are acting in part by recruiting the PKA pathway. By using GST fusions of PPARs, we demonstrate that PKA is able to phosphorylate different subdomains of PPARs *in vitro*, the DBD being the main phosphorylation target. Finally, using hepatocytes isolated from WT and PPARα KO mice, we show that PKA activators can enhance the expression of certain PPARα target genes and that PKA inhibitors repress WY 14, 643 dependent stimulation of these genes. These findings highlight the involvement of the PKA pathway in PPAR action and furthermore suggest that it is essential for the regulation of gene activation by PPAR ligands.

RESULTS

PKA activators enhance PPARα activity on PPRE containing promoters. To evaluate the effect of PKA on PPAR activity, we transfected HEK-293 cells with a mPPARα expression vector along with the PPRE-containing 2CYPA6-TK-CAT reporter construct or the TK-CAT construct as a control (Fig 1). Cells were treated or not with WY 14,643 (a PPARα ligand) and/or cholera toxin (CT) as a PKA activator. In the absence or the presence of cotransfected PPARα, expression of the TK-CAT construct was not significantly affected by WY 14, 643 nor by CT. In the absence of PPARα, the 2CYPA6-TK-CAT construct, which contains functional PPREs (PPAR response element), was not stimulated by WY 14,643 nor by CT. When the PPARα expression vector was cotransfected, PPARα stimulated the activity of the 2CYPA6-TK-CAT construct in the presence of WY 14,643 (up to 7 fold compared to PPARα in the absence of ligand). Addition of CT to the WY 14,643 treatment produced a further enhancement of PPARα activity of about 2 fold compared to WY 14,643 treatment alone. In the absence of WY 14,643, CT by itself had also a stimulatory effect of about 2 fold. These results demonstrate that PKA activators can enhance PPARα activity.

Dose dependent activation of mouse PPARs by their ligands. To further evaluate the effect of PKA on mouse PPAR activity, we transfected mPPARα, β and γ expression vectors along with the 2CYPA6-TK-CAT reporter construct (Fig 2). Cells were treated or not with PPAR ligands (WY 14,643 for mPPARα, bromopalmitate for mPPARβ and BRL 49,653 for mPPARγ) and/or CT. PPARα stimulated the activity of the reporter construct in a WY 14,643 dose dependent manner. Addition of CT to the WY 14,643 treatment produced a further enhancement of PPARα activity even at saturating concentrations of WY 14,643. The same enhancement of activity by CT in the presence of ligand was obtained with PPARβ and with PPARγ. Since these observations were made with mouse PPARs, we tested whether

amphibian PPARs would behave similarly. We observed that amphibian PPAR activity was also enhanced by PKA in a ligand-independent and ligand-dependent manner (data not shown), thus suggesting that the PPAR response to the PKA pathway is well conserved throughout evolution.

PPAR activity enhancement by PKA can be obtained with PKA activators and mediated by different PPREs. To ensure that the results obtained above were not dependent on the 2CYPA6-TK-CAT reporter construct only, we tested the effect of PKA on the PPAR-dependent stimulation of the ACO-TK-CAT reporter construct (Fig. 3). This construct, which contains the PPRE containing region of the ACO gene promoter was inducible by mouse PPARα, β and γ in the presence of exogenous ligands, eventhough the effect was less pronounced than with the 2CYPA6-TK-CAT reporter gene. Addition of CT was able to increase PPAR activity 2 to 3 times. Interestingly, the ligand-independent effect of CT on PPAR activity was more pronounced on the ACO-TK-CAT reporter than on the 2CYPA6-TK-CAT reporter. These data suggest that the enhancement by PKA of PPAR activity can be mediated by distinct PPREs.

To determine whether other PKA activators (see Fig. 4A) had similar effects as CT (a Gs protein activator), we tested 8-Br-cAMP (a cAMP analog), forskolin (an adenylate cyclase activator) and IBMX (a phosphodiesterase inhibitor) in transient transfection with the mouse PPARα expression vector and the 2CYPA6-TK-CAT reporter construct (Fig. 4B). CT and forskolin had roughly the same activation ability, while 8 Br-cAMP and IBMX had no significant effect even when higher concentrations of these activators were used (data not shown). These results suggest that PKA activators acting directly on cAMP production are the major stimulators of PPAR activity.

