

# Liver Glucokinase Can Be Activated by Peroxisome Proliferator-Activated Receptor- $\gamma$

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**Thiazolidinediones (TZDs), synthetic ligands of peroxisome proliferator-activated receptor (PPAR)- $\gamma$ , are known to decrease hepatic glucose production and increase glycogen synthesis in diabetic animals. Recently it was reported that glucokinase (GK) expression was increased by TZDs in the liver of diabetic ZDF rats. However, the mechanism whereby TZDs increase GK expression is not yet studied. We have assumed that liver type glucokinase (LGK) induction by TZDs could be achieved by direct transcriptional activation. Thus, we have dissected the LGK promoter to explore the presence of a PPAR response element (PPRE) in the promoter. From this study, we were able to localize a PPRE in the -116/-104 region of the rat LGK gene. The PPAR- $\gamma$ /retinoid X receptor- $\alpha$  heterodimer was bound to the element and activated the LGK promoter. The LGK promoter lacking the PPRE or having mutations in the PPRE could not be activated by PPAR- $\gamma$ . Furthermore, troglitazone increased endogenous GK mRNA in primary hepatocytes. These results indicate that PPAR- $\gamma$  can directly activate GK expression in liver and may contribute to improving glucose homeostasis in type 2 diabetes. *Diabetes* 53 (Suppl. 1):S66-S70, 2004**

**T**he regulation of hepatic glucose metabolism is important in glucose homeostasis because the liver stores or produces glucose depending on metabolic needs. Enzymes involved in the major metabolic pathways in liver are largely regulated by glucose or its metabolites at transcriptional levels and are known to contain carbohydrate response elements in their promoters (1). Although the mechanism whereby glucose affects the transcription of these genes is not well understood, it is evident that glucokinase (GK) enables glucose

to regulate the expression of glucose-responsive genes (1). GK functions as a glucose sensor in pancreatic  $\beta$ -cells, hepatocytes, enterocytes, and specialized hypothalamic neurons (2). GK has been thought to be essential for liver in maintaining metabolic function because it exerts a strong influence on glucose utilization by stimulating glycolysis and glycogen synthesis in liver. Thus, even a small change in GK expression can lead to a measurable impact on the blood glucose concentration (3,4).

GK is acutely regulated by the GK regulatory protein (GKRP), which is mainly localized in the nucleus (5). At low glucose concentrations, GKRP is associated with GK in the nucleus. Acute glucose challenge induces dissociation of the GK-GKRP complex, and then GK is translocated into cytoplasm, resulting in increased GK activity (6,7). However, the long-term role of GKRP is thought to be a GK stabilizer because GKRP knockout mice showed decreased GK activity and GK protein level (8,9). Another mechanism for controlling GK activity involves transcription and/or translation levels. The GK gene contains two distinctive promoters, initially believed to be specific for pancreatic  $\beta$ -cells and hepatocytes, but used to explain differential expression in the variety of cells where GK is expressed (10). Of these two promoters, the downstream promoter regulates liver type GK (LGK) expression.

Peroxisome proliferator-activated receptor (PPAR)- $\gamma$  is a member of the nuclear hormone receptor that contains the ligand-dependent activation domain (AF-2) (11). Upon ligand binding, PPAR- $\gamma$  heterodimerizes with retinoid X receptor (RXR)- $\alpha$ , binds to the PPAR response element (PPRE), and activates target gene transcription. Thiazolidinediones (TZDs) are a class of antidiabetic agents that improve insulin sensitivity in various animal models of diabetes (12-14). Patients with a dominant-negative mutation in the PPAR- $\gamma$  gene show severe hyperglycemia, which provides a genetic link between PPAR- $\gamma$  and type 2 diabetes (15). In liver, TZDs increase glucose utilization and decrease glucose production, but the mechanism whereby TZDs affect hepatic glucose metabolism is not yet clear. Thus, we have assumed that PPAR- $\gamma$  can activate the genes of the glucose-sensing apparatus in the liver.

In this study, we identified a PPRE in the LGK promoter and demonstrated that TZDs induced LGK expression in primary hepatocytes. The presence of this functional PPRE in the promoter suggested that LGK could be a direct target of PPAR- $\gamma$ .

