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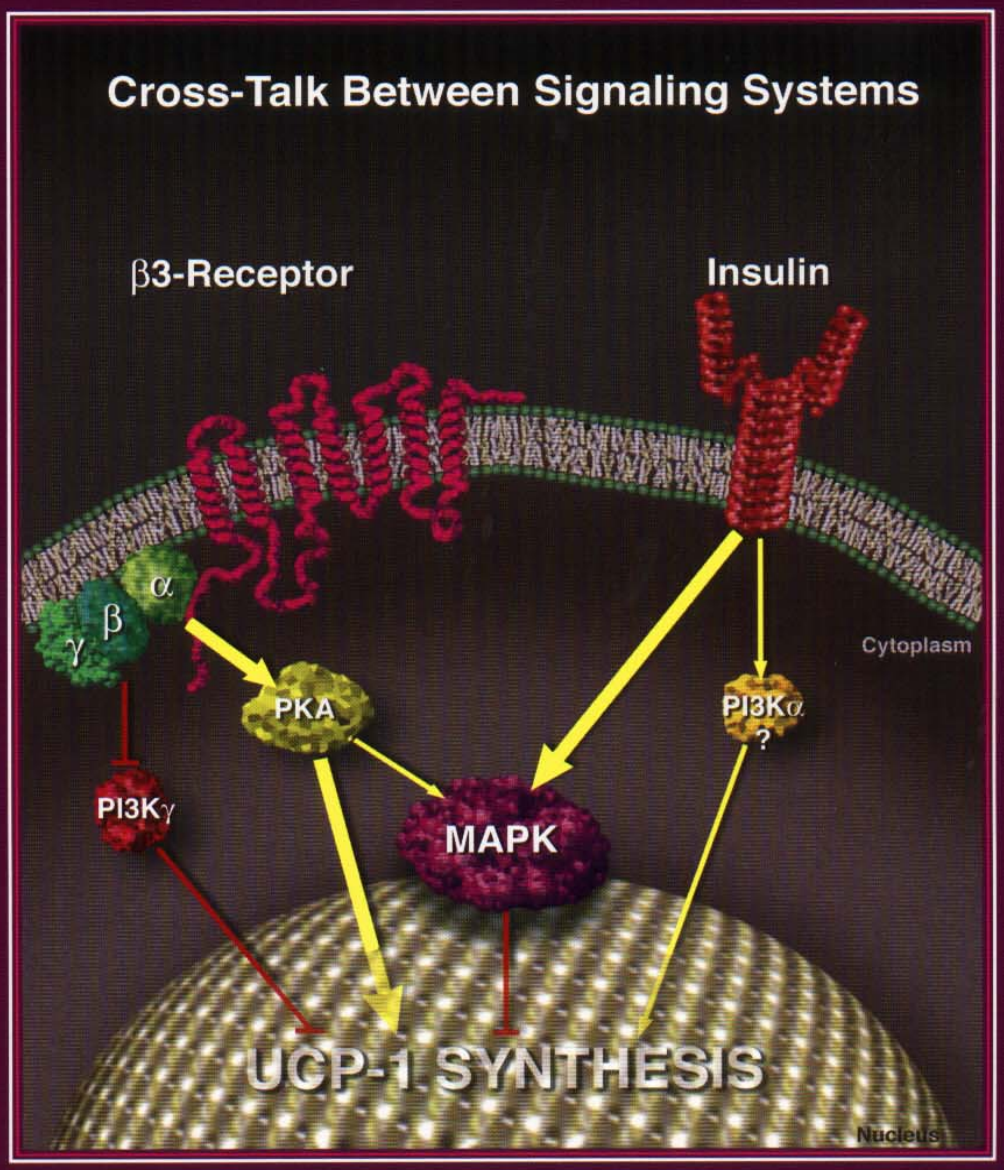

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SPECIAL FOCUS ON DIABETES

Insulin and the β3-Adrenoceptor Differentially Regulate Uncoupling Protein-1 Expression

Johannes Klein*, Mathias Fasshauer*, Manuel Benito, and C. Ronald Kahn

Research Division Joslin Diabetes Center (J.K., M.F., C.R.K.)
Harvard Medical School
Boston, Massachusetts 02215

Department of Internal Medicine I (J.K.)
Medical University of Lübeck
23538 Lübeck, Germany

Facultad de Farmacia (M.B.)
Universidad Complutense
28040 Madrid, Spain

Cross-talk between insulin and the adrenergic system is important in the regulation of energy homeostasis. In cultured, differentiated mouse brown adipocytes, β3-adrenergic stimulation induced a 4.5-fold increase in uncoupling protein-1 (UCP-1) expression, which was diminished by 25% in the presence of insulin. β3-Adrenergic stimulation also activated mitogen-activated protein (MAP) kinase by 3.5-fold and caused a decrease in basal phosphoinositide (PI) 3-kinase activity detected in p110γ- and Gβ-subunit-immunoprecipitates in a time-dependent manner, whereas insulin stimulated p110α- and phosphotyrosine-associated PI 3-kinase activity. Inhibition of MAP kinase or PI 3-kinase potentiated the β3-adrenergic effect on UCP-1 expression, both alone and in the presence of insulin. Thus, insulin inhibits β3-adrenergic stimulation of UCP-1, and both MAP kinase and PI 3-kinase are negative regulatory elements in the β3-adrenergic control of UCP-1 expression. Cross-talk between the adrenergic and insulin signaling systems and impaired regulation of UCP-1 might contribute to the development of a reduced energy balance, resulting in obesity and insulin resistance. (Molecular Endocrinology 14: 764–773, 2000)

INTRODUCTION

Cross-talk between hormonal stimuli promoting energy storage, such as insulin, and those increasing energy expenditure, such as activation of the sympathetic nervous system, may play a crucial role in the pathophysiology of obesity and the insulin resistance syndrome (1).