Ligand activation of PPARs involves the PKA pathway. Next, we tested whether ligand activation of PPARs was also involving the PKA pathway, even in the absence of added PKA

activators. To answer to this question, we used the H89 compound (a specific inhibitor of PKA at low concentrations) (27) in transient transfection experiments (Fig 5A). We observed that H89 was able to repress not only CT-dependent induction of PPARα, β and γ activity but also the ligand-dependent activation as well as the activation due to CT and ligand used together. In addition, the basal receptor activity, in the absence of exogenous ligand, was also repressed. These data suggest that PKA affects both the basal and ligand-regulated activities of the PPARs. To ensure that the effects observed with CT or H89 were not due a change in PPAR expression, we checked by western blot the levels of expression PPARα after treatment with WY 14,643, CT or H89 (Fig. 5B). PPARα protein was only detected after transfection of PPARα expression vector. Moreover, PPARα protein levels were unchanged following treatment with WY 14,643, CT or H89, alone or in combination. These data demonstrate that the effects observed in transfection assays are not due to changes in PPAR expression levels. We then checked whether these drugs could affect PPAR DNA binding. The same extracts as in Fig. 5B were used to perform a gel shift assay using the PPRE from the ACO gene (Fig. 5C). We observed a strong binding with PPARα WCE in the absence of added drug. Surprisingly, WY 14,643 treatment decreased PPARα DNA binding ability. CT also diminished PPARα DNA binding ability but to a lesser extent. More interestingly, the combination of WY 14,643 and CT enabled PPAR to bind to DNA more strongly than WY 14,643 or CT treatment alone. This in agreement with the situation observed for ERα for which PKA inhibits the dimerization of the receptor in the absence of ligand (28). Finally H89 had the same ability to reduce PPAR DNA binding as did WY 14,643. These data suggest that CT acts by stabilizing the decreased DNA binding ability of the liganded receptor in this *in vitro* assay.

RXR contributes to PPAR activation by PKA. As RXR is an obligate heterodimerization partner of the PPARs for DNA binding and transactivation, we determined whether RXR

could be involved in the PKA activation of PPARα (Fig 6A). In the absence of transfected RXR or PPAR, the activity of the 2CYPA6-TK-CAT construct was very low and not modulated by the WY 14,643 and CT. In the absence of transfected RXR, transfected PPAR was active and modulated by PKA in HEK-293 cells, as these cells express low levels of endogenous RXR. In contrast, transfection of RXR alone in these cells had almost no effect on the expression of the 2CYP4A6-TK-CAT reporter gene even in the presence of 9-cis-retinoic acid (9cRA, is a ligand of RXR). However, we observed an enhancement of PPARα activity in the presence of 9cRA and CT both in the absence and in the presence of WY 14,643. Indeed, enhancement of the PPARα activity was even more potent with CT + 9cRA than with WY 14,643 + 9cRA. On the contrary, in the presence of WY 14,643, 9c-RA had only a minor effect. By overexpressing simultaneously RXR and PPARα, in the absence of 9cRA, we observed an increase by about 30% of PPARα activation by WY 14,643, and a 2 fold activity enhancement in the presence of CT and without ligand compared to PPARα without cotransfected RXR. RXR affected only moderately PPARα activation (about 20%) by WY 14, 643 + CT. In the presence of RXR and 9cRA and in the absence of WY 14,643 and CT, we observed a 3 fold enhancement of PPARα activity compared to cells without 9cRA. In the presence of WY 14,643 or WY 14,643 + CT, 9cRA only increased by 30% the activity seen in the absence of 9cRA. Finally, 9cRA was unable to affect CT induction of PPARα in the absence of WY 14,643. These data suggest that RXR cooperates with PPARα in the absence of exogenous ligand to increase both the basal and CT-induced activity of PPARα on PPREs. We next checked whether RXR was itself the target of PKA when bound to its preferred binding site (DR1). To do so, we used the DR1-TK-CAT construct containing a strong RXR binding site (Fig. 6B). We observed a strong activation of the construct by RXR in the presence of RA. CT treatment increased both ligand-independent and ligand-dependent

activity of RXR. Thus, RXR by being itself the target of PKA can enhance PPAR activity on PPREs.

AF-1 is dispensable for PPAR stimulation by PKA activators. In order to explore which domain of PPAR is the target of the PKA pathway, we created 3 truncated versions of PPARα (Fig 7A): one was deleted of the entire AB region (lacking AF-1, ΔAB mPPARα), and the 2 others lacked the AF-2 domain as the entire LBD (ΔLBD mPPARα) or the last 13 C-terminal residues (ΔAF2 mPPARα) were deleted (Fig. 7A). Surprisingly, the ΔAB mPPARα construct had roughly the same transactivation ability and exhibited the same enhancement of activity by CT as the wild-type mPPARα. On the contrary, the ΔLBD and ΔAF2 constructs were totally insensitive to WY 14,643 or CT treatments. These data suggest that the AF-2 region is the major mediator of the effects of PKA activators and that the AF-1 region is not essential. As a control, we checked the DNA binding ability of the mutants by performing a gel shift assay using ACO PPRE (Fig. 7B). We *in vitro* translated the different mutants and checked first that they were produced in similar amounts (data not shown). We observed that ΔAB had the same ability to bind to DNA as wild-type PPARα, whereas ΔAF2 and ΔLBD had a weak or not detectable binding ability respectively. This lack of binding of ΔLBD mutant is in agreement with previous reports (29). However, the weaker DNA binding ability of the ΔAF2 mutant cannot explain totally the lack of responsiveness to CT and we rather hypothesized that the AF2 function was involved in PKA stimulation. To confirm this hypothesis, we constructed GAL4 chimera comprising either the AB domain or the LBD of PPARα fused to the GAL4 DNA binding domain (Fig. 7C). In transfection assays in HEK-293 cells, the GAL-AF1 chimera exhibited a strong ligand independent activity. This product was insensitive to WY 14,643 and CT alone or in combination, whereas the activity of the GAL-AF2 chimera was synergistically enhanced by WY 14,643 and CT treatments.