## RESEARCH DESIGN AND METHODS

**Materials.** Troglitazone and expression plasmids pCMX-mPPAR- $\gamma$  and pCMX-mRXR- $\alpha$  were described earlier (16). The rat LGK promoter-reporter

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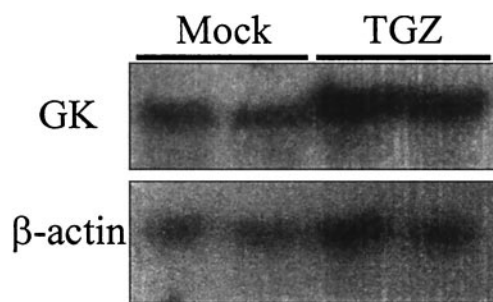
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EMSA, electrophoretic mobility shift assay; GK, glucokinase; GKRP, GK regulatory protein; LGK, liver type glucokinase; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; RXR, retinoid X receptor; SSC, sodium chloride-sodium citrate; TZD, thiazolidinedione.

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**FIG. 1.** LGK transcription was increased by troglitazone. Primary hepatocytes were cultured for 48 h in the presence or absence of troglitazone (20  $\mu\text{mol/l}$ ) and 9-*cis* retinoic acid (1  $\mu\text{mol/l}$ ). Total RNA (20  $\mu\text{g}$ ) was separated on 1% agarose gels and transferred onto a nylon membrane. The membrane was hybridized with  $^{32}\text{P}$ -labeled GK and  $\beta$ -actin cDNA probes. The  $\beta$ -actin gene was used as an internal control.

construct pRGL-1448 was described by Kim et al. (17). 5' Serial deletion of LGK promoter reporter constructs pRGL-238, pRGL-120, and pRGL-76 were constructed by amplifying rat LGK promoter regions of  $-238/+127$ ,  $-120/+127$ , and  $-76/+127$ , respectively, and subcloning into the pGL3 basic vector. For construction of truncated promoter reporter constructs pRGL-120/-77 and pRGL-89/-63, *KpnI* sites were introduced into the appropriate region by site-directed mutagenesis and *KpnI*-digested fragments were excised out. Mutant constructs pRGL-120M1, pRGL-120M2, and pRGL-120M3 were produced by introducing substitution mutations into pRGL-238 using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). DNA sequences of all constructs were confirmed by T7 DNA sequencing. The primers used in site-directed mutagenesis were also used as probes in the electrophoretic mobility shift assay (EMSA).

**Cell culture and transient transfection.** Alexander cells were maintained as described earlier (18). Transient transfections were performed using the Lipofectamine Plus reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol, and the luciferase assay was performed as described previously (16).

**In vitro transcription and translation.** In vitro translated PPAR- $\gamma$  and RXR- $\alpha$  were produced using TNT Quick Coupled Transcription/Translation systems (Promega, Madison, WI) following the manufacturer's protocol (17). **EMSA.** EMSA was performed as described earlier (17). The oligonucleotides used in EMSA were as follows: RGL-120/-77, 5'- CCCTGTCCCCTGTGGCCTTTGTCAAACCCGACCCACAGTGGTTCTTTGTCTGGC-3'; RGL-120/-77M1, 5'- CCCTcTagaTGTTGGCCTTTGTCAAACCCGACCCACAGTGGTTCTTTGTCTGGC-3'; RGL-120/-77M2, 5'- CCCTGTCCCCTGTctagaTGTCAAACCCGACCCACAGTGGTTCTTTGTCTGGC-3'; RGL-120/-77M3, 5'- CCCTGTCCCCTGTGGCCTTcTagAACCCGACCCACAGTGGTTCTTTGTCTGGC-3'. The PPRE sequence is underlined, and mutated bases are shown in lowercase letters (19).