In small mammals and infants, brown adipose tissue is important in the regulation of energy homeostasis by virtue of expression of the uncoupling protein-1 (UCP-1), which uncouples mitochondrial oxidative phosphorylation, thereby dissipating energy as heat (2–4). Recent studies with selective agonists for the β3-adrenergic receptor, a β-adrenergic receptor expressed mainly in brown adipocytes and mediating induction of UCP-1, have found significant positive effects on energy expenditure and fat metabolism in adult humans and primates (5, 6). Moreover, mutations in the gene for UCP-1 alone (for a recent review see Ref. 7) or in combination with mutations in the β3-adrenergic receptor (8, 9) have been reported to be associated with changes in energy homeostasis favoring weight gain.

Obesity is an important component of type 2 diabetes. Obesity induces insulin resistance and hyperinsulinemia, both of which, in turn, may contribute to alterations in energy expenditure (10–12). In the present study, we have investigated mechanisms of cross-talk between insulin and the β3-adrenergic receptor controlling UCP-1 expression in a mouse brown adipocyte cell model. We find that insulin exerts a mitogen-activated protein (MAP) kinase-dependent bidirectional effect on the β3-adrenergic control of UCP-1. Moreover, we provide evidence that phosphoinositide (PI) 3-kinase and MAP kinase are regulatory elements for UCP-1 expression in both insulin and β3-adrenergic signaling pathways. Imbalance in the regulation of these signaling intermediates might have pathophysiological implications for the development of obesity, insulin resistance, and type 2 diabetes.

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RESULTS

β3-Adrenergic Stimulation of UCP-1 Expression Is Protein Kinase A (PKA) Dependent and Inhibited by Insulin Treatment

Adrenergic stimulation increases UCP-1 expression via a protein kinase A-dependent pathway (13–16). In differentiated, cultured brown adipocytes, Western blot analyses with a UCP-1-specific antibody showed a 4.5-fold increase of UCP-1 protein levels by β3-adrenergic stimulation using the β3-selective agonist CL316243 at a concentration of 10 μM. This effect was dose-responsive with half-maximal stimulation at a concentration of 100 nM (data not shown). The β3-adrenoceptor-induced increase was reduced by about 40% using the membrane-permeable PKA-inhibitor H-89 (Fig. 1, A and B). The efficacy of H-89 was demonstrated in PKA activity assays, which revealed a more than 90% decrease in endogenous PKA activity after treatment of adipocytes with this compound for 19 h (Fig. 1C). Furthermore, the β3-receptor-mediated increase of UCP-1 could be mimicked by the PKA-activating compound forskolin (Fig. 1D). This increase was reduced by more than 60% after pretreatment of adipocytes with H-89 (Fig. 1D). These experiments further confirm the importance of a PKA-dependent signaling pathway for UCP-1 induction. When the cells were incubated with 100 nM insulin alone for 6 h before stimulation with CL316243, there was a significant decrease of about 25% in β3-adrenoceptor-induced UCP-1 protein levels (Fig. 2, A and B). Further time course studies showed that this decrease was first detectable after 3 h of insulin pretreatment and was most prominent at 6 h (data not shown). Similar results were found using epidermal growth factor at 20 ng/ml during the prestimulation period (data not shown). Insulin stim-

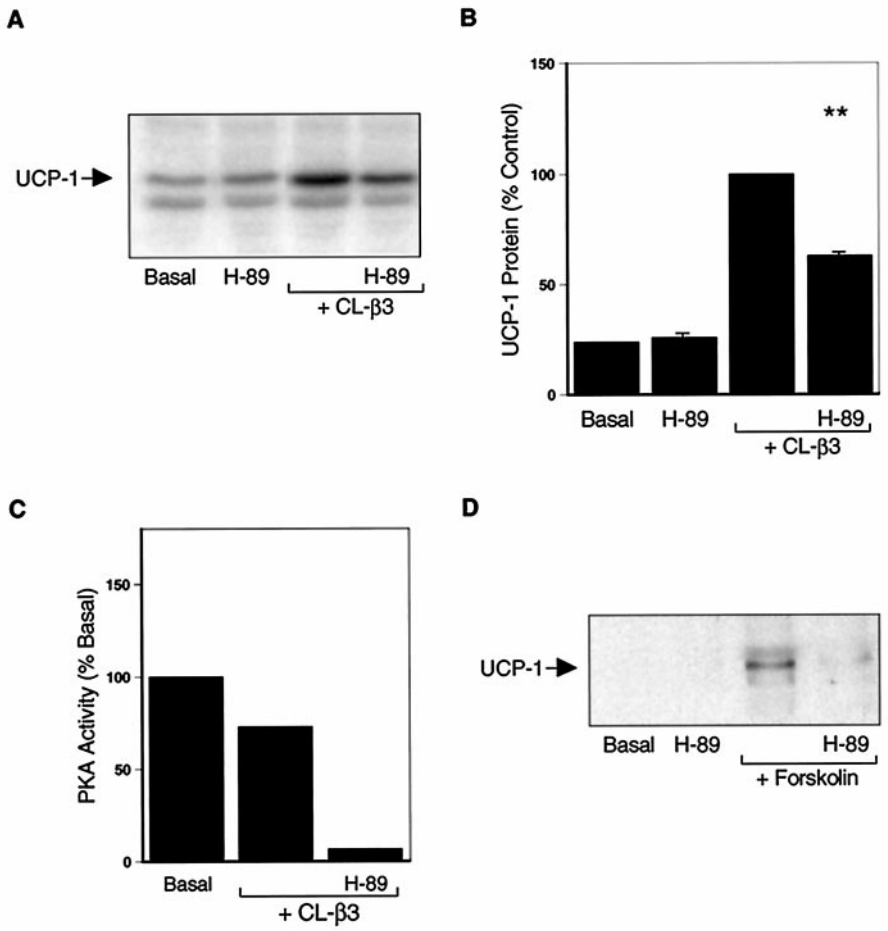

Fig. 1. β3-Adrenergic Induction of UCP-1 Expression Is PKA Dependent

Differentiated brown adipocytes were either nontreated or stimulated for 18 h with CL316243 (CL-β3, 10 μM) alone or in combination with the PKA inhibitor H-89 (10 μM, 1 h pretreatment). Cell lysates were prepared as described in *Materials and Methods*. A, UCP-1 was visualized by blotting with a specific antibody and autoradiography of a representative experiment is shown. B, UCP-1 protein levels relative to CL316243 induction alone (Control, mean ± SEM) are shown as determined by PhosphorImager quantitation of two independent experiments. Statistical significance was calculated with Student's *t* test comparing CL-β3 treatment alone with CL-β3 + H-89; ** denotes differences of high statistical significance ($P \leq 0.01$). C, Representative PKA activity assay. D, Cells were stimulated with forskolin (50 μM) for 18 h, either with or without prior H-89-treatment for 1 h (10 μM). Representative blot probed with UCP-1 antibody.