Interestingly, the GAL-AF2 chimera was not significantly affected by CT treatment alone, suggesting that other domains are involved in the effects of CT.

The main phosphorylation target of PKA activators is the DBD. To investigate which regions of PPARs were phosphorylated by PKA, we constructed a set of different GST fusion proteins corresponding either to the AB, DNA-binding (DBD) or ligand-binding (LBD) domains of the mouse PPARα and β. The fusion proteins produced in bacteria were then purified on GSH columns and submitted to PKA treatment with recombinant enzyme in the presence of γ-[^{32}P]-ATP (Fig. 8A). We observed a strong phosphorylation of the DBD and a weaker labelling of the LBD from PPARα and PPARβ, suggesting that the DBD is the main phosphorylation target. These data are in agreement with the effects of CT on DNA binding of PPARα (see above). Interestingly, the AB domain of mPPARα was also phosphorylated at a low level. We then mapped the putative sites of PKA phosphorylation (Fig. 8B). The most likely sites were mapped in the AB, C and E domain confirming that several domains of PPAR are potential targets of PKA phosphorylation.

PKA modulates PPARα target genes. It was of interest to determine the effect of the PKA pathway on endogenous genes regulated by PPARα *in vivo*, and particularly in liver which is one of the main sites of PPARα action. To this end, isolated hepatocytes either from wild-type mice or from PPARα KO mice (30) were cultured *in vitro* and treated with PKA activators or inhibitors in the absence or the presence of WY 14,643. The expression of the ACO (peroxisomal acylCoA oxidase) and the FABP (fatty acid binding protein) genes, two PPARα target genes was analysed by northern blot (Fig. 9A). Under the conditions used, the ACO gene was not induced by WY 14,643 alone in the cultured wild-type hepatocytes, possibly because of an unidentified limiting factor was lost, due to *in vitro* partial dedifferentiation of the hepatocytes (31). However, CT was able to weakly stimulate ACO gene expression in the

absence of WY 14,643, but CT was a strong activator in the presence of WY 14,643. These data underline the ability of PKA activators to potentiate PPARα ligand-dependent activation. In contrast, PPARα KO hepatocytes were not sensitive to CT nor to WY 14,643 treatment, demonstrating that PPARα was required for the WY 14,643 and CT synergism in the stimulation of the ACO gene. The expression of the FABP gene was strongly enhanced by WY 14,643 in wild-type hepatocytes but not in PPARα KO hepatocytes, suggesting that distinct factors are required for ACO and FABP stimulation by PPARα. In wild-type hepatocytes, addition of CT did not significantly affect FABP expression in the absence and in the presence of WY 14,643. On the contrary, H89 reduced by about 90% the WY 14,643 induction of FABP expression, which is in agreement with our transfection experiments. In PPARα KO hepatocytes, the expression of the FABP gene was not significantly affected by WY 14,643, CT or H89, demonstrating that PPARα was required for FABP induction. In conclusion, our data suggest that ligand and PKA activation of PPARα converge in the stimulation of the PPAR target genes in hepatocytes.

DISCUSSION

Among the different stimuli known to phosphorylate and modulate nuclear receptor activity, the PKA pathway is certainly one of the best studied. However, no data are yet available concerning the potential effect of PKA on PPAR activity. This is in contrast with several studies focusing on PPAR phosphorylation by other stimuli. The scope of this work was to evaluate the role of PKA in modulating PPAR activity.

Early work from Shalev *et al.* (14) has shown that insulin treatment can phosphorylate PPARα. Insulin can also increase PPARα, and PPARγ2 activity in transient transfections. Insulin stimulation of PPARγ involves MAP kinases (15, 19). On the contrary, other pathways stimulated by EGF (epidermal growth factor) and PDGF (platelet-derived growth factor) also involving MAP kinase have a negative effect on mPPARγ1 activity by phosphorylating serine 82 in the AB domain, which corresponds to serine 112 of mPPARγ2 (16, 17, 20). Further studies have demonstrated that this negative effect of MAP kinase was due to the inhibition of ligand binding consequent to an alteration of the three-dimensional structure of the receptor (32).