**RNA preparation and Northern blot analysis.** Total RNA was isolated from primary hepatocytes using the TRIzol reagent following the manufacturer's protocol (Life Technologies). Total RNA (20  $\mu\text{g}$ ) was separated on 1% agarose gels containing 0.66 mol/l formaldehyde. After electrophoresis, RNA was transferred to a nylon membrane by capillary transfer in the presence of 20  $\times$  sodium chloride-sodium citrate (SSC). Then the membrane, which was ultraviolet-cross-linked, was hybridized with  $^{32}\text{P}$ -labeled GK or  $\beta$ -actin cDNA probes in Rapid-hyb buffer (Amersham Pharmacia, Buckinghamshire, U.K.) at 65°C for 4 h. After hybridization, the membrane was rinsed with 2 $\times$  SSC, 0.1% SDS, followed by 0.2 $\times$  SSC, 0.1% SDS, and exposed to X-ray film at  $-70^\circ\text{C}$  with an intensifying screen.

**Statistical analysis.** All transfection studies were performed in triplicate and repeated more than three times. The data were represented as means  $\pm$  SD. Statistical analysis was carried out using Excel software (Microsoft, Redmond, WA).

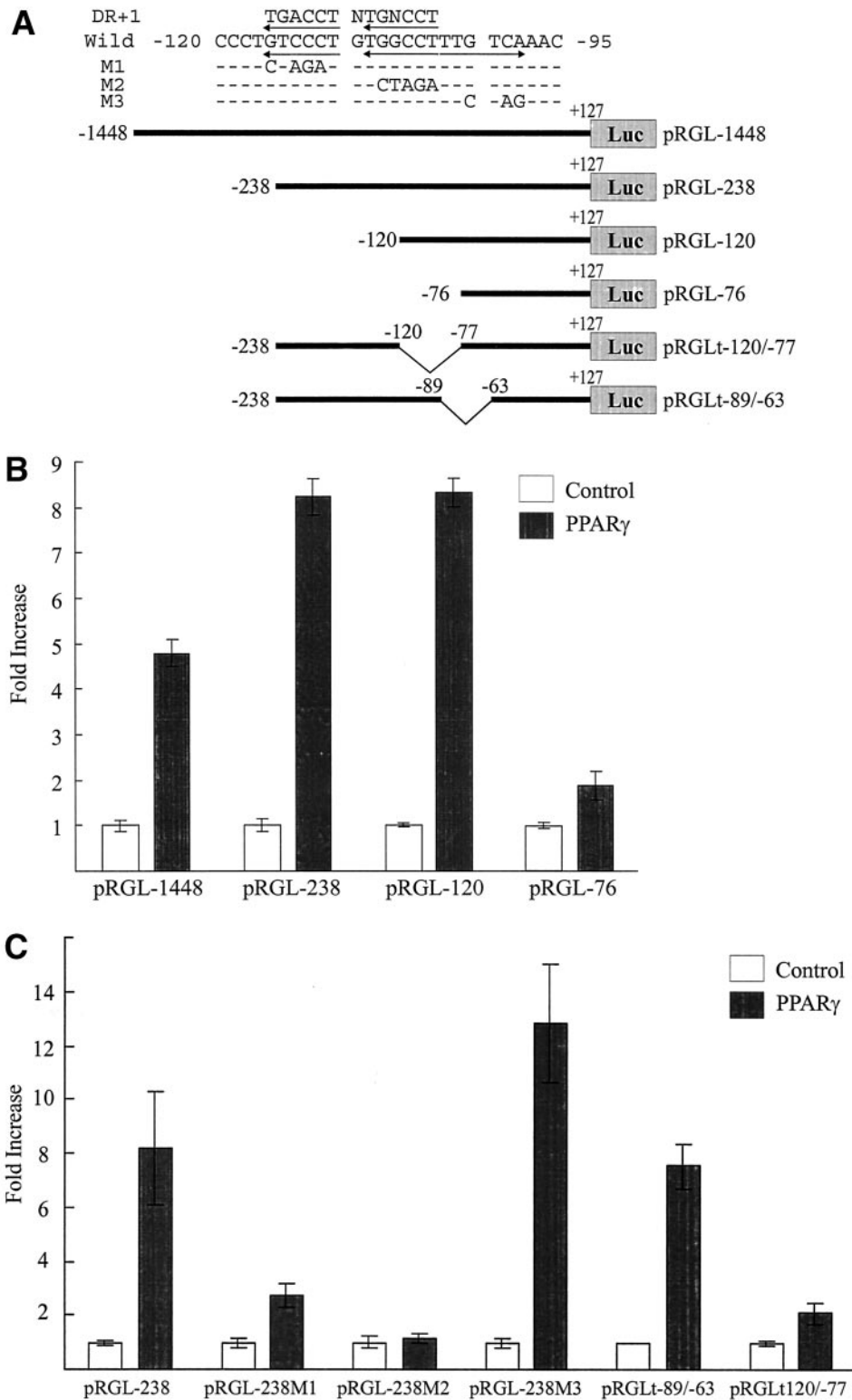
## RESULTS

**Troglitazone can increase GK expression in primary hepatocytes.** To test whether PPAR- $\gamma$  agonists can directly activate GK gene expression in an isolated system, we treated troglitazone with primary hepatocytes isolated from male Sprague-Dawley rats (200–250 g) and measured the mRNA levels by Northern blot analysis. The transcription of the GK gene was increased by troglitazone (Fig. 1).

This result supports the observation that TZDs increase GK expression in the liver of diabetic ZDF rats (20) and suggests the presence of PPRE in the LGK promoter.