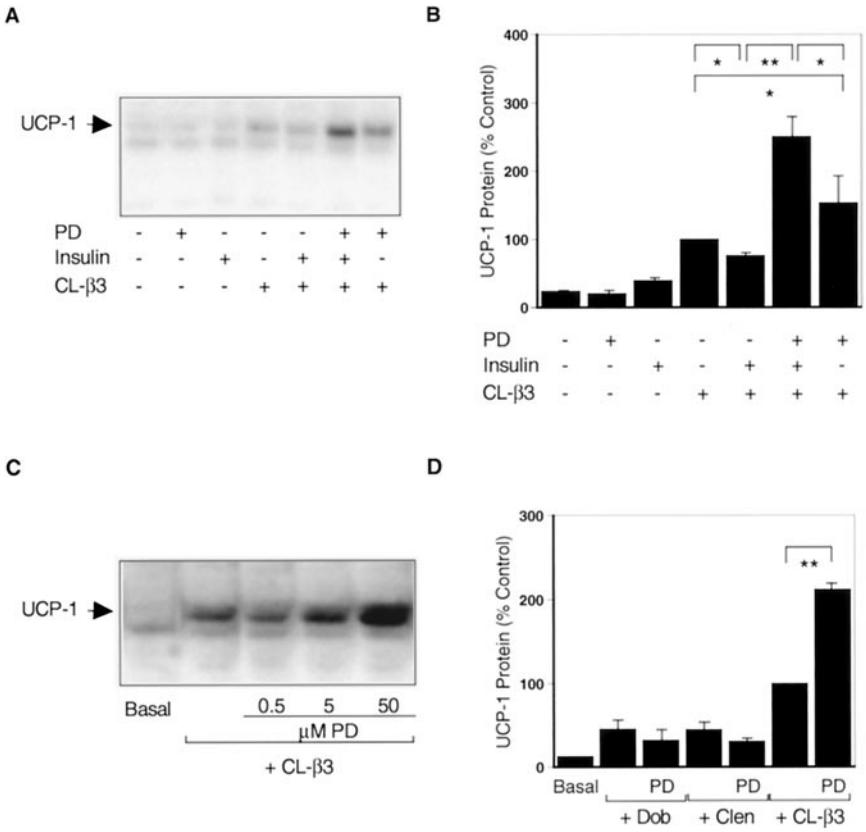

Fig. 2. Bidirectional Effect of Insulin and Influence of MAP Kinase Inhibition on β3-Adrenergic Induction of UCP-1 Expression

A, Differentiated brown adipocytes were either nontreated or treated with insulin (24 h, 100 nM), the MEK inhibitor PD098059 (PD, 19 h, 50 μM), or CL316243 (CL-β3, 18 h, 10 μM) alone or in the combinations indicated. UCP-1 immunoblots were prepared as described in *Material and Methods*. Representative autoradiography. B, The statistical analysis of at least three independent experiments with the SEM is shown. C, Autoradiography of cells treated with the indicated concentrations of PD. D, Brown adipocytes were stimulated with 10 μM dobutamin (Dob), clenbuterol (Clen), or CL316243 (CL-β3) for 16 h alone or in combination with PD098059 (PD, 1 h pretreatment, 50 μM). Statistical significance in panels B and D was calculated with Student's *t* test comparing CL-β3 treatment alone (Control) with either insulin + CL-β3, or PD + CL-β3, or comparing treatments with insulin + PD + CL-β3 and PD + CL-β3, or insulin + CL-β3, respectively; * Denotes differences of statistical significance ($P \leq 0.05$); ** denotes high statistical significance ($P \leq 0.01$).

ulation alone produced no significant effect, but there was a trend ($P = 0.08$) toward a positive effect over basal UCP-1 protein levels (Fig. 2, A and B). Furthermore, short-term stimulation with 100 nM insulin significantly increased tyrosine phosphorylation of the insulin receptor but not insulin-like growth factor I receptor (data not shown), indicating that the observed effects of insulin are mediated primarily by the insulin receptor.

The Role of MAP Kinase in the Regulation of UCP-1 Expression

Stimulation of different G protein-coupled receptors, including Gs-coupled β-adrenergic receptors (17), has been shown to regulate both MAP kinase and PI 3-kinase pathways (for a recent review see Ref. 18). These two pathways are also major contributors to insulin signaling (19, 20). In the differentiated brown adipocytes, the β3-selective agonist CL316243 increased MAP kinase activity in a time-dependent manner with a 3.5-fold maximum after 20 min, as assessed with a phosphospecific antibody against the activated MAP kinase isoforms p42 and p44 (Fig. 3, A and B). Inhibition of PKA with the membrane-permeable PKA-specific inhibitor H-89 produced an almost 50% decrease in β3-adrenergic activation of MAP kinase (Fig. 3, C and D). Conversely, the adenylyl-cyclase-activating compound forskolin and the cAMP-analog $(Bu)_2$cAMP increased MAP kinase activation in a time-dependent manner (Fig. 3E). As expected, the MAP kinase kinase (MEK) inhibitor PD098059 completely abolished MAP kinase activation (Fig. 3, C and D), whereas pharmacological inhibition of PI 3-kinase (LY294002), protein kinase C (GF109203X), and G_i-coupled receptors (pertussis toxin) had no significant effect on MAP kinase activation in these cells (data not shown). Furthermore, LY294002 pretreatment had no significant effect on insulin-induced MAP kinase phosphorylation (Fig. 3F).

