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Inhibitory roles of protein kinase B and Peroxisome Proliferator-activated receptor gamma coactivator on hepatic HMG-CoA Reductase promoter activity

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ABSTRACT

Since we had previously demonstrated that siRNAs to tristetraprolin (TTP) markedly inhibited insulin stimulation of hepatic HMG-CoA reductase (HMGR) transcription, we investigated the effects of transfecting rat liver with TTP constructs. We found that transfecting diabetic rats with TTP did not increase HMGR transcription but rather led to modest inhibition. We then investigated whether co-transfection with protein kinase B, hepatic form (AKT2), might lead to phosphorylation and result in activation of HMGR transcription. We found that this treatment resulted in near complete inhibition of transcription. Transfection with peroxisome proliferator-activated receptor γ coactivator (PGC-1α) also inhibited HMGR transcription. These results show that although TTP is needed for activation of HMGR transcription, it cannot by itself activate this process. AKT2 and PGC-1α, which mediate the activation of gluconeogenic genes by insulin, exert the opposite effect on HMGR.

Keywords: *In Vivo* Electroporation; HMG-CoA Reductase; Insulin; Protein Kinase B; Peroxisome Proliferator-Activated Receptor γ Coactivator; Tristetraprolin

1. INTRODUCTION

The rate of transcription of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) is rapidly, within 1 hr increased in response to insulin [1]. This leads to correspondingly rapid rises in HMGR mRNA, immunoreactive protein and enzyme activity levels [2-4]. The increase in hepatic HMGR expression promotes resistance to the serum cholesterol raising action of dietary cholesterol [5,6].

One of the characteristics of rapid response genes is the presence of AU-rich sequences in the 3'-untranslated regions of their mRNAs [7]. These sequences generally serve as binding sites for proteins that act either to accelerate the rate of degradation of the mRNA or to stabilize it. However some of these AU-rich RNA binding proteins act as translational repressors [8] or act at the level of transcription [9,10]. In a recent study, we examined the effects of siRNAs to several of these AU-rich RNA binding proteins on hepatic HMGR promoter activity *in vivo* by introducing an HMGR luciferase promoter construct directly into localized sites in livers of rats by electroporation [11]. Strikingly, siRNAs to one of these, tristetraprolin (TTP), completely eliminated the transcription of HMGR. The TTP siRNAs also prevented the stimulation of HMGR transcription caused by insulin. It is known that insulin treatment of NIH 3T3 fibroblasts overexpressing human insulin receptors causes a dramatic increase in TTP mRNA within 10 minutes [12]. We have observed a 6-fold increase in TTP protein in liver nuclear extracts from diabetic rats treated with insulin [11].

Thus, we sought to determine whether co-transfection of TTP with HMGR promoter construct by *in vivo* electroporation would stimulate HMGR transcription independent of insulin treatment. As phosphorylation of TTP has been reported to impair the mRNA degradation normally promoted by TTP [13], we also performed transfections with and without protein kinase B/Akt2. Since peroxisome proliferator, activated receptor-co activator 1α (PGC-1α) which regulates hepatic metabolism during fasting is phosphorylated and inactivated by Akt2 [14], we also examined the effects of transfecting liver with PGC-1α and the effects of siRNAs to PGC-1α.

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2. MATERIALS AND METHODS

2.1. Experimental Animals

Male Sprague-Dawley rats weighing 100 - 125 g were purchased from Harlan (Madison, WI). The rats were housed in a reversed lighting cycle room (12 h dark/12 h light) and fed Harland Teklad 22/5 rodent chow. A single subcutaneous injection of streptozotocin (Sigma), 65 mg/kg in 0.1 M sodium citrate, pH 5.5, was given to the rats to induce diabetes. Clinistix (Bayer) were used to detect the presence of urinary glucose to confirm the induction of diabetes in the rats. *In vivo* electroporation experiments were preformed 4 days after induction of diabetes. Insulin treatment consisted of giving a single subcutaneous injection of recombinant human insulin (3.0 units/100 g of Novolin 70/30 from Novo Nordisk) 2 h prior to taking tissue samples. All procedures were carried out according to protocol 3571 approved by the University of South Florida Institutional Animal Care and Use Committee.