**Localization of PPRE in the LGK promoter.** To delineate a functional PPRE in the LGK promoter, we constructed an LGK promoter-luciferase reporter construct that contained the  $-1448/+127$  region of the LGK promoter and performed a transient transfection assay. The LGK promoter was activated by fivefold when transfected into Alexander cells with overexpression of PPAR- $\gamma$  and RXR- $\alpha$  in the presence of their ligands. This result suggested that functional PPRE is present in the LGK promoter (Fig. 2). To localize the PPRE in the  $-1448/+127$  region, we prepared 5' deletion constructs and performed deletion study. In this experiment, pRGL-238 and pRGL-120 were still activated by PPAR- $\gamma$ , but deletion down to the  $-76$  region of the LGK promoter resulted in loss of PPAR- $\gamma$  responsiveness. These results suggested that putative LGK-PPRE could be present between the  $-120$  and  $-76$  region. To narrow down the functional PPRE, we prepared mutant promoter-reporter constructs pRGL-120/-77 and pRGL-89/-63, where the  $-120/-77$  region and  $-89/-63$  region were removed from pRGL-238, respectively. Of the two truncated promoters, pRGL-120/-77 did not respond to PPAR- $\gamma$ , whereas pRGL-89/-63 still retained responsiveness, suggesting that PPRE could be located in the  $-120/-89$  region. The consensus sequence of PPRE was known to be DR+1, a hexameric consensus sequence (AGGTCA) in a direct repeat spaced by one nucleotide (19). But there were three AGGTCA sequences in the  $-120/-89$  region. Thus, we prepared three mutants to further localize the PPRE. Substitution mutations were introduced into putative AGGTCA sequences of minimal rat LGK promoter reporter construct pRGL-238 and named pRGL-238M1, pRGL-238M2, and pRGL-238M3, respectively. Among these three mutants, pRGL-238M3 was activated by PPAR- $\gamma$ , whereas pRGL-238M1 and pRGL-238M2 were not. These data indicate that the first two reverse "AGGTCA" sequences constitute DR+1. Thus, we could define the  $-116/-104$  region containing the sequence of GTCCCTGTGGCCT as LGK-PPRE, which is responsible for the functional activation of the LGK promoter by PPAR- $\gamma$ .