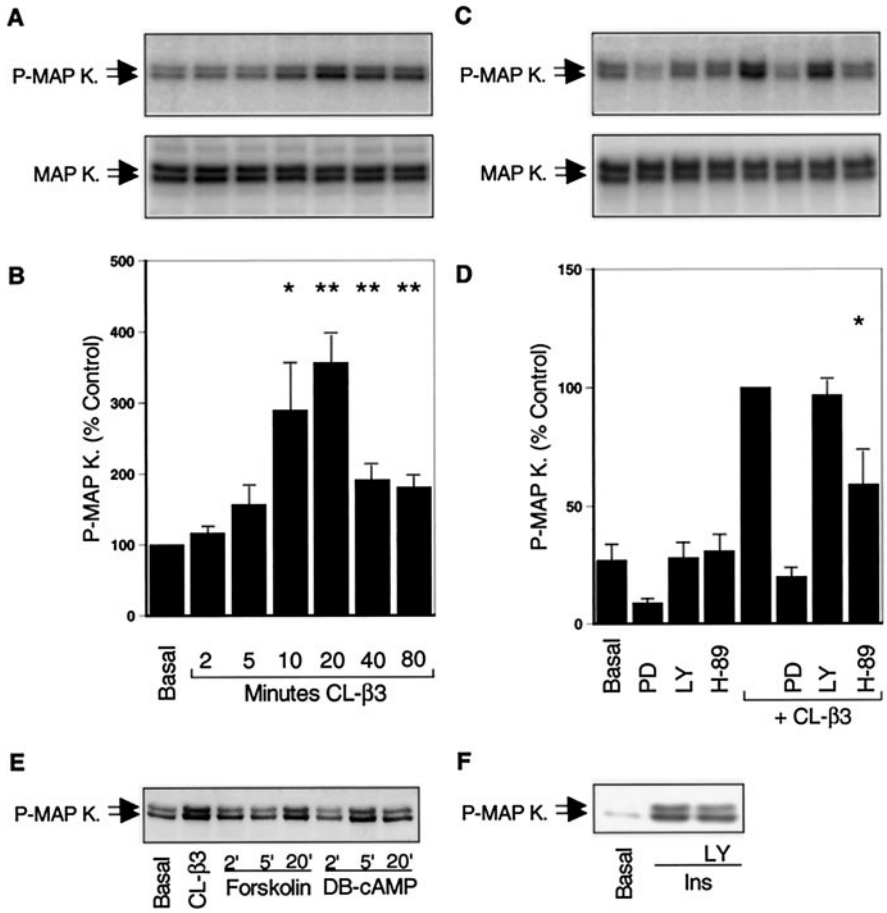

Fig. 3. β3-Adrenergic Stimulation Leads to Activation of MAP Kinase in a Time- and PKA-Dependent Manner

A, Fully differentiated brown adipocytes were either nontreated or treated with CL316243 (CL-β3, 10 μM) for the indicated times. Cell lysates and MAP kinase immunoblots were prepared as described in *Material and Methods*. Representative autoradiography. After incubating immunoblots with a phosphospecific MAP kinase antibody (P-MAP K., *upper panel*) blots were stripped and reprobed with MAP kinase antibody to control for equal loading (MAP K., *lower panel*). B, The statistical analysis of four independent experiments with the SEM is shown. Statistical significance was calculated with the Student' s *t* test comparing nontreated cells (Control) with CL-β3 treatment; * denotes statistical significance ($P \leq 0.05$); ** denotes high statistical significance ($P \leq 0.01$). C, Brown adipocytes were either nontreated or treated with CL316243 (CL-β3, 20 min, 10 μM), the MEK inhibitor PD098059 (PD, 1 h, 50 μM), the PI 3-kinase inhibitor LY294002 (LY, 1 h, 10 μM), the PKA inhibitor H-89 (1 h, 10 μM), alone or in different combinations as indicated. MAP kinase immunoblots were prepared as described in panel A. D, The statistical analysis of four independent experiments with the SEM is shown. Statistical significance was calculated using Student' s *t* test comparing CL-β3 treatment (Control) with H-89 + CL-β3; * denotes statistical significance ($P \leq 0.05$). E, Brown adipocytes were nontreated, treated with CL316243 (CL-β3, 20 min, 10 μM), or treated for the indicated times with forskolin (50 μM) and $(Bu)_2$cAMP (DB-cAMP, 1 mM). Immunoblots were prepared as described in panel A with a phosphospecific MAP kinase antibody. F, Brown adipocytes were either nontreated or stimulated with insulin (Ins, 5 min, 100 nM) alone or in combination with LY294002 (LY, 1 h, 10 μM). Immunoblots were prepared as described in panel A with antiphospho MAP kinase antibody.

As both insulin and epidermal growth factor are also activators of MAP kinase, we investigated whether inhibition of MAP kinase would modify the effects of either insulin or β3-adrenergic agents on UCP-1 expression. Surprisingly, inhibition of MAP kinase by PD098059 resulted in a significant 1.5-fold increase in the β3-adrenergic stimulation of UCP-1 (Fig. 2, A and B). The positive effect of MAP kinase inhibition on β3-adrenoceptor-induced UCP-1 expression followed a dose-response curve (Fig. 2C). The inhibitor alone had no effect on the basal level of UCP-1 expression (Fig. 2, A and B). Interestingly, insulin pretreatment, in the presence of a pharmacological MAP kinase inhibitor (MEK inhibitor PD098059) further enhanced the β3-adrenergic stimulation of UCP-1 levels by approximately 2.5-fold (Fig. 2, A and B).