2.2. Materials

The TTP clone was kindly provided by Dr. Perry J. Blackshear [12]. FLAG-tagged TTP was provided to us by Dr. Jens Lykke-Andersen [13]. A synthetic TTP clone was purchased from GeneCopoeia. Dr. Denise Cooper and N. Patel kindly provided the AKT 2 clone [15] and Dr. Edwards A. Parks generously furnished the peroxisomeproliferator activated receptor gamma coactivator (PGC-1α) [16]. siRNAs to PGC-1α were purchased from Dharmacon.

2.3. *In Vivo* Electroporation

The rats are anaesthetized with 5% isoflurane and maintained under 3% isoflurane. The hair on the abdomen is trimmed closely using a number 10 blade. The surgical area is scrubbed with 70% isopropyl alcohol followed by scrubbing with a betadine solution. A subcutaneous injection of Ketoprofen (5 mg/kg) is given for analgesia. The surgical area is drapped. Sterile instruments and electrode are used for the *in vivo* electroporation. A transverse incision over the sterum is made. The underlying muscle is then cut taking care to not cause any organ damage. Thus, the left, right and median lobes are exposed. The clones and siRNA in 50 µl of sterile saline are injected subcapusularly using a 26 gauge, 3/8 inch length needle. The 0.5 cm six-needle hexagonal array electrode is then placed over the site and six 150 ms pulses of 75 V with 150 ms rests between pulses are delivered. Duplicates are placed in each lobe. Ten µg of the −325/+70 HMGR promoter in pGL3-Basic [1] with and without ten µg of TTP, AKT2 or PGC-1α or PGC-1α siRNA is introduced. The abdominal muscle layer is sutured with Surgilene. Then the skin layer is closed with surgical staples. The rats are up in two minutes following surgery.

2.4. Luciferase Assays

One day after surgery, the sites in each liver lobe are removed using a 5 mm cork-borer. The light scarring from the six acupuncture needles helps to identify the electroporated sites. The liver pieces, approximately 0.1 g are homogenized in passive lysis buffer (Promega) using a Polytron homogenizer. The samples are processed for luciferase activity using the dual luciferase assay kit from Promega [17]. Luciferase activity was calculated as the ratio of firefly (reporter) to *Renilla* (transfection control).

3. RESULTS

3.1. Effect of Co-Transfecting TTP Clones on Hepatic HMGR Promoter Activity

Since we have previously shown [11] that introduction of siRNAs to TTP into liver by *in vivo* electroporation completely inhibits hepatic HMGR promoter activity and the insulin mediated stimulation of promoter activity we tested whether transfecting with a TTP clone might stimulate HMGR promoter activity without insulin. Transfecting livers of diabetic rats with the wild type HMGR luciferase construct and a TTP clone did not stimulate HMGR promoter activity; but rather produced decreases of about 50% (**Figure 1**). This same result was also obtained with FLAG-tagged TTP and the synthetic TTP clones. Surprisingly, co-transfection of insulin-treated diabetic rats with TTP resulted in a marked decrease in HMGR promoter activity (**Figure 1**).

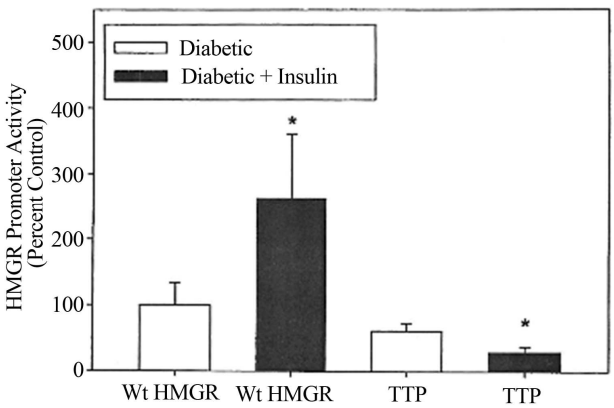

Figure 1. Effect of transfecting TTP on hepatic HMGR promoter activity in diabetic and insulin-treated diabetic rats. Diabetic and insulin-treated diabetic rats were transfected with wild type HMGR with and without the TTP clone from Dr. Blackshear. The data are expressed as means ± SD. $^{*}p < 0.01$ compared with insulin-treated rats transfected with only WT HMGR. Five rats were used in each group.