**Binding of the PPAR- $\gamma$ /RXR- $\alpha$  heterodimer to LGK-PPRE.** The binding of the PPAR- $\gamma$ /RXR- $\alpha$  heterodimer to LGK-PPRE was analyzed by EMSA using in vitro translated PPAR- $\gamma$  and RXR- $\alpha$ . As shown in Fig. 3A, the heterodimer of PPAR- $\gamma$  and RXR- $\alpha$  could bind to RGL-120/-77 and RGL-120/-77M3 probes. However, RGL-120/-77M1 and RGL-120/-77M2 were not bound by the proteins. The identity of the PPAR- $\gamma$ /RXR- $\alpha$  heterodimer bound to LGK-PPRE was further confirmed by supershift assay using the anti-PPAR- $\gamma$  antibody, which could block the DNA binding of PPAR- $\gamma$ . In addition, we confirmed the specificity of the binding by competition assay (Fig. 3B). Fifty-molar excess cold competitors were cocubated with a wild-type probe. Wild competitor and RGL-120/-77M3 could compete out the binding, but RGL-120/-77M1 and RGL-120/-77M2 could not. These results demonstrated that the LGK promoter could be activated by PPAR- $\gamma$  through the binding of the PPAR- $\gamma$ /RXR- $\alpha$  heterodimer to LGK-PPRE.

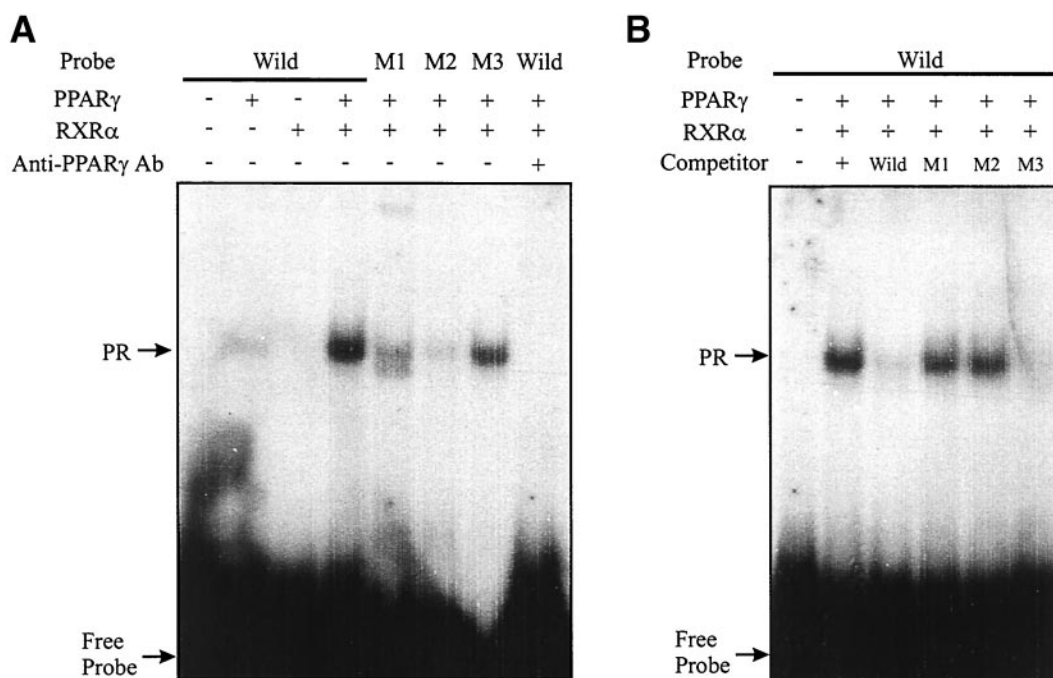


**FIG. 2.** The LGK promoter contained functional PPRE. **A:** Structures of LGK promoter luciferase reporter constructs. DNA sequences of the wild-type and mutant versions of the promoter element were shown to define the PPRE. The hexameric consensus sequences are underlined with arrows, and mutated nucleotides are shown. **B:** Luciferase reporter constructs under the control of the rat LGK promoter spanning from -1448, -238, -120, or -76 to +127, respectively, were co-transfected into Alexander cells with (■) or without (□) the indicated PPAR- $\gamma$ /RXR- $\alpha$  expression vector. **C:** Luciferase reporter constructs under control of the LGK minimal promoter or its mutants were transfected into Alexander cells with (■) or without (□) the indicated PPAR- $\gamma$ /RXR- $\alpha$  expression vector. The cells were incubated for 18 h after transfection in the presence of appropriate ligands: 20  $\mu$ M troglitazone for PPAR- $\gamma$  and 1  $\mu$ M 9-*cis* retinoic acid for RXR- $\alpha$ . Normalized luciferase activities are shown as means  $\pm$  SD of three independent experiments in triplicate and are expressed as the fold increase relative to the basal activity, respectively, in the absence of the expression vectors and ligands.