Furthermore, we determined whether UCP-1 protein was also up-regulated by β1-adrenergic stimulation with dobutamin or β2-receptor activation with clenbuterol. Both compounds were able to increase UCP-1 protein levels about 3-fold over basal; however, the same concentration of Cl-β3 was about 2 times as effective in inducing UCP-1 (Fig. 2D). Surprisingly, no significant change in β1- or β2-adrenoceptor-

induced UCP-1 protein could be observed after inhibition of MAP-kinase, whereas β3-adrenergic stimulation of UCP-1 protein was again significantly enhanced by PD098059 pretreatment (Fig. 2D). Furthermore, forskolin-induced UCP-1 expression was not altered by MAP kinase inhibition (data not shown).

The Role of PI 3-Kinase in the Regulation of UCP-1 Expression

To determine whether PI 3-kinase-dependent signaling pathways might play a role in the β3-adrenergic control of UCP-1 expression, differentiated cells were pretreated with an inhibitor of PI 3-kinase (LY294002) before β3-adrenergic stimulation. Inhibitor treatment resulted in a 2-fold increase in UCP-1 protein over the levels achieved by β3-adrenergic treatment alone (Fig. 4, A and B). Insulin pretreatment did not have a significant effect on this increase (Fig. 4, A and B). The inhibitor alone had no effect on the basal level of UCP-1 expression (data not shown). The positive effect of PI 3-kinase inhibition on β3-adrenoceptor-induced UCP-1 expression followed a dose-response curve (Fig. 4C). Furthermore, in contrast to β3-adrenergic treatment, β1- and β2-adrenoceptor- as well as forskolin-induced UCP-1 protein was not significantly changed after inhibition of PI 3-kinase by LY294002 pretreatment (Fig. 4D and data not shown).

As inhibition of PI 3-kinase enhanced the β3-adrenoceptor-induced UCP-1 protein expression, we performed *in vitro* PI 3-kinase assays to identify relevant isoforms involved in the mediation of this effect. Previous studies have shown that PI 3-kinase activity associated with p110α is stimulated by insulin (20), whereas G protein-coupled receptors have been shown to stimulate PI 3-kinase activity associated with p110γ acting via βγ-subunits (21). As expected, in

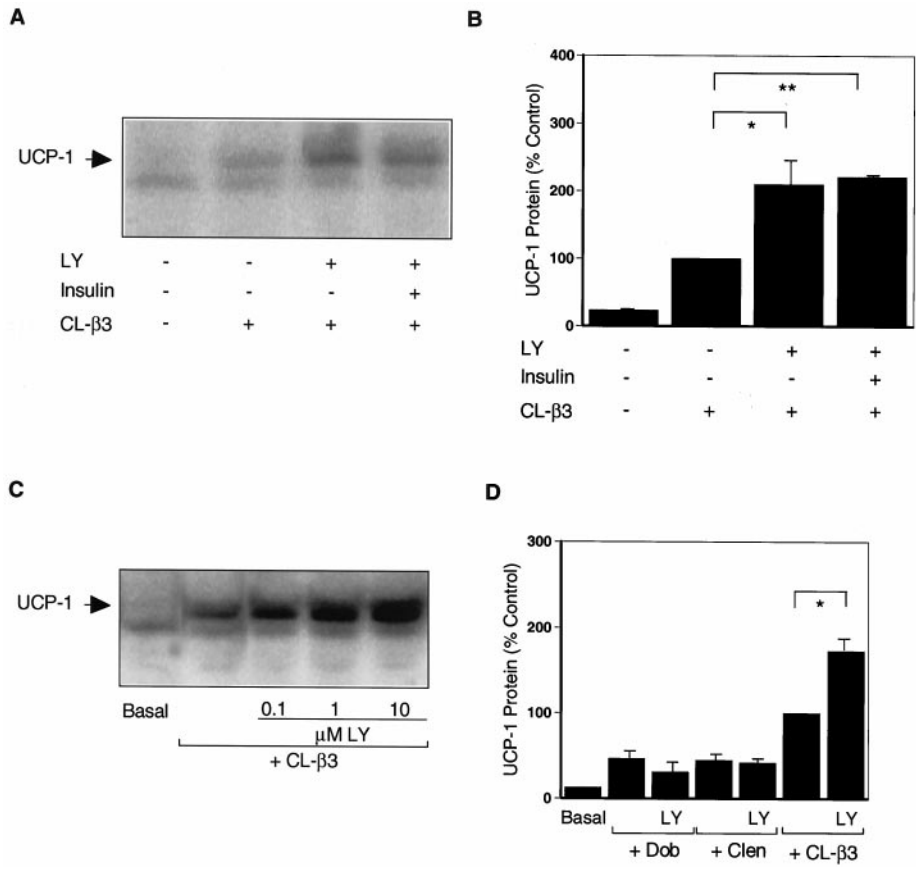

Fig. 4. β3-Adrenergic Induction of UCP-1 Expression Is Regulated by PI 3-Kinase-Dependent Pathways

A, Fully differentiated brown adipocytes were either nontreated or treated with the β3-selective agonist CL316243 (CL-β3, 18 h, 10 μM), the PI 3-kinase inhibitor LY294002 (LY, 19 h, 10 μM), or insulin (24 h, 100 nM) alone or in the combinations indicated. UCP-1 immunoblots were prepared as described in *Materials and Methods.* Representative autoradiography. B, *Bar graph* depicting UCP-1 protein levels relative to CL316243 induction alone (mean ± SEM) as determined by PhosphorImager quantitation of at least three independent experiments. C, Autoradiography of cells treated with the indicated concentrations of LY294002. D, Brown adipocytes were nontreated or incubated with 10 μM dobutamin (Dob), clenbuterol (Clen), or CL316243 (CL-β3) for 16 h alone or in combination with LY294002 (LY, 1 h pretreatment, 10 μM). Statistical significance in panels B and C was calculated with Student's *t* test comparing CL-β3 treatment alone with either CL-β3 + LY or CL-β3 + LY + insulin treatments, respectively; * denotes statistical significance ($P \leq 0.05$); ** denotes high statistical significance ($P \leq 0.01$).

brown adipocytes, insulin produced a 3-fold increase in p110α-associated PI 3-kinase activity and a more than 10-fold increase in PI 3-kinase activity in antiphosphotyrosine immunoprecipitates. β3-Adrenergic stimulation produced no change in either of these fractions (Fig. 5, A and B). By contrast to effects of other G-protein-coupled receptors, β3-adrenergic stimulation failed to increase, and actually reduced, PI 3-kinase activity in both p110γ and Gβ-subunit immunoprecipitates. This effect was time dependent and represented more than a 60% decrease by about 40 min. Insulin had no significant effect on PI 3-kinase associated with either p110γ or Gβ-subunits (Fig. 5, C and D).