Here we demonstrate by transient transfection experiments that PKA activators can stimulate PPAR activity in an exogenous ligand-independent manner. Moreover, a combination of PKA activators and PPAR ligands leads to an increased activation of PPAR target genes. Interestingly, this effect was obtained even at saturating concentrations of PPAR ligands. Moreover, we show that these effects are not due to change in PPAR expression. This stimulatory effect of PKA is in agreement with the results obtained with other nuclear receptors. Most studies report an activation of nuclear receptors such as estrogen receptor (ERα), glucocorticoid receptor (GR),mineralocorticoid receptor (MR), progesterone receptor (PR), androgen receptor (AR) or steroidogenic factor-1 (SF-1) by PKA (28, 33-38), whereas HNF4 has been shown to be down-regulated by PKA (39). Moreover, PKA activated PPARs

were able to stimulate two types of PPREs, even though the amplitude of response was different. Interestingly, PKA activators were nearly as effective as PPAR ligands to activate the ACO PPRE, whereas they could only activate the CYPA6 PPRE to levels corresponding to 25-30% of those obtained with PPAR ligands. Such differences were also found with ERα according to the cell-type and the promoter used (40-42).

Using different PKA pathway activators, we observed that the most potent ones were those affecting adenylate cyclase activity and not those leading to a direct increase of cAMP such as supplementation with cAMP analogs or inhibition of phosphodiesterase activity. To address the question whether ligands by themselves would activate the PKA pathway, we treated cells with H89, an inhibitor of PKA (27). Interestingly, we observed that H89 could reduce not only the CT induction of PPARs but also their induction by ligands such as WY 14,643, bromopalmitate and BRL 49,653, suggesting that these ligands act in part through the PKA pathway. This result was not due to a change in PPAR levels as shown by western blot. An attractive hypothesis would be that the ligands can also act indirectly by modulating intracellular cAMP levels. Such observations have indeed been reported for estrogens, which are able to increase intracellular cAMP levels, which in turn activate PKA and increases ERα activity (34).

As PPARs act essentially as heterodimers, it was of particular interest to determine whether its heterodimer partner (RXR) could be involved in PPAR activation by PKA. Surprisingly, in the absence of 9-cis-retinoic acid (RXR ligand), cotransfection of RXR moderately affected PPARα activity in the presence of WY 14,643, but increased PPARα activity by about 2 fold without any ligand, leading to an activation by CT equal to the one with WY 14,643. In the presence of RA, RXR conferred an even stronger ligand-independent activity to PPARα. This could be explained by the fact that RXR is itself the target of PKA as shown clearly on DR1-TK-CAT construct. RAR and RXR activation by PKA have been previously reported (43-45).

Thus, in the absence of WY 14,643 but in the presence of RA, it seems that the PKA action on RXR heterodimerized with PPAR leads to a major enhancement of the activity of PPAR/RXR heterodimers. However, we cannot exclude the possibility that other factors such as coactivators could also be the target of PKA.

Using truncated PPARα constructs, we determined that the AF-2 domain was most important for transactivation by PKA. Deletion of the AB domain from PPARα only slightly affected its basal and ligand induced activity, which is in agreement with previous data (29). Interestingly, CT was still able to potentiate WY 14,643 induction of the truncated PPARα, demonstrating that the AF-1 function was not involved in the potentiation by PKA. On the contrary, AF-2 deletion completely abolished WY 14,643 as well as CT activation of PPARα. Our data obtained with the GAL4 chimeras clearly confirm that AF2 but not AF1 is the target of PKA action. The demonstration that AF2 is the target of PKA is in agreement with the results found for PKA activation of ERα (42, 46) or SF-1 (38). However, for AR (37) and MR (47), the N-terminal portion seemed to be involved in PKA effects. Interestingly, CT had no effect on the ligand-independent activity of the GAL-AF2 chimera suggesting that other domains of the receptors are necessary. To better characterize the domains involved, we analysed the phosphorylation of different domains of PPAR by using GST-PPAR fusions. We observed a very strong phosphorylation of the DBD, a weaker one for the AB domain and a faint one for the LBD, again suggesting that several domains are involved in the activation of PPARs by the PKA pathway. This result is confirmed by the mapping of the most conserved putative PKA sites which are present in the A/B, DBD and LBD domains. As the main phosphorylation site is present in the DBD, we analysed whether the drugs used could modulate the DNA binding ability of PPARα *in vitro*. To our surprise, WY 14,643 and CT strongly inhibited PPARα DNA binding. However, cotreatment with WY 14,643 and CT led only to a limited decreased binding. We thus propose that CT acts in part by preventing the

decreased binding of PPAR liganded with WY 14,643. This stabilization would in turn increase PPAR activity. This in agreement with a previous report (28), which shows that ERα DNA binding is inhibited by PKA only in the absence of estradiol. This report also shows that the target of PKA is in the DBD of ERα. Rangarajan et al. (33) have also demonstrated that PKA enhancement of liganded GR required specific residues of the DBD. In this case, however, they observed an enhancement of GR binding in the presence of PKA. This suggested that depending on the receptors, the mechanisms of enhancement of the activity by PKA requires different functions of the receptor.