## DISCUSSION

Several hepatic genes that are involved in glycolysis, glycogen synthesis, and lipogenesis are regulated by glucose, and GK expression is essential to this process (1). In type 2 diabetic subjects, hepatic glucose production is increased and glycogen synthesis is decreased because of insulin resistance and increased free fatty acid. PPAR- $\gamma$  agonists were known to increase peripheral insulin sensi-

tivity and increase glucose-sensing ability in pancreatic  $\beta$ -cells (11). They also decrease free fatty acid levels by repartitioning the fat to adipocytes and hepatic glucose production by decreasing gluconeogenic gene expression and increasing GK expression in diabetic ZDF rats (20). It has been a matter of debate whether the antidiabetic effects of TZDs are due to the improved insulin sensitivity or direct transcriptional activation of genes involved in



**FIG. 3.** Binding of PPAR- $\gamma$ /RXR- $\alpha$  heterodimer to LGK-PPRE. **A:** EMSA of RGL-120/-77 (wild) using in vitro translated PPAR- $\gamma$  and RXR- $\alpha$ .  $^{32}$ P-labeled double-strand oligonucleotides were incubated with in vitro translated PPAR- $\gamma$  (2  $\mu$ l) and/or RXR- $\alpha$  (2  $\mu$ l) as indicated. Anti-PPAR- $\gamma$  antibody (2  $\mu$ l) was added into the reaction mixture. **B:** For competition experiments, 50-molar excess of RGL-120/-77 (wild), M1, M2, and M3 was included in the binding reaction. PR indicates the shifted band by the PPAR- $\gamma$ /RXR- $\alpha$  heterodimer.

glucose homeostasis. However, the effects of PPAR- $\gamma$  on liver have been regarded as rather indirect, because the ligand-induced decrease in free fatty acid levels preceded that in glucose and the change of hepatic gene expressions (20).

In the present study, we have identified a functional PPRE in the rat LGK promoter. The LGK-PPRE was localized within -116/-104, which contained the sequence GTCCCTGTGGCCT, by promoter analysis performed in Alexander cells. PPAR- $\gamma$  heterodimerized with RXR- $\alpha$  could bind to LGK-PPRE and activate the LGK transcription. Also, we observed the activation of LGK gene expression by PPAR- $\gamma$  in primary hepatocytes. Through this study, we demonstrate that LGK is a direct target of PPAR- $\gamma$  in liver. Given that GK is controlling the influx of glucose and accompanying glycolysis in the liver and enables glucose to regulate the enzymes involved in the major metabolic pathway of the liver, the activation of LGK expression by PPAR- $\gamma$  seems to have an important role in improving systemic glucose homeostasis.

Recently, evidence supporting the direct action of PPAR- $\gamma$  on glucose homeostasis has been accumulating. We reported that GLUT2 and  $\beta$ GK are the direct targets of PPAR- $\gamma$  in pancreatic  $\beta$ -cells. Here, we present the evidence that LGK could be directly activated by PPAR- $\gamma$ . It is the first demonstration that LGK could be a direct target of PPAR- $\gamma$ , and the activation may contribute to improved glucose homeostasis independent of the systemic effects of PPAR- $\gamma$ .