3.2. Effect of Co-Transfecting with TTP and Akt2 Clones on Hepatic HMGR Promoter Activity

Since it is generally felt that insulin signaling is mediated through Akt kinase phosphorylations [18] and TTP is a phosphoprotein [13], we performed transfections with TTP and Akt2 by *in vivo* electroporation. Introduction of an Akt2 clone together with the TTP clone into livers of diabetic rats resulted in near complete elimination of hepatic HMGR promoter activity (**Figure 2**). A recent study showed that insulin signaling was normal in mice with hepatic deletion of both Akt1 and Akt2 and Foxo 1 [19]. The authors concluded that Akt is not an obligate intermediate for insulin signaling.

3.3. Effects of Peroxisome Proliferator-Activated Receptor-Coactivator 1α on Hepatic HMGR Promoter Activity

Since Akt/PKB is known to phosphorylate and thus inactivate PGC-1α [14], we decided to test the effects of co-transfecting PGC-1α on HMGR promoter activity. PGC-1α co-activates transcription of key gluconeogenic enzymes in fasting such as PEPCK and G6Pase [20]. Insulin acts to repress these genes and promotes utilization of incoming dietary carbohydrate in the fed state. Co-transfection of liver sites in diabetic rats with PGC-1α markedly inhibited HMGR promoter activity (**Figure 3**). Co-transfection with both PGC-1α and TTP also resulted in very low levels of promoter activity. Since PGC-1α transfection acted to inhibit HMGR transcription, we tested the effect PGC-1α siRNA. As would be expected, this treatment increased hepatic HMGR transcription in diabetic rats treated with insulin (**Figure 4**). For comparison, transfection with Akt2 by itself was performed.

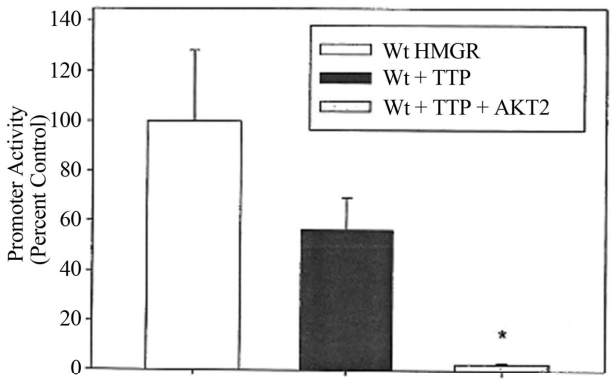

Figure 2. Effect of transfectingwith both TTP and AKT2 on hepatic HMGR promoter activity in diabetic rats. Diabetic rats were transfected with wild type HMGR with TTP or with both TTP and AKT2. The data are expressed as means ± S.D. for five rats in each group. $^{*}p = 0.01$ as compared with diabetic rats tansfected with only wild type HMGR.

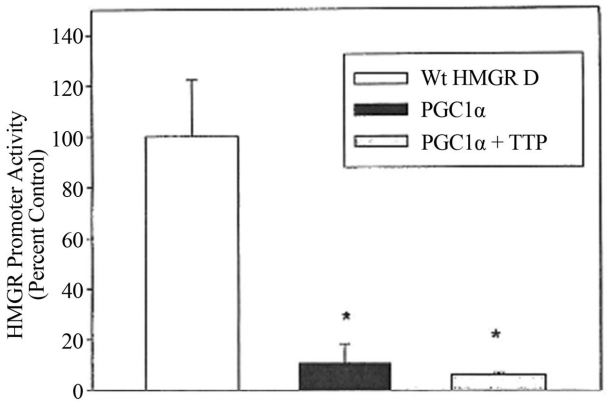

Figure 3. Effect of transfecting with PGC-1α and TTP on hepatic HMGR promoter activity in diabetic rats. Diabetic rats were transfected with wild type HMGR with PGC-1α or with both PGC-1α and TTP. The data are expressed as means ± S.D. for three rats in each group. $^{*}p = 0.01$ as compared with diabetic rats transfected with only wild type HMGR.