A crucial question was whether PKA does modulate the expression of PPAR target genes? To answer this question, we focused on the role of PPARα in the liver and took advantage of PPARα KO mice. We analysed the expression of two target genes, ACO and FABP (48, 49). In wild-type mice hepatocytes cultured *in vitro*, we demonstrated that cotreatment with WY 14,643 and CT led to a synergistic activation of the ACO gene expression. On the other hand, the FABP gene was only weakly affected by addition of exogenous PKA activators, but addition of PKA inhibitors strongly diminished its induction by WY 14,643, confirming the results obtained in transient transfection experiments. In PPARα KO mice, ACO was not subjected to stimulation by WY 14,643 or CT alone or in combination, confirming that PPARα was essential to PKA activation of the ACO gene. In these mice, FABP induction by WY 14,643 was completely abolished. FABP which is involved in fatty acid (FA) binding in hepatocytes and ACO in β-oxidation of FA could therefore be integrated in the following model involving the PKA pathway (Fig 9B): Under conditions of stress, fasting or exercise, the limiting factor for brain and muscles rely on increased energy fuel availability, essentially glucose and ketone bodies. One way for the organism to meet these needs is to stimulate gluconeogenesis and ketogenesis. The adipose tissue hydrolyses triglycerides (TG) to liberate free non esterified fatty acids, which are released into the blood circulation and are then

rapidly taken up by the liver to be transformed into ketone bodies. PPARα and PPARγ are directly involved in the regulation of several key enzymes of these pathways. Stress, fasting or exercise are also associated with an increased glucagon production (one of the key factors increasing cAMP levels in cells and thus activating PKA) by the adrenal gland (50). PKA increases PPARα activity in liver, which in turn stimulates the β-oxidation and in particular the conversion of FA into acetyl-CoA used in the production of ketone bodies (51). Results from our laboratory (52) have also demonstrated that fasting did not affect FABP expression in wild-type mice. On the contrary, ACO gene expression has been shown to be up-regulated by fasting in wild-type mice but not in PPARα KO mice (24, 26), confirming the scheme of regulation we propose. In addition, PPARα KO mice exhibited an increased accumulation of FA in liver, due to impaired β-oxidation (53).

In conclusion, our results suggest that under conditions of stress, fasting and exercise, PPARα activity is increased by the PKA pathway and leads to an enhancement of β-oxidation, production of glucose and ketone bodies, which serve as fuel for muscles and brain.

MATERIALS AND METHODS

Chemicals.

Bromopalmitate, CT (cholera toxin), Forskolin, 8-Br-cAMP (8-Bromoadenosine 3':5'-cyclic monophosphate), IBMX (isobutyl methylxanthine) were from Sigma (St Louis, MO). WY 14,643 was from Chemsyn Science Laboratories (Kansas, USA). BRL 49,653 was a kind gift from Parke Davis. H89 (N-[2-((p-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl) was from Calbiochem (La Jolla, CA). PKA catalytic subunit was from Promega (Madison, WI).

Oligonucleotide Sequences.

ACO1: GCCACCGCCTATGCCTTCCACTTT

ACO2: CGGCTTGCACGGCTCTGTCTTGA

LPL1: CCTGCGGGCCCTATGTTTG

LPL2: CTCGCCGATGTCTTTGTCCAGT

mFABP1: CAATTGCAGAGCCAGGAGAACTTT

mFABP2: CAATGTCGCCCAATGTCA

Plasmids.

The reporter plasmid 2CYP-TK-CAT contains two copies of the CYP4A6 PPRE cloned in opposite orientation upstream of the minimal herpes simplex virus thymidine kinase promoter in the pBLCAT8+ plasmid (54). ACO-TK-CAT plasmid corresponds to the Acyl-CoA oxidase promoter PPRE cloned in PBLCAT8+ as described by Dreyer et al. (6). DR1-TK-CAT corresponds to the perfect DR1 sequence (AGCTTCATTCT<u>AGGTCA</u>A<u>AGGTCA</u>TCCCCT) cloned in the pBLCAT8+ plasmid. pG5CAT reporter plasmid (Clontech) corresponds to 5 Gal4 binding sites upstream of the E1b minimal promoter. Mouse and Xenopus PPAR α, β and γ cDNAs were cloned into the BamHI site of the pSG5 mammalian expression vector. For PKA *in vitro* assays, portions of mouse