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#### REFERENCES

1. Girard J, Ferre P, Foufelle F: Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. *Annu Rev Nutr* 17:325-352, 1997
2. Bell GI, Cuesta-Munoz A, Matschinsky FM: Glucokinase. In *Encyclopedia of Molecular Medicine*. New York, Wiley, 2002, p. 1437-1442
3. Ferre T, Riu E, Bosch F, Valera A: Evidence from transgenic mice that glucokinase is rate limiting for glucose utilization in the liver. *FASEB J* 10:1213-1218, 1996
4. Niswender KD, Shiota M, Postic C, Cherrington AD, Magnuson MA: Effects of increased glucokinase gene copy number on glucose homeostasis and hepatic glucose metabolism. *J Biol Chem* 272:22570-22575, 1997
5. Vandercammen A, Van Schaftingen E: Species and tissue distribution of the regulatory protein of glucokinase. *Biochem J* 294:551-556, 1993
6. Vandercammen A, Van Schaftingen E: The mechanism by which rat liver glucokinase is inhibited by the regulatory protein. *Eur J Biochem* 191:483-489, 1990
7. Van Schaftingen E, Dethoux M, Veiga da Cunha M: Short-term control of glucokinase activity: role of a regulatory protein. *FASEB J* 8:414-419, 1994
8. Farrelly D, Brown KS, Tieman A, Ren J, Lira SA, Hagan D, Gregg R, Mookhtiar KA, Hariharan N: Mice mutant for glucokinase regulatory protein exhibit decreased liver glucokinase: a sequestration mechanism in metabolic regulation. *Proc Natl Acad Sci U S A* 96:14511-14516, 1999
9. Grimby J, Coffey JW, Dvorozniak MT, Magram J, Li G, Matschinsky FM, Shiota C, Kaur S, Magnuson MA, Grippo JF: Characterization of glucokinase regulatory protein-deficient mice. *J Biol Chem* 275:7826-7831, 2000
10. Magnuson MA: Glucokinase gene structure: functional implications of molecular genetic studies. *Diabetes* 39:523-527, 1990
11. Picard F, Auwerx J: PPAR(gamma) and glucose homeostasis. *Annu Rev Nutr* 22:167-197, 2002
12. Nolan JJ, Ludvik B, Beerdsen P, Joyce M, Olefsky J: Improvement in glucose tolerance and insulin resistance in obese subjects treated with troglitazone. *N Engl J Med* 331:1188-1193, 1994
13. Cavaghan MK, Ehrmann DA, Byrne MM, Polonsky KS: Treatment with the oral antidiabetic agent troglitazone improves beta cell responses to glucose in subjects with impaired glucose tolerance. *J Clin Invest* 100: 530-537, 1997

14. Sreenan S, Sturis J, Pugh W, Burant CF, Polonsky KS: Prevention of hyperglycemia in the Zucker diabetic fatty rat by treatment with metformin or troglitazone. *Am J Physiol* 271:E742–E747, 1996
15. Barroso I, Gurnell M, Crowley VE, Agostini M, Schwabe JW, Soos MA, Maslen GL, Williams TD, Lewis H, Schafer AJ, Chatterjee VK, O'Rahilly S: Dominant negative mutations in human PPARgamma associated with severe insulin resistance, diabetes mellitus and hypertension. *Nature* 402:880–883, 1999
16. Kim HI, Kim JW, Kim SH, Cha JY, Kim KS, Ahn YH: Identification and functional characterization of the peroxisomal proliferator response element in rat GLUT2 promoter. *Diabetes* 49:1517–1524, 2000
17. Kim HI, Cha JY, Kim SY, Kim JW, Roh KJ, Seong JK, Lee NT, Choi KY, Kim KS, Ahn YH: Peroxisomal proliferator-activated receptor-gamma upregulates glucokinase gene expression in beta-cells. *Diabetes* 51:676–685, 2002
18. Moon YA, Lee JJ, Park SW, Ahn YH, Kim KS: The roles of sterol regulatory element-binding proteins in the transactivation of the rat ATP citrate-lyase promoter. *J Biol Chem* 275:30280–30286, 2000
19. Palmer CN, Hsu MH, Griffin HJ, Johnson EF: Novel sequence determinants in peroxisome proliferator signaling. *J Biol Chem* 270:16114–16121, 1995
20. Way JM, Harrington WW, Brown KK, Gottschalk WK, Sundseth SS, Mansfield TA, Ramachandran RK, Willson TM, Klier SA: Comprehensive messenger ribonucleic acid profiling reveals that peroxisome proliferator-activated receptor gamma activation has coordinate effects on gene expression in multiple insulin-sensitive tissues. *Endocrinology* 142:1269–1277, 2001