DISCUSSION

The characteristic function of brown adipose tissue is to regulate adaptive thermogenesis by virtue of expression of the mitochondrial protein UCP-1 (2–4). Brown fat is also a target for both insulin and β-adrenergic regulation. In immortalized brown adipocytes, we find that cross-talk between insulin and the β3-adrenergic receptor impacts on the regulation of UCP-1 expression. The approximately 4- to 5-fold induction of UCP-1 expression in response to β3-adrenergic stimulation is blocked by inhibition of PKA, consistent with a classical signaling pathway from β-adrenergic receptors via $G\alpha_s$-mediated activation of

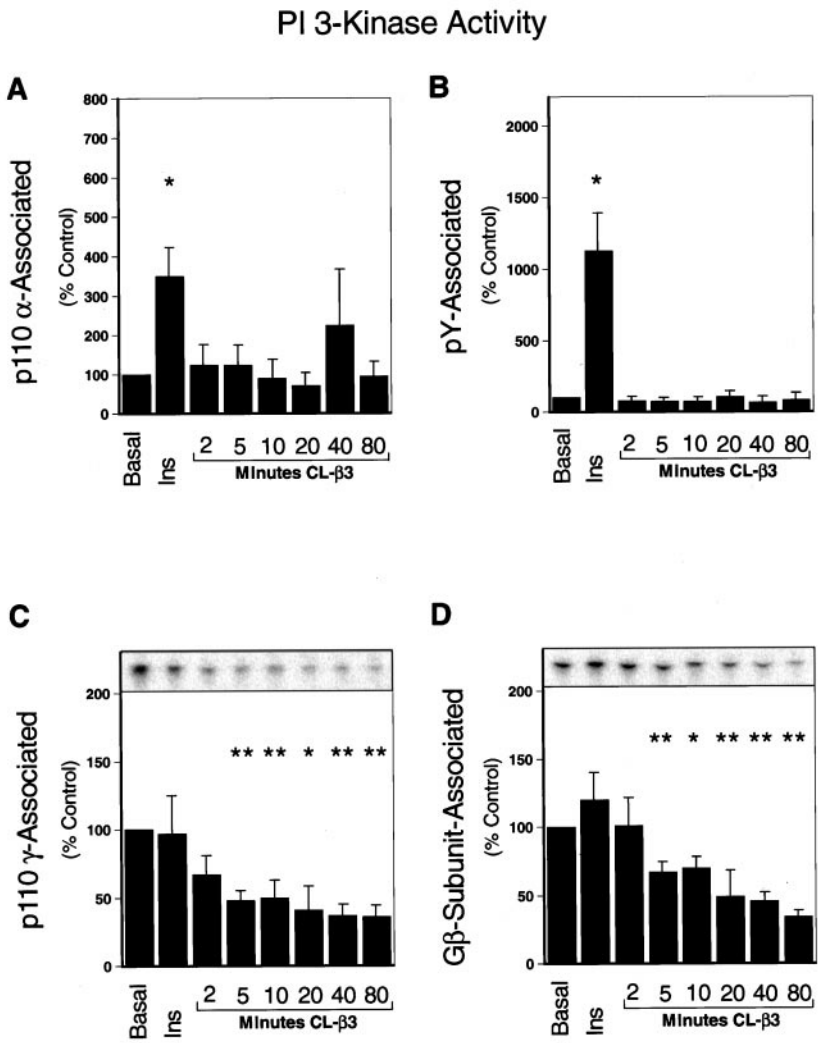

Fig. 5. Stimulation of β3-Adrenergic Receptors Inhibits PI 3-Kinase p110γ Activity

Fully differentiated brown adipocytes were either nontreated, treated with CL316243 (CL-β3, 10 μM) for the indicated periods of time, or treated with insulin (Ins, 5 min, 100 nM). Protein lysates were subjected to immunoprecipitation with antibodies against p110α (A), phosphotyrosines (B), p110γ (C), and Gβ-subunit (D), respectively, and PI 3-kinase activities were measured as described in *Materials and Methods*. The statistical analysis of four independent experiments with the SEM is shown. * Denotes statistical significance ($P \leq 0.05$); ** denotes high statistical significance ($P \leq 0.01$) comparing nontreated cells (Control) with CL-β3 treatment. Representative experiments are shown as *insets on top of the respective bar graph* analyses in panels C and D.

PKA (13–16). Furthermore, specific activation of β1- and β2-adrenoceptors by dobutamin and clenbuterol, as well as direct activation of PKA by forskolin, was also able to increase UCP-1 protein levels, confirming results obtained by other investigators (16). Insulin pretreatment diminishes the β3-adrenergic induction of UCP-1. This direct inhibitory effect of insulin on β3-adrenergic stimulation of UCP-1 may have important pathophysiological implications. Indeed, in several animal and human studies, hyperinsulinemia has been found to precede increased body weight gain and obesity (10–12, 22, 23). Although the negative effect of insulin on the β3-adrenergic UCP-1 induction is small, in the context of chronic hyperinsulinemia, a small reduction in energy expenditure caused by diminished UCP-1 expression could represent a potential mechanism favoring a significant cumulative weight gain over time.