PPAR cDNAs were amplified by PCR and then subcloned into the BamHI site of the prokaryotic expression vector pGEX1. mPPARα AB (aa 1-101) was cloned into the SmaI/BamHI sites of pGEX1. mPPARα DBD domain (aa 98-203), mPPARβ DBD domain (aa 68-129), mPPARα LBD (aa 202-468) and mPPARβ LBD (aa 136-396) were cloned into the BamHI site of pGEX1. pSG5-mPPARα ΔAB was obtained by removing aa 1 to 101 from WT mPPARα by PCR and pSG5-mPPARα ΔLBD by removing sequences downstream from residue 247. pSG5-mPPARα ΔAF2 was obtained by removing the last 13 residues from WT mPPARα. GAL-AF1 and GAL-AF2 expression plasmids correspond to AB domain (aa 1 to 100) LBD domain (165-468) respectively cloned in Gal4 DBD pM vector (Clontech).

***In vitro* Translation**

In vitro translation was performed using the TNT Promega kit. Briefly, 1 µg of expression vector was mixed to 25 µl of TNT rabbit reticulocyte lysate, 2 µl of TNT buffer, 1 µl of mix containing all amino acids, 1 µl of RNAsin (50 U/µl), 1 µl of T7 RNA polymerase (20 U/µl). A control reaction was performed under the same conditions but [^{35}S]-methionine (15 µCi/µl) was used to label the protein produced. The final reaction volume was 50 µl. The reaction was performed for 1.5 h at 30 °C. The translation efficiency was checked by loading 1 µl of labelled lysate on an SDS-PAGE gel.

Gel Mobility Shift Assays.

Gel mobility shift assays were carried out as previously described (55). Briefly, [^{32}P]-labeled ACoA (GATCCCGAACGTGACCTTTGTCCTGGTCCCGATC) double strand oligonucleotide , (56) was combined with *in vitro* translated PPAR or HEK-293 WCE and when indicated mouse RXRβ_2 Sf9 cellular extract. Protein-DNA complexes were separated from the free probe by non-denaturating gel electrophoresis with 4% polyacrylamide (29/1) gels in 0.5 X TBE .

Cell Culture and Transient Transfection.

HEK-293 cells (human embryonic kidney cells) were cultured in 10% FCS (foetal calf serum) DMEM-F12 with 5% CO_2. Cells were plated in 24-well plates in 10% CDFCS, phenol-free DMEM 24 h before transfection. Transfections were performed by lipofection (lipofectamine, Life Technologies, Rockville, MA) using 200 ng of CAT reporter construct, 400 ng of the internal reference ß-galactosidase reporter plasmid (pCH110) and 100 ng of pSG5-PPAR or pSG5-hRXRα expression vectors per well. After lipofection, the cells were grown in 10% CDFCS, DMEM in the presence of different ligands for 36 h. Transactivation ability was determined by CAT activity on the whole cell extract as previously described (55).

Hepatocyte Isolation and Culture

Hepatocytes were isolated from liver of adult male wild-type (SV129) or PPARα KO mice using a two-step *in situ* portal vein collagenase A (Boehringer Mannheim) perfusion method (57). Freshly isolated hepatocytes were filtered through nylon membrane to remove tissue debris and cell clumps. The cell suspension was washed in Leibovitz's L-15 medium and resuspended twice after centrifugation. The isolated hepatocytes were suspended in William's medium E supplemented with 10% FCS, 100 µg/ml streptomycin and 100 µg/ml penicillin and seeded in dishes at the density of 5.10^5 cells/ml medium. The medium was renewed 4 hr later to remove dead cells. Cells were then treated with or without WY 14,643 (10 µM) and cholera toxin (1 µg/ml), in presence or not of H89 (10 µM). Cultures were maintained at 37°C in a humidified air / CO_2 incubator (5% CO_2, 95% air) for 24 h.

Whole cell extract preparation and western blot.

HEK-293 cells were harvested, washed in PBS, and resuspended in TEG (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, and 10% glycerol)/ 0.4 M KCl containing 5 µg/ml aprotinin, leupeptin and pepstatin A and 0.1 mM phenylmethylsulfonyl fluoride. Then, cells were sonicated and the cellular debris were pelleted by centrifugation at 14000 rpm for 20 minutes

in microfuge tubes. Thirty µg of whole cell extract proteins were subjected to SDS-PAGE followed by electrotransfer onto a nitrocellulose membrane. The blot was probed with anti PPARα AB antibody (1/1000) (polyclonal rabbit antibody produced in our laboratory and directed against mouse PPARα AB region) and then incubated with rabbit anti-rabbit IgG horseradish peroxydase conjugated antibody (1 µg/ml). ECL kit from Amersham (Arlington, IL, USA) was used for protein detection.

RNA Isolation and Northern Blot.