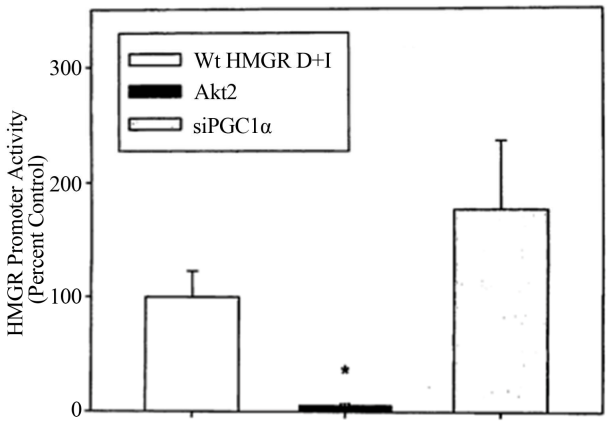

Figure 4. Effects of PGC-1α siRNA as compared with AKT2 on hepatic HMGR promoter activity in insulin-treated diabetic rats. Insulin treated diabetic rats transfected with wild type HMGR were also given either AKT2 or PGC-1α siRNA. The data are expressed as means ± S.D. for three rats in each group. $^{*}p < 0.01$ for AKT2 transfected rats as compared with insulin-treated diabetic transfected with only wild type HMGR.

This treatment markedly inhibited HMGR transcription.

4. DISCUSSION

Although it has been established that the insulin mediated rapid increases in hepatic HMGR mRNA and protein levels are due to activation of transcription [1] and that this effect does not require protein synthesis [21], the signaling pathway from insulin binding to the insulin receptor to an increased rate of transcription has not been established. The insulin signaling pathways that have been investigated have focused on PEPCK and G6Pase, (key enzymes of gluconeogenesis), glucokinase, glycollysis, fatty acid oxidation, fatty acid synthesis, bile acid synthesis, protein translation and cell growth [14,20,22,

23]. None of these studies have addressed the signaling pathway leading to activation of cholesterol biosynthesis in normal liver. One study reported that activation of Akt promoted accumulation of HMGR mRNA in tumor cells associated with increased SREBP-1 [24].

Our recent finding that siRNAs to TTP abolished transcriptional activation of hepatic HMGR by insulin [11] raised the possibility that TTP might be involved in this signaling pathway. However, *in vivo* transfection of liver with TTP clones did not stimulate transcription in diabetic rats and markedly inhibited transcription of HMGR in insulin treated diabetic rats (**Figure 1**). Thus, it appears that TTP is required for activation of hepatic HMGR transcription; however TTP alone does not activate HMGR transcription. Further increases in TTP expression resulted in inhibition of HMGR transcription.

It is generally held that insulin signaling following interaction with its receptor involves phosphorylation of the insulin receptor substrate family that initiates a linear cascade that culminates in phosphorylation of Akt kinases [19]. These kinases then phosphorylate mediators that activate or inactivate key proteins to decrease hepatic glucose output, increase lipogenesis etc. However, transfecting liver sites with Akt2 in addition to TTP resulted in near complete elimination of HMGR transcription rather than stimulation (**Figure 2**). Also transfecting with PGC-1α inhibits HMGR transcription. These results are opposite to those found for the gluconeogenic genes [20], which are elevated under fasting conditions while hepatic HMGR is increased under fed conditions. Thus, this result would actually be anticipated.

It has also been reported that fenofibrate induction of LDL receptor involves protein kinase B (Akt) and peroxisome proliferator-activated receptor α [25]. In many situations hepatic HMGR and LDL receptor activation go together. However, in this case they do not.

5. CONCLUSION

We demonstrated in the investigation reported here that tristetraprolin (TTP) alone cannot mediate insulin's stimulation of HMGR transcription. We also showed that two of the leading candidates that might assist TTP, namely protein kinase B and peroxisome proliferator-activated receptor γ coactivator, actually cause marked inhibition. It is our hope that other investigators will use this knowledge and perhaps employ the *in vivo* electroporatic approach used here to study other potential regulatory factors and define the molecular mechanism by which insulin acts to increase hepatic HMGR expression and thereby confer resistance to dietary induced increases in serum and tissue cholesterol levels.

6. ACKNOWLEDGEMENTS

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