As MAP kinase and PI 3-kinase are important signaling elements common to both insulin and G protein-coupled receptor-signaling pathways (18–20), we have explored their role as potential mediators of β3-adrenergic and insulin effects on UCP-1. In brown adipocytes, MAP kinase is activated by β3-adrenergic stimulation via a PKA-dependent pathway (this study and Ref. 24). Although this might not be the only route of activation with respect to the β3-adrenergic receptor (25), studies in several other systems, including 3T3-F44 preadipocytes (26), cardiac myocytes (27), and PC12 pheochromocytoma cells (28, 29), have also demonstrated a PKA-dependent MAP kinase activation. On the other hand, inhibition of PI 3-kinase, protein kinase C, and G_i-coupled receptors does not significantly reduce the β3-adrenergic activation of MAP kinase, indicating that, in this cell model, these signaling intermediates are not upstream of MAP kinase. Interestingly, inhibition of MAP kinase enhances the β3-adrenergic stimulation of UCP-1 and reverses the inhibitory effect of insulin. These observations suggest a negative role for MAP kinase in both β3-adrenergic and insulin pathways. This is the first study to show the relevance of a MAP kinase-mediated pathway in the β3-adrenergic regulation of UCP-1. A negative role for MAP kinase on UCP-1 expression has also been described in insulin-like growth factor-mediated pathways in fetal brown adipocytes (30).

Another interesting finding of this study is that inhibition of MAP kinase unmasks a second stimulatory effect of insulin on the β3-receptor-induced UCP-1 expression. Although we cannot present direct evidence in this study, a possible candidate mediating this positive effect of insulin is PI 3-kinase p110α. PI 3-kinase activity appears to also play a role in β3-adrenergic pathways, as indicated by the fact that β3-adrenergic induction of UCP-1 is enhanced by PI 3-kinase inhibition. In G_i-coupled muscarinic receptors, the catalytic PI 3-kinase isoform p110γ has been shown to be activated by direct binding to βγ-subunits (21, 31). We detect PI 3-kinase activity in both Gβ-subunit- and p110γ-immunoprecipitates. In contrast to effects of G_i-coupled muscarinic receptors, however, activation of G_s-coupled β3-adrenergic receptors decreases PI 3-kinase activity in both fractions in a time-dependent manner. The mechanism responsible for this inhibition of p110γ activity by β3-adrenergic stimulation is unknown at present. A possible explanation for this novel observation might be a divergent effect of different $G_{\beta\gamma}$-subunits on their effector.

Furthermore, MAP kinase and PI 3-kinase appear to be signaling intermediates specific in the β3-adrenergic control of UCP-1 inasmuch as β1- and β2-adrenoceptor- as well as forskolin-induced UCP-1 protein content is not significantly altered by pharmacological inhibition of either molecule. It has been proposed that each β-adrenoceptor subtype may be coupled to one or more specific adenylyl cyclase isoforms leading to compartmentalization of the functional adrenergic responses (16). Furthermore, it has been shown that β-adrenergic receptors can interact with different G proteins (25). Further studies will be needed to clarify this issue.

Finally, both animal and cell culture studies have indicated a pretranslational control of UCP-1 (32). Depending on conditions, half-life of UCP-1 protein is between 1 and 7 days (32, 33). Since the stimulation period for these experiments was only 16–18 h, the observed effects of MAP kinase and PI 3-kinase inhibition are most likely caused by transcriptional upregulation, as an effect mainly mediated by changes of the protein half-life would be expected to take much longer to be this prominent. Furthermore, β1- and β2-selective stimulation of UCP-1 could not be enhanced using PD098059 or LY294002, demonstrating a specific effect of β3-selective stimulation and making it unlikely that the observed augmentation after β3-selective stimulation is due to an effect of the pharmacological inhibitors on the protein half-life.

Taken together, we propose a model in which the β3-adrenoceptor stimulates UCP-1 expression via a PKA-mediated pathway. β3-Adrenoceptor-mediated inhibition of the p110γ isoform of PI 3-kinase adds to this effect, while the PKA-dependent activation of MAP kinase provides a concurrent autoinhibition. Activation of MAP kinase also functions as an important switch for mediating bidirectional cross-talk with the insulin receptor (Fig. 6). Dysregulation of these cross-talking signaling elements, notably in hyperinsulinemic states, might contribute to a decrease in energy expenditure resulting in obesity and insulin resistance.

MATERIALS AND METHODS

Materials

The selective β3-receptor agonist CL316243 was a generous gift of Dr. Kurt Steiner (Wyeth-Ayerst Research, Princeton, NJ). The UCP-1 specific antibody was purchased from Alpha Diagnostic International (San Antonio, TX) and tested for

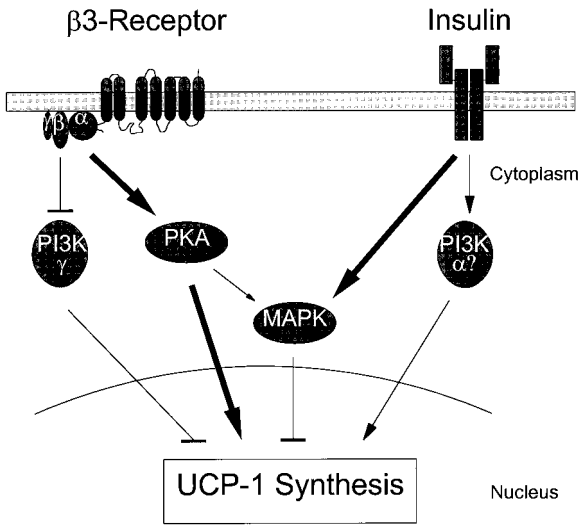

Fig. 6. Model of Regulatory Elements for UCP-1 Expression

UCP-1 expression is regulated both by G protein-coupled β3-adrenergic receptors and signals from the insulin receptor. A main stimulatory pathway leads from $G\alpha_s$-coupled β3-adrenergic receptors to activation of PKA, as inhibition of PKA causes a decrease of UCP-1 protein expression. Activation of β3-adrenergic receptors results in decreased activity of the PI 3-kinase isoform p110γ. As pharmacological inhibition of PI 3-kinases leads to enhanced β3-induced UCP-1 expression, we suggest that the β3-induced decrease of p110γ activity diminishes the negative effect of this PI 3-kinase isoform on UCP-1 expression, thereby promoting UCP-1 protein expression. On the other hand, β3-adrenergic activation of PKA also mediates MAP kinase activation, which provides an autoinhibitory pathway as inhibition of MAP kinase increases β3-induced UCP-1 expression. Similarly, activation of MAP kinase by insulin pretreatment in differentiated brown adipocytes primarily diminishes β3-adrenoceptor-stimulated UCP-1 protein levels. As MAP kinase inhibition together with insulin pretreatment further enhances β3-adrenoceptor-stimulated UCP-1 protein expression, there is likely to be another stimulatory pathway in which the insulin-regulated PI 3-kinase p110α might mediate positive effects from the insulin receptor.