Total RNA was isolated from isolated hepatocytes using the Trizol reagent from Life Technologies (Rockville, MA) as described by the manufacturer. ACO and FABP probes were amplified by RT-PCR. The amplifying primers were: ACO1 and ACO2 primers for mouse peroxisomal acylCoA oxidase (ACO) probe (1036-1690), mFABP1 and mFABP2 for mouse fatty acid binding protein (FABP) probe (61-394) (see above).

For northern blot analysis, 20 µg of total RNA was electrophorized in a 2.2 M formaldehyde-1% agarose gel in MOPS buffer and then hybridized with the different probes as previously described (58).

Production of GST Fusion Proteins

Production of GST fusion proteins was performed as previously described (13). Protein concentration was estimated by the Bradford method. The levels of expressed fusion proteins were determined by an *in vitro* binding assay followed by a SDS-PAGE gel and a Coomassie Blue staining.

***In vitro* PKA Assays with Glutathione Sepharose**

Glutathione Sepharose (Pharmacia Biotech, Uppsala, Sweden) was equilibrated with NET binding buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA). Crude bacterial extract containing GST fusion proteins was incubated at 4 °C with 25 µl of beads for 2.5h . After 2 washes with NETN (NET + 0.5% NP40), the beads were washed 2 times with PKA

buffer (50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 10% glycerol). The beads were then incubated in a mix containing 50 µl of PKA buffer , 45U of PKA catalytic subunit, 0.5 µl γ-[^{32}P]-ATP and 0.5 µl ATP 2.5 mM for 45 min at 30 °C. After 2 washes with NETN, beads were boiled in SDS loading buffer, and a quarter of the proteins were run on SDS-PAGE. The gel was then stained with coomassie blue. After extensive washes with a solution containing 20% methanol and 10% acetic acid, the gel was submitted to autoradiography.

FIGURE LEGENDS

Fig 1: CT increases mPPARα activity on a PPRE containing promoter

Mouse PPARα was transfected along with TK-CAT or 2CYPA6-TK-CAT constructs in HEK-293 cells. Transfections were with 200 ng of TK-CAT or 2CYPA6-TK-CAT reporter construct and without or with 100 ng of pSG5-mPPARα expression vector per well. Cells were grown for 36 h in the presence of 1 μM of WY 14,643 (WY), with or without 1μg/ml CT. Results are shown as the mean ± SD (n = 6) of CAT activity after normalization for β-galactosidase activity.

Fig 2: CT increases mPPAR activity at various concentrations of PPAR ligands

Mouse PPARα, β and γ were tested for their ability to respond to CT in HEK-293 cells. Transfections were with 200 ng of 2CYPA6-TK-CAT reporter construct and 100 ng of pSG5-mPPARα, β and γ expression vectors per well. Cells were grown for 36 h in the presence of various concentrations of WY 14,643 (WY), Bromopalmitate (Bro) and BRL 49,653 (BRL), with or without 1μg/ml CT. Results are shown as the mean ± SD (n = 6) of CAT activity after normalization for β-galactosidase activity.

Fig 3: PKA enhancement of PPAR activity via the ACO PPRE

Mouse PPARα, β and γ cDNAs were cotransfected in HEK-293 cells with ACO-TK-CAT reporter construct instead of the 2CYPA6-TK-CAT reporter construct in the same conditions as in fig 1. Cells were grown for 36 h in the presence of 1 μM WY 14,643 (WY), 50 μM Bromopalmitate (Bro) and 5 μM BRL 49,653 (BRL), with 1μg/ml CT when indicated. Results are shown as the mean ± SD (n = 6) of CAT activity after normalization for β-galactosidase activity.

Fig 4: various PKA activators stimulate PPAR activity

A. Schematic representation of the PKA pathway. cAMP is produced from ATP by a membrane-bound adenylate cyclase (Ac). Transmembrane receptors (R) for numerous hormones, neurotransmitters and other stimuli (H) are coupled to adenylate cyclase via heterotrimeric G-proteins (α, β and γ subunits). This interaction promotes the exchange of GDP, bound to the α-subunit, for GTP and the subsequent dissociation of the α subunit form the βγ heterodimer. The Gα-GTP then binds to adenylate cyclase and modulates its activity. PDE: phosphodiesterase; FSK: forskolin; CT: cholera toxin; . **B.** 100 ng of pSG5-mPPARα was cotransfected in HEK-293 cells with 2CYPA6-TK-CAT reporter construct under the same conditions as in Fig 1. Cells were grown for 36 h in the presence of 1 μM of WY 14,643 (WY), with 1μg/ml Cholera toxin (CT), 50 mM 8-BrcAMP, 200 μM forskolin (FSK) or 10 μM IBMX when indicated. Results are shown as the mean ± SD (n = 3) of CAT activity after normalization for β-galactosidase activity.

Fig 5: PKA inhibitors can repress PPAR activity

A. Mouse PPARα, β and γ were tested for their ability to respond to CT in HEK-293 cells using 200 ng of 2CYPA6-TK-CAT reporter construct and 100 ng of pSG5-PPAR expression vectors. After lipofection, 10 μM of H89 was added to the medium 1 h before ligands. Cells were grown for 36 h in the presence of 1 μM WY 14,643 (WY), 50 μM Bromopalmitate (Bro) and 5 μM BRL 49,653 (BRL), with 1μg/ml CT when indicated. Results are shown as the mean ± SD (n = 6) of CAT activity after normalization for β-galactosidase activity. **B.** 30 μg of whole cell extracts from transfected cells were loaded on SDS-PAGE and probed by western blot using mPPARα antibody. The first lane corresponds to HEK-293 cells transfected with the empty pSG5 vector and the remaining lanes correspond to 293 cells

transfected with pSG5-mPPARα vector and treated or not (-) with WY, CT or H89 under the same conditions as in Fig. 5A. **C.** 5 μg of the same WCE were used in gel shift assays using the ACoA probe.

Fig 6: RXR modulates PPAR activity in the presence of PKA activators

A. 100 ng of pSG5, pSG5-mPPARα and pSG5-mRXRβ2 expression vectors per well in combination or alone were cotransfected in HEK-293 cells with 200 ng of 2CYPA6-TK-CAT reporter construct. After lipofection, cells were grown for 36 h with or without 1 μM of WY 14,643 (WY), and 1 μM 9-cis retinoic acid (RA), with 1μg/ml CT when indicated. Results are shown as the mean ± SD (n = 6) of CAT activity after normalization for β-galactosidase activity. **B.** 100 ng of pSG5 or pSG5-mRXRβ2 expression vectors were cotransfected in HEK-293 cells with 200 ng of DR1-TK-CAT reporter construct per well. After lipofection, cells were grown for 36 h with or without 1 μM 9-cis retinoic acid (RA) and 1μg/ml CT when indicated. Results are shown as the mean ± SD (n = 6) of CAT activity after normalization for β-galactosidase activity.

Fig 7: The AB domain is dispensable for PPAR response to PKA

A. 100 ng of pSG5-mPPARα WT, or pSG5-mPPARα ΔAB, pSG5-mPPARα ΔLBD, or pSG5-mPPARα ΔAF2 constructs were transfected in HEK-293 cells with 200 ng of 2CYPA6-TK-CAT reporter construct per well. After lipofection, cells were grown for 36 h with or without 1 μM of WY 14,643 (WY) with or without 1μg/ml CT. Results are shown as the mean ± SD (n = 6) of CAT activity after normalization for β-galactosidase activity. **B.** Equivalent amounts of *in vitro* translated mPPARα WT, mPPARα ΔAB, mPPARα ΔLBD, or mPPARα ΔAF2 receptors were used in gel shift assays in combination with the ACoA probe.

Lane 1 corresponds the probe alone and lane C to mock lysate. In addition, mouse RXRβ_2 Sf9 cellular extract was eventually added (RXR)**.** The arrow indicated the position of RXR retarded complex and the star the position of a non specific complexes. **C.** 100 ng of GAL, GAL-AF1, or GAL-AF2 constructs were transfected in HEK-293 cells with 200 ng of pG5CAT reporter construct. After lipofection, cells were grown for 36 h with or without 1 µM of WY 14,643 (WY) with or without 1µg/ml CT. Results are shown as the mean ± SD (n = 6) of CAT activity after normalization for β-galactosidase activity.

Fig 8: PPAR phosphorylation in response to PKA occurs mainly in the DBD

A. Regions encoding AB (ABα), DBD (DBDα) and LBD (LBDα) domains of mPPARα as well as DBD (DBDβ) and LBD (LBDβ) of mPPARβ were cloned in pGEX1 prokaryotic expression vector as GST fusions. *In vitro* PKA assay was performed as described in the Materials and Methods. The upper panel corresponds to the gel autoradiogramm and the lower panel to the protein expression levels after coomassie blue staining of the gel. The arrow labelled NS corresponds to a non specific band. **B.** Mapping of the main putative PKA sites of phosphorylation on mPPARα.

Fig 9: PKA modulates some PPAR target genes

A. Hepatocytes were isolated from wild-type and PPARα KO mice and cultured in Williams medium supplemented with 10% CDFCS. Cells were then treated for 24 h with or without 10 µM WY 14,643 (WY) and 1 µg/ml cholera toxin (CT). When H89 was used (H: 10 µM), it was added to the medium 1 h before treatment with WY or CT. After RNA extraction, 10 µg of total RNA were used for northern blotting and then hybridized sequentially with ACO, FABP and 28S probes. The figure shows a representative experiment. **B.** General model of

cross-talk between fatty acids and PKA signalling involving PPARs. TG: triglycerides, FA: fatty acids, Glucocor: glucocorticoids, β OX: β oxidation, LPL: lipoprotein lipase.

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