specificity using a yeast recombinant mouse UCP-1 protein (kindly provided by Dr. Bradford Lowell, Beth Israel Deaconess Medical Center, Boston, MA). Antiphosphotyrosine 4G10 antibody was kindly provided by Dr. Morris White (Joslin Diabetes Center, Boston, MA). Polyclonal anti-PI 3-kinase p110γ, anti-Gβ, anti-p110α, and anti-MAP kinase antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); phosphospecific MAP kinase antibody and PD098059 (PD) were from New England Biolabs, Inc. (Beverly, MA); LY294002 (LY), H-89, GF109203X, and pertussis toxin were obtained from Calbiochem (La Jolla, CA). BSA was from Arnel Products Co., Inc. (New York, NY); the PKA assay system was from Life Technologies, Inc. (Gaithersburg, MD); [γ^{32}P]-ATP was purchased from NEN Life Science Products (Boston, MA); protein A-Sepharose was from Pharmacia Biotech (Piscataway, NJ); and [^{125}I]-protein A was from ICN Biochemicals, Inc. (Costa Mesa, CA). Phosphoinositol was purchased from Avanti Polar Lipids (Alabaster, AL); TLC plates were obtained from VWR Scientific Products (Bridgeport, NJ); nitrocellulose was from Schleicher & Schuell, Inc. (Keene, NH); and electrophoresis supplies were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). All other supplies were from Sigma (St. Louis, MO).

Cell Isolation And Culture

Interscapular brown adipose tissue was isolated from newborn FVB mice (Taconic Farms, Inc.) as previously described (34). Preadipose cells were immortalized by infection with the retroviral vector pBabe encoding SV 40 T antigen (kindly provided by Dr. J. DeCaprio, Dana Farber Cancer Institute, Boston, MA) and selected with puromycin (1 μg/ml) for at least 3 weeks. For differentiation, selected preadipocytes were grown to confluence in culture medium supplemented with 20 nM insulin and 1 nM T3 (differentiation medium). After induction of differentiation in confluent cells for 24 h in differentiation medium further supplemented with 0.5 mM isobutylmethylxanthine, 0.5 μM dexamethasone, and 0.125 mM indomethacin, cells were maintained in differentiation medium for 4–5 days until exhibiting a fully differentiated phenotype with massive accumulation of multilocular fat droplets. The different stimulation experiments were carried out after starving the cells in serum-free medium for 16–18 h.

Western Blot Analysis

At the end of the stimulation period, cells were washed twice with ice-cold PBS and lysed in extraction buffer (50 mM HEPES, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Na pyrophosphate, 10 mM NaF, 2 mM EDTA, 10% glycerol, 1% Igepal CA-630, 2 mM vanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM PMSF, pH 7.4). Cell lysates were centrifuged at 12,000 × *g* for 10 min at 4 C, and the protein amount in the supernatant was determined by the Bradford method (35) using BSA as standard and the dye reagent concentrate from Bio-Rad Laboratories, Inc. (Richmond, CA). Equal amounts of protein (100 μg) were directly solubilized in Laemmli sample buffer and separated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking with TBS (10 mM Tris, 0.15 M NaCl, 0.05% Tween, pH 7.2) containing 3% BSA for 30 min, membranes were incubated with the appropriate antibodies for 2 h, washed three times for 5 min each in TBS, and incubated with [^{125}I]protein A for 45 min. After an additional three washes, the immunoblots were exposed on a PhosphorImager screen, and signals were quantified using a densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

PI 3-Kinase Assays

Lysates were obtained as described above. Supernatants containing 300 μg protein were immunoprecipitated for 2 h at 4 C with the appropriate antibodies, and the immune complexes were collected by adding 50 μl of a 50% slurry of protein A-Sepharose in PBS for 1 h at 4 C. After washing the immune complexes twice with PBS containing 1% Igepal-CA 630, twice with 0.5 M LiCl-0.1 M Tris (pH 7.5) and twice in reaction buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA), Sepharose beads were resuspended in a mixture containing 50 μl of reaction buffer, 10 μl of 100 mM $MgCl_2$, and 10 μl of phosphatidylinositol (2 μg/μl). Reactions were initiated by adding 5 μl of reaction mixture [880 μM ATP, 20 mM $MgCl_2$, and 10 μCi of [γ^{32}P]ATP (3,000 Ci/mmol)] per tube and stopped after 10 min by adding 20 μl of 8 N HCl and 160 μl of $CHCl_3$-methanol (1:1). After a brief centrifugation, 50 μl of the lower organic phase of each sample were spotted on a silica gel TLC plate. The plate was developed in $CHCl_3$-methanol-H_2O-NH_4OH (120:94:23:2.4), dried, exposed to a PhosphorImager screen, and quantitated with a densitometer (Molecular Dynamics, Inc.).

PKA Assays

Lysates were obtained after 18 or 19 h (H-89-pretreated cells) of stimulation, respectively, as described above, and PKA activity was measured according to the manufacturer's instructions.

Statistical Analysis

Student's *t* tests were used for analysis of differences between treatments. P values < 0.05 are considered statistically significant and values <0.01 are considered highly significant. The SEM is indicated in all *bar graphs.*

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Address requests for reprints to: C. Ronald Kahn, M.D., Joslin Diabetes Center, One Joslin Place, Boston, Massachusetts 02115; E-mail: c.ronald.kahn@joslin.harvard.edu.

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* These authors equally contributed to this work